

GREEN CHEMISTRY-DRIVEN DESIGN AND SYNTHESIS OF SULPHONYL PYRIMIDINONES AS DUAL CHOLINESTERASE INHIBITORS FOR ALZHEIMER'S DISEASE

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Received: July 20, 2025; Revised: December 28, 2025; Accepted: January 22, 2026

Abstract

In this investigation, a green chemistry strategy was used to synthesize novel sulphonyl-Pyrimidinone derivatives as dual inhibitors of Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), enzymes, which are involved in the development of Alzheimer's disease. A series of sulphonyl pyrimidinone derivatives was synthesized via an eco-friendly method, i.e., microwave-assisted synthesis, affording high yields (89-93%) with minimal waste. The compounds were systematically characterized using physicochemical and spectral techniques and screened for in vitro AChE and BChE inhibition by Ellman's method. All the synthesized compounds showed comparable inhibitory activity against both AChE and BChE, with IC₅₀ values ranging from 23.00 nM to 71.66 nM and 36.00 nM to 81.00 nM, respectively. The most promising activity was demonstrated by 3b (AChE, IC₅₀ = 23.00 nM; BChE IC₅₀ = 36.00 nM), which was better than the standard drug, Donepezil (AChE, IC₅₀ 37.00 nM, BChE IC₅₀ = 46.00 nM). Furthermore, Molecular docking simulations supported that the compound 3b binds through π - π stacking with Tyr124 and Tyr133 and other residues located in the PAS and CAS region of AChE and H-bonding with Thr120 in BChE. Such interactions revealed that these compounds might inhibit both catalytic activity and AChE-induced A β aggregation in AChE. The results indicate that electron-donating methyl substitution strengthens dual-site binding and improves inhibitory potency, which may modulate the amyloid- β aggregation. Collectively, this work demonstrates the potential of microwave-assisted, green synthetic methods for generating sulphonyl-pyrimidinone derivatives as sustainable dual cholinesterase inhibitors for Alzheimer's disease therapy.

Keywords: AChE and BChE Inhibitory Activity; Alzheimer's Disease; Green Chemistry Approach; Molecular Docking; Sulphonyl-Pyrimidinone Derivatives

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DOI: <https://doi.org/10.55766/sujst10819>

Introduction

Alzheimer's disease (AD) is an irreversible psychiatric disorder that affects the brain and which is characterized by behavioral abnormalities, impaired cognitive function and memory disturbances (Shaikh *et al.*, 2020). The cholinergic hypothesis is one of the most widely recognized pathogenic mechanisms of AD, though several other pathogeneses have been advanced. According to this theory, the cognitive impairments observed in AD patients are directly triggered by considerable loss of cholinergic neurons and decreased levels of the neurotransmitter acetylcholine (ACh). In this regard, two primary enzymes that deal with the hydrolysis of ACh: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are relevant therapeutic targets, with blocking AChE and BChE being a key approach (Ibrahim *et al.*, 2020; Chen *et al.*, 2022).

Even though the main enzyme that breaks down ACh in the healthy brain is AChE, BChE contributes to the metabolism of ACh, especially during the later phases of AD, at which point, the activity of BChE tends to rise as the content of AChE decreases (Wu *et al.*, 2021). As such, this complementary role has led to increased interest in designing dual inhibitors capable of targeting both AChE and BChE in tandem and may provide better neuroprotection and symptomatic relief. Moreover, AChE-mediated β -amyloid ($A\beta$) aggregation involves the interaction of AChE with $A\beta$ peptides via its peripheral anionic site (PAS), promoting the nucleation and accelerated formation of fibrillar $A\beta$ aggregates. This non-enzymatic function of AChE contributes to neurotoxicity and the progression of AD. Consequently, dual site inhibition of AChE, at the catalytic anionic site (CAS) and peripheral anionic site (PAS), has been shown to enhance cholinergic signaling while simultaneously reducing AChE-mediated- $A\beta$ -aggregation (Bhardwaj *et al.*, 2025; Grabowska *et al.*, 2025).

At this time, AD is predominantly treated with Acetylcholinesterase inhibitors (AChEIs), of which there are only FDA-approved medications: Tacrine, Donepezil, Rivastigmine, and galantamine (Bortolami *et al.*, 2021). The first-generation AChEI, such as Tacrine, is limited in clinical use due to its hepatotoxicity (Cacabelos, 2020). However, these medications only target AChE and do not address the potential benefits of dual inhibition. Thus, the development of new drugs that target both AChE and BChE concomitantly

may be an effective intervention in Alzheimer's disease treatment.

Numerous pharmacophores are integrated into the structure-based rational design of sulphonyl-pyrimidine derivatives to improve AChE inhibition. The pyrimidine scaffold was selected as the core structure due to its desirable heterocycle framework with a wide range of biological properties, including cholinesterase inhibition, neuroprotection, anti-inflammatory, and cognitive enhancement properties. Additionally, the planar aromatic structure of pyrimidine, along with its nitrogen atoms serving as basic and nucleophilic centers, makes it appropriate for interaction with the catalytic anionic site (CAS) of AChE (Manzoor *et al.*, 2021). The Schiff base linker also has the benefit of additional hydrogen-bond donor/acceptor properties and provides conformational flexibility, in addition to antioxidant and neuroprotective properties. The sulphonamide group also enhances affinity to bind the enzyme by its strong bond connection and enhances better metabolic stability and bioavailability (Aytac *et al.*, 2023). The aromatic ring is part of the hydrophobic domain and facilitates π - π interaction with aromatic residues within the AChE gorge. The phenyl ring in this scaffold contains variable substituents, enabling adjustment of lipophilicity, electronic effects, and steric bulk to optimize enzyme binding (Mishra *et al.*, 2025). These strategically integrated motifs in combination are aimed to achieve the best possible positioning of the molecule within the catalytic gorge, enhance binding affinity, and hence facilitate the development of effective anti-cholinesterase agents.

This study involves the investigation of novel sulphonyl-pyrimidinone derivatives as cholinesterase inhibitors by employing a green chemistry approach (Figure 1). The research work highlights the limitations of conventional drug design in the development of a cholinesterase inhibitor. It combines rational drug design with *in vitro* cholinesterase inhibition assay and molecular docking studies to analyze the binding interactions of cholinesterase enzyme at both the CAS and PAS sites. Additionally, a structure-activity relationship (SAR) analysis was conducted to identify key structural features contributing to potency and selectivity, aiding in the development of potential lead candidates for the treatment of Alzheimer's disease.

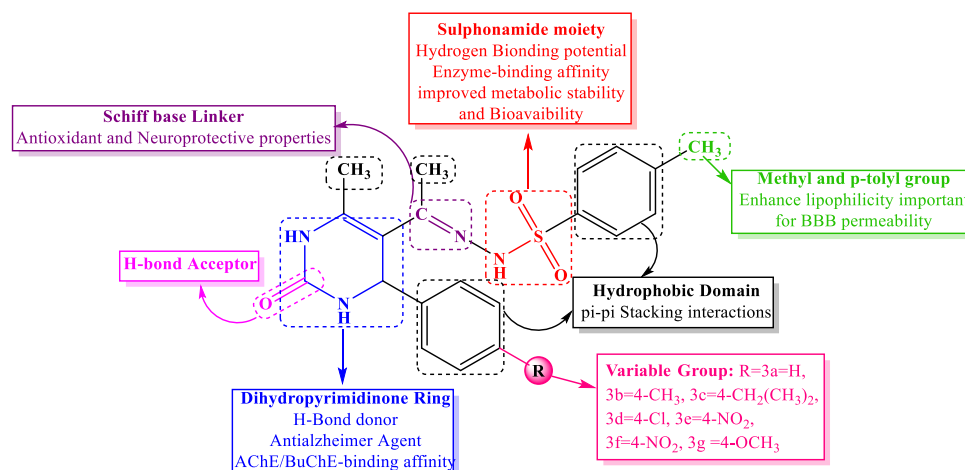


Figure 1. Designed sulphonyl-pyrimidinone derivatives (3a–g)

Results and Discussions

Chemistry

The novel sulphonyl pyrimidinone compounds (3a–g) were synthesized by a multistep synthetic route (Figure 2). The first intermediate dihydropyrimidinone core (compound 1) was obtained via one-pot, three-component, and Biginelli condensation of *p*-tolualdehyde, acetylacetone, and urea in ethanol under microwave irradiation in

the presence of a catalytic amount of hydrochloric acid. The microwave synthesis afforded an improved yield 94% with significantly reduced reaction time (15 min), showing better efficiency than the conventional Biginelli reaction, which has been reported to have a low yield (78%) and more reaction time (Chandravarkar *et al.*, 2024). The IR spectrum confirmed the formation of the Dihydropyrimidinone scaffold, where the characteristic IR absorptions at 3,424.34 cm⁻¹ for N-H stretching frequency and two carbonyl

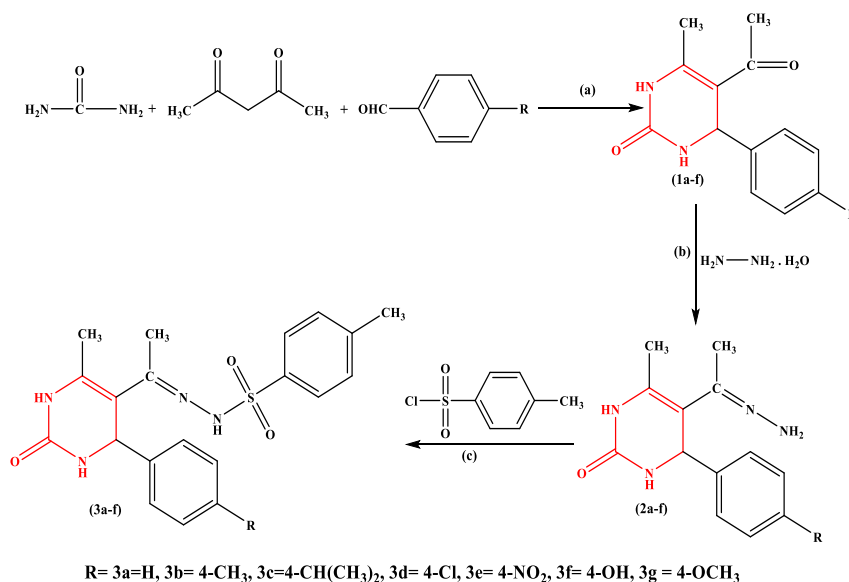


Figure 2. General synthetic route for the synthesis of sulphonyl pyrimidinone derivatives

Reagents and Reaction Conditions: (a) EtOH, a few drops Conc. HCl, 100–120°C, 240 W, stirred for 10–15 min. (b) 10% NaOH, Ethanol, stir for 3–4 h at 20–25°C, (c) Toluene sulphonyl chloride, 10% NaOH, and stir for 3–4 h.

groups (C=O) stretching frequencies observed at 1,691.72 cm^{-1} and 1,618.32 cm^{-1} . Based on the ^1H NMR spectrum of compound 1b, singlets at the δ 2.13, 2.19, and 2.26 ppm were observed in relation to the methyl, acetyl, and pyrimidine methyl groups. The methine proton at the C-4 position of the pyrimidine ring is attributed a singlet at δ 5.13 ppm. The aromatic ring displayed anticipated doublets at δ 7.09 and 7.23 ppm, indicating the para-substitution in the phenyl ring. The singlets at δ 7.56 and 9.09 ppm refer to the $-\text{NH}-$ protons of the pyrimidinone core. The structure was additionally supported by the ^{13}C NMR spectrum, where the signals for methyl (17.77–27.03 ppm), methine (51.42 ppm), aromatic (126–140 ppm), and carbonyl carbons (150.23 and 192.22 ppm) were observed. It was then followed by Nucleophilic attack of hydrazine hydrate on the ketone (acetyl group) at the C-5 position of compound 1 to produce a hydrazone linkage with high yield (92%) compared to the conventional method (56–67%) (Ismaeel *et al.*, 2024). The ^1H and ^{13}C NMR spectra of compound 2 closely resembled those of compound 1, with slight changes due to hydrazone formation. There have been minor downfield shifts indicating functional group conjugation with the newly formed hydrazone moiety. Additional NMR signals attributed specifically to the hydrazone proton were not clearly distinguished and may overlap or be broadened due to exchangeable NH signals. Furthermore, in the final step, sulfonamide derivatives (3a–g) were synthesized via condensation of compound 2 with p -toluenesulphonyl chloride in the presence of triethylamine under microwave-assisted conditions. The tosyl group reacted selectively with the terminal NH_2 group of the hydrazone to afford the corresponding sulphonohydrazone in good yield. Notably, the microwave-assisted protocol afforded these products in higher yields (89–93%) and substantially reduced reaction times compared to the conventional heating method (74%), highlighting the superior efficiency of the green synthetic approach (Thakur *et al.*, 2018). The IR spectrum of

compound 3a showed new absorptions at 1,167.61 cm^{-1} and 1,109.34 cm^{-1} , corresponding to the asymmetric and symmetric stretching of the SO_2 group, confirming successful tosylation. The N–H (3,320.71 cm^{-1}) and C=O (1,714.85, 1,691.17 cm^{-1}) stretches were found to show slight changes. In the ^1H NMR, additional signals included a singlet at δ 10.50 ppm, corresponding to the $\text{SO}_2\text{--NH}$ proton, and aromatic protons of the tosyl group appearing between δ 7.28–7.29 ppm, along with corresponding aromatic carbons at δ 128.35–129.38, 137.58, and 143.06 ppm in the ^{13}C NMR. The molecular ion peak at m/z 398.27[M $^+$] in the mass spectrum was consistent with the expected molecular formula. Elemental analysis data matched well with the calculated values, further confirming the structure of compound 3a. Overall, the spectroscopic data obtained strongly supported the proposed structure of compounds 3a–g. The results were in good agreement with the expected chemical composition and structural features of the compound. Representative spectra for all synthesized compounds are provided in the Supplementary Data (Figures S1–S33) for reference.

In-vitro Anti-acetylcholinesterase activity

The synthesized compounds 3a–g were evaluated for their dual inhibitory activity against AChE and BChE. The IC_{50} values for AChE inhibition ranged from 23.00 nM to 71.66 nM and for BChE inhibition from 36.00 nM to 81.00 nM (See Table 1 and Figure 3). These were compared with the standard AChE inhibitor Donepezil (AChE IC_{50} = 37.00 nM, BChE IC_{50} = 46.00 nM). The results revealed that substitution at the para position of the aromatic ring significantly influenced enzyme inhibition. Among all, compound 3b (4- CH_3) exhibited the most potent dual inhibitory activity (AChE IC_{50} = 23.00 nM, BChE IC_{50} = 36.00 nM), outperforming the standard in both assays, highlighting the favorable impact of a small, hydrophobic electron-donating methyl group on the binding affinity of enzymes. The isopropyl-substituted compound 3c (4-CH (CH_3) $_2$) also

Table 1. Anti-cholinesterase Potential of Sulphonyl Pyrimidinone Derivatives (3a–g)

S. No.	Compounds	R	^a IC_{50} values	
			AChE	BChE
1.	^b Standard	-	37.33 $\pm$ 0.882	46.00 $\pm$ 1.155
2.	3a	H	43.00 $\pm$ 1.000	47.33 $\pm$ 0.882
3.	3b	4- CH_3	23.00 $\pm$ 1.528	36.00 $\pm$ 1.155
4.	3c	4- CH_2CH_3	32.66 $\pm$ 1.453	42.33 $\pm$ 0.882
5.	3d	4-Cl	71.66 $\pm$ 1.202	77.16 $\pm$ 0.441
6.	3e	4- NO_2	58.00 $\pm$ 0.577	81.33 $\pm$ 1.202
7.	3f	4-OH	60.66 $\pm$ 1.202	65.66 $\pm$ 0.882
8.	3g	4- OCH_3	52.66 $\pm$ 1.202	74.66 $\pm$ 1.202

^a IC_{50} Value expressed in nM $\pm$ SEM

^b Standard: Donepezil (DNZ) used as the standard AChE inhibitor

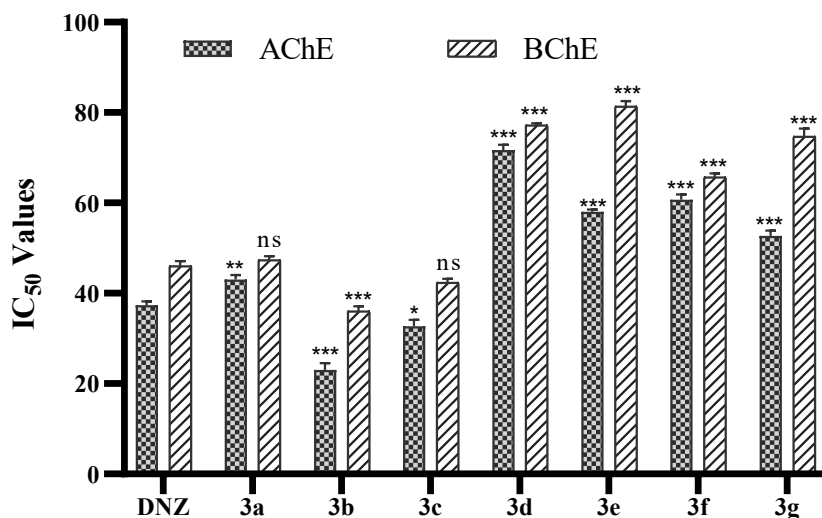


Figure 3. *In vitro* anti-AChE and anti-BChE potential of compound (3a-g). All values are expressed in mean $\pm$ SEM, n=3. One-way ANOVA and Dunnett's t-test were performed for statistical analysis. Data indicates *p<0.05, **P< 0.01, *** p<0.001, and ns indicates not significant when compared to the standard

showed moderate dual inhibition, while the unsubstituted 3a (H) demonstrated weaker activity than the standard, indicating that the presence and nature of the substituent are critical for activity, and that slightly larger alkyl substituents are still tolerated but less effective than methyl. In contrast, compounds bearing electron-withdrawing groups (3d: -Cl, 3e: -NO₂) and polar substituents (3f: -OH, 3g: -OCH₃) displayed significantly reduced inhibition of both enzymes, likely due to steric hindrance or unfavorable electronic effects. These observations are consistent with previous reports on cholinesterase inhibitors, where small lipophilic and electron-donating substituents (especially methyl) were found to enhance enzyme binding affinity through improved hydrophobic interactions within the active site (Jameel *et al.*, 2017; Das *et al.*, 2022). Overall, the data suggest that the methyl group at the para position favors inhibition of cholinesterase enzymes and also may interfere with AChE-mediated β -amyloid aggregation. This supports compound 3b emerging as the most promising lead for further development in the treatment of neurodegenerative disorders such as Alzheimer's disease.

Molecular Docking Simulation

Molecular docking simulations were conducted for the sulphonyl pyrimidinone derivatives (3a-g) against AChE (PDB ID: 4EY7) and BChE (PDB ID: 4TPK) using Molegro Virtual

Docker v.6.0 software, and the docking result was compared with the standard drug Donepezil. The compounds displayed the binding affinities as measured by their MolDock score, ranging from -129.574 kcal/mol to -146.967 kcal/mol, with compound 3b consistently showing the highest affinities for AChE (-146.967 kcal/mol) and BChE (-146.967 kcal/mol), outperforming Donepezil (AChE: -122.197 kcal/mol; BChE: -126.208 kcal/mol). Detailed docking data are represented in Table 2. The strong binding affinities of sulphonyl-pyrimidinone derivatives are demonstrated towards AChE, due to the formation of hydrogen bonds, π - π interactions, and hydrophobic contacts within the catalytic gorge. A consistent finding for all ligands was their stable interaction with Tyr133, a key residue of the CAS, suggesting ligands' ability to anchor effectively within the active site of AChE. Additionally, most compounds also interacted with the PAS residue, which facilitates AChE-mediated β -amyloid aggregation. This dual binding behavior indicates that the derivatives, particularly compound 3b, not only effectively inhibit AChE but may also modulate β -amyloid aggregation. Compound 3b demonstrated favorable interactions with residues spanning both the CAS and PAS, conforming dual-site binding mode. Within AChE, 3b interacted with PAS residues (Tyr124, Trp86) along with CAS residues involved in substrate, as illustrated in Figure 4, indicating its ability to occupy the entire active-site gorge. As shown in Figure 4, in BChE,

Table 2. Molecular docking simulations study of the synthesized sulphonyl-pyrimidinone derivatives (3a-g) and Donepezil with target proteins

Compound ID	R	AChE (PDB ID: 4EY7)				BuChE (PDB ID: 4TPK)			
		MolDock Score (kcal/mol)	No. of H-bonds	H-bond Interaction	S-bond Inter-action	MolDock Score (kcal/mol)	No. of H-bonds	H-bond Interaction	S-bond Inter-action
Donepezil	-	-122.197	2	Tyr133	Trp86, Gly120, Gly121, Gly122, Ser125, Gly126, Ala127, Tyr133, Ser203, Trp236, Phe297, Phe338, His447	-126.208	1	Thr120	Gly115, Glu197, Phe329
3a	-H	-142.669	2	Ser203, Tyr124	Trp86, Gly120, Tyr124, Ser203, Phe297, Phe338	-131.273	6	Trp82, Thr120, Trp430, His438, Tyr440	Trp82, Ser198, Ala328, Tyr440, His438
3b	4-CH ₃	-146.967	2	Tyr124, Tyr133	Trp86, Gly122, Ser125, Tyr133, Glu202, Phe297, Phe338, His447	-147.054	7	Gly115, Thr120, Tyr128, Glu197	Trp82, Thr120, Tyr128, Glu197
3c	4-CH(CH ₃) ₂	-144.201	2	Tyr124, Tyr133	Trp86, Gly122, Tyr124, Ser125, Tyr133, Glu202, Phe297, Phe338, His447	-143.961	6	Trp82, Thr120, Trp430, His438, Tyr440	Trp82, Ser198, Met437, His438
3d	4-Cl	-139.068	2	Tyr133	Trp86, Gly120, Gly121, Gly122, Tyr133, Glu202, Tyr337, Phe338, His447, Gly448	-129.574	6	Trp82, Thr120, His438, Tyr440	Trp82, Met437, His438, Tyr440
3e	4-NO ₂	-139.832	3	Gly121, Tyr133, Ser203	Trp83, Trp86, Tyr124, Tyr133, Glu202, Phe338	-130.554	6	Trp82, Thr120, Tyr128, Tyr440	Trp82, Tyr128, Tyr440
3f	4-OH	-143.017	4	Trp86, Gly120, Tyr133	Trp86, Gly120, Gly121, Gly122, Ser125, Glu202, Phe297, Phe338, His447, Gly448	-137.744	7	Trp82, Thr120, Ser198, Trp430, His438, Tyr440	Trp82, Ser198, Met437, His438, Tyr440
3g	4-OCH ₃	-143.581	3	Gly120, Ser125, Tyr133	Trp86, Gly120, Gly121, Gly122, Glu202, Ser203, Phe338, His447, Gly448	-134.247	4	Trp82, Tyr128, Glu197, Tyr440	Trp82, Met437, His438, Gly439

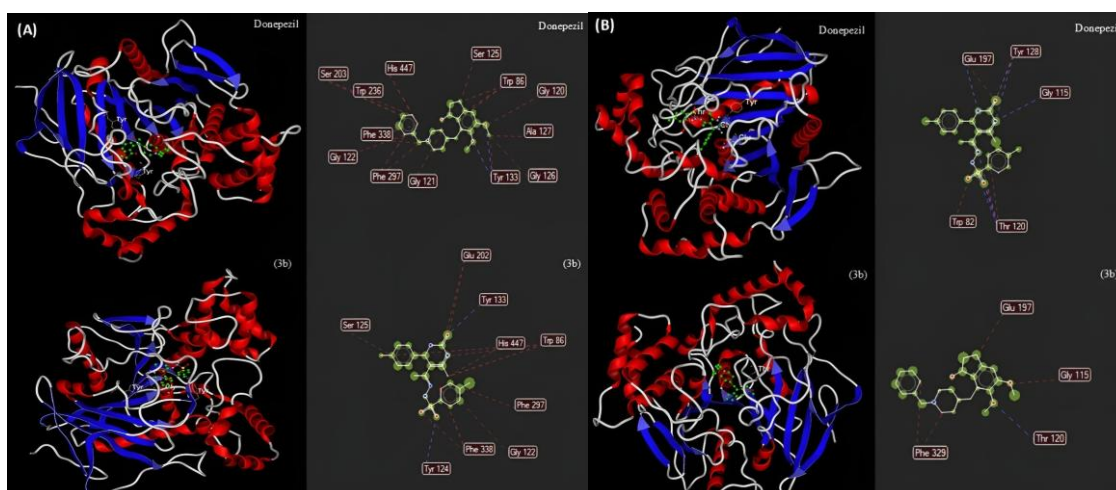


Figure 4. Molecular docking visualization of the synthesized sulphonyl pyrimidinone compound 3b and standard drug Donepezil with target proteins (A) acetylcholinesterase (AChE) and (B) butyrylcholinesterase (BChE), performed using Molegro Virtual Docker v6.0. Left images represented the docking view where ligands are shown as colored ball-and-stick models, interacting amino acid residues as thin sticks, and the protein backbone in red and blue-ribbon structures. The right-side images illustrate molecular interactions, with blue dotted lines indicating hydrogen bonds (H-bonds) and red dotted lines denoting steric bonds (S-bonds) between the ligands and active site residues.

both 3b and Donepezil formed hydrogen bonds with Thr120 and hydrophobic interactions with Glu197, demonstrating their compatibility with the enzyme's binding pocket. These interactions collectively form the dual cholinesterase inhibition potential of this series of compounds.

These findings further validate the rational design of the sulphonyl pyrimidinone scaffold for dual cholinesterase inhibition. Among the series, compound 3b demonstrated the strongest binding affinity towards both AChE and BChE with key interactions that extend into the PAS region, supporting both potent cholinesterase inhibition and the potential modulation of AChE-induced amyloid- β aggregation, offering dual therapeutic benefits. These docking results are consistent with the *in-vitro* inhibitory data and align with previously reported binding modes of AChE inhibitors (Evren *et al.*, 2024), where aromatic π - π interactions with Trp86 and Phe338 and hydrophobic interactions around the catalytic site significantly enhance potency (Bagri *et al.*, 2020; Damuka *et al.*, 2020). Together, the biological evaluation and molecular docking analysis showed that the para-methyl substitution in compound 3b provides an optimal balance of electron-donating substituents, enhancing binding affinity through optimal hydrophobic interaction of the active site gorge, facilitating enhanced affinity, dual site interaction, and more complementary binding orientation than bulkier, polar, or electron-withdrawing groups.

AChE: acetylcholinesterase; BChE: butyrylcholinesterase; H-bond: Hydrogen bond; S-bond: Steric bond

Structure-activity Relationship

The biological evaluation and molecular docking simulation of sulphonyl-pyrimidinone derivatives (3a-g) revealed a comprehensive structure-activity relationship (SAR) that explains the way that these molecules achieve their cholinesterase inhibition. SAR of compounds (3a-g) indicated that the cholinesterase inhibition is influenced by the nature of the para-substituent on the phenyl ring. The greater inhibitory activity was observed for the compound bearing a methyl group 3b, indicating that small hydrophobic and electron-donating groups increase the binding interactions within the catalytic gorge of AChE and BChE. Replacement of the methyl group with a bulkier alkyl substituent, as in 3c (4-isopropyl), resulted in a moderate inhibitory effect, suggesting that hydrophobic character is favorable, and increasing steric bulk slightly reduces binding efficiency. The unsubstituted compound 3a showed an intermediate effect, highlighting the significance of the para substitution for enzyme interaction.

In contrast, compounds with electron-withdrawing substituents (3d: 4-Cl; 3e: 4-NO₂) demonstrated the lower inhibitory effect, probably due to diminished electron density and weaker aromatic interaction with key active-site residues. Similarly, polar substituents in 3f (4-OH) and 3g (4-OCH₃) reduced the inhibitory effect, indicating that polar substituents were not well compatible with the hydrophobic enzyme gorge. Overall, the SAR reveals that small hydrophobic electron-donating groups and steric compatibility at the para position are essential for achieving a strong inhibitory effect against both AChE and BChE, with compound 3b emerging as the most favorable structural modification.

Material and Methods

General Information

The analytical graded chemicals and solvents used in the synthesis were of Merck chemicals, Loba Chemie, and CDH chemicals. Acetylcholinesterase from *Electrophorus electricus* (electric eel), Type VI-S lyophilized powder, Butyrylcholinesterase, acetylcholine chloride, DTNB, and Phosphate buffer were purchased from Sigma-Aldrich and SLS chemicals. The reaction progress and completion were followed by means of thin layer chromatography (TLC) on silica gel G (E, Merk) with Chloroform: Methanol (7:3) as the mobile phase. The structural characterization was done by NMR on a Bruker Advance Neo 400 MHz spectrometer with solvent DMSO-d₆, and spectral reference to tetramethylsilane (TMS). The infrared (IR) spectra were taken using a Perkin Elmer Spectrum IR version 10.6.2 FTIR spectrophotometer, and mass spectra using a G2-XS QT quadrupole time-of-flight (QTOF) mass spectrometer. An elemental analysis was carried out using the Thermo Scientific Flash 2000 analyzer.

Chemistry

General procedure for the synthesis of 5-acetyl-6-methyl-4-(p-tolyl)-3,4-dihydropyrimidin-2(1H)-one (1a-g)

An equimolar mixture of methyl benzaldehyde (10 mmol, 1.06 g), acetylacetone (10 mmol, 1.00 g), and urea (10 mmol, 0.60 g) was dissolved in 30 mL of ethanol. The reaction mixture was acidified by adding 3-4 drops of hydrochloric acid (35%) to maintain the pH 3-4 under continuous stirring. After that reaction mixture was transferred to a microwave synthesizer and irradiated at 100-120°C, 240 W for 10 -15 min. Upon completion, the reaction was cooled to room temperature and poured slowly into 100 g crushed ice with constant stirring, followed by

gradual neutralization using a 10% aqueous sodium hydroxide solution to induce complete precipitation. The product was collected by vacuum filtration, washed with cold water, and dried.

Color: Yellow powder; yield: 94%; IR(KBr, cm^{-1}): 3,424.34 (N-H), 2,826.81 (Ar C-H), 1,691.72 (C=O), 1,618.32 (C=O), 1,469.81 (Ar C=C); ^1H NMR (400 MHz, DMSO- d_6): δ ppm 2.13 (3H, s, -COCH₃), 2.19 (3H, Ar-CH), 2.26 (3H, s, CH₃), 5.13 (1H, s, -CH-), 7.09 (2H, d, ArCH), 7.23 (2H, d, ArCH), 7.56 (1H, s, -NH-CO-NH-), 9.09 (1H, s, -NH-CO-NH-); ^{13}C NMR (125 MHz, DMSO- d_6), δ ppm: 17.77 (1C, ArCH₃), 21.33 (1C, ArCH₃), 27.03 (1C, -COCH₃), 51.42 (1C, pyrimidinone), 106.72 (1C, pyrimidinone), 126.82-128.84 (4C, ArCH), 136.44 (1C, Ar-CH), 140.36 (1C, ArCH), 145.04 (1C Pyrimidinone), 150.23 (1C, C=O), 192.22 (1C, C=O).

General procedure for the synthesis of 4-(4-substitutedphenyl)-5-(1-hydrazineylideneethyl)-6-methyl-3,4-dihydropyrimidin-2(1H)-one (2a-g)

An equimolar amount of compound 1 (10 mmol) and hydrazine hydrate (10 mmol) was dissolved in 10 mL of ethanol. The solution was acidified slightly using 2-3 drops of concentrated acetic acid. The reaction mixture was stirred and subjected to microwave irradiation at a power of 280 W, maintaining a temperature of 70-75°C. For two successive cycles of 10-15 min each. The reaction mixture was poured onto 100 g of crushed ice to induce precipitation. The solid precipitate formed was collected by vacuum filtration, washed thoroughly with cold water, and dried under reduced pressure.

Color: Yellow powder; yield: 92%; IR (KBr, cm^{-1}): 3,424.34 (N-H), 2,826.81 (Ar C-H), 1,691.72 (C=O), 1,618.32 (C=O), 1,469.81 (Ar C=C);

^1H NMR (125 MHz, DMSO- d_6): δ ppm 2.07 (3H, s, -CH₃), 2.26 (3H, s, CH₃), 5.13 (1H, s, -CH-), 5.98 (-NH₂), 7.28 (4H, d, ArCH), 7.29 (2H, d, ArCH), 7.56 (1H, s, -NH-CO-NH-), 9.09 (1H, s, -NH-CO-NH-); ^{13}C NMR (125 MHz, DMSO- d_6), δ ppm: 17.77 (1C, ArCH₃), 21.33 (1C, ArCH₃), 27.03 (1C, -COCH₃), 51.42 (1C, pyrimidinone), 106.72 (1C, pyrimidinone), 126.82-128.84 (4C, ArCH), 136.44 (1C, Ar-CH), 140.36 (1C, ArCH), 145.04 (1C Pyrimidinone), 150.23 (1C, C=O), 192.22 (1C, C=O).

General procedure for synthesis of N-(1-(4-(4-substitutedphenyl)-6methyl-2-oxo-1,2,3,4-tetrahydropyrimidin-5yl)ethylidene)-4-methylbenzenesulphonohydrazide (3a-g)

In a microwave reaction vessel, 1.0 mmol of compound 2 was dissolved in 10-15 mL ethanol,

followed by the addition of p-toluenesulphonyl chloride (10 mmol, 1.9 g) and triethylamine (12 mmol, 1.7g). The mixture was stirred for 5 min, then irradiated in a microwave synthesizer at 90-100°C, 280 W for 10-15 min. The reaction mixture was cooled to room temperature and poured into 100 g of crushed ice with gentle stirring to precipitate the product. The resulting solid was collected by vacuum filtration, washed with cold water, and dried.

4-methyl-N'-(1-(6-methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidin-5-yl)ethylidene)benzenesulphonohydrazide (3a)

Color: yellowish white powder; % yield: 90%; m.p.: 204-206°C; IR (KBr, cm^{-1}): 3,320.71 (N-H), 3,022.91 (Ar C-H), 2,917.73 (Al C-H), 1,691.17 (C=O), 1,568.09 (C=N), 1,440.81 (Ar-C=C), 1,302.74 (S=O), 1,167.61 (S=O); ^1H NMR (125 MHz, DMSO- d_6): δ 2.07 (3H, s, -CH₃), 2.26 (3H, s, -CH₃) 2.43 (3H, s, Ar-CH₃), 5.13 (1H, s, -CH-), Ar-CH), 7.28 (4H, d, ArCH), 7.29 (1H, d, ArCH), 7.39 (2H, d, ArCH), 7.56 (1H, s, -NH-CO-NH-), 9.09 (1H, s, -NH-CO-NH-), 10.50 (1H, s, -SO₂NH-); ^{13}C NMR (125 MHz, DMSO- d_6), δ ppm: 18.37 (1C, CH₃), 21.37 (1C, -CH₃), 22.12 (1C, -CH₃), 51.76 (1C, pyrimidinone), 107.49 (1C, pyrimidinone), 126.76-126.76 (3C, ArCH), 128.35-129.38 (6C, ArCH), 137.58 (1C, ArCH), 141.12 (1C, pyrimidinone), 143.06-143.36 (2C, ArCH), 150.24 (1C, >C=O), 155.63 (1C, >C=N-), MS: calculated for [M⁺] m/z: 398.48, found: 398.27 [M⁺]; Elemental Analysis calculated for C₂₀H₂₂N₄O₃S: C, 60.28; H, 5.57; N, 14.06; O, 12.04; S, 8.05; Found: C, 60.46; H, 5.62; N, 14.23; O, 12.31, S, 8.24.

4-methyl-N'-(1-(6-methyl-2-oxo-4-(p-tolyl)-1,2,3,4-tetrahydropyrimidin-5-yl)ethylidene)benzenesulphonohydrazide (3b)

Color: yellow powder; % yield: 96%; m.p.: 185-187°C; (KBr, cm^{-1}): 3,415.79 (N-H), 3,123.88 (Ar C-H), 2,971.12 (Al C-H), 1,618.73 (C=O), 1,523.57 (C=N), 1,445.75 (Ar-C=C), 1,233.88 (S=O), 1,173.56 (S=O), 1,056.75 (N-N); ^1H NMR (125 MHz, DMSO- d_6): δ 2.07 (3H, s, -CH₃), 2.19 (3H, s, -CH₃), 2.26 (3H, s, -CH₃) 2.43 (3H, s, Ar-CH₃), 5.13 (1H, s, -CH-), 7.08 (2H, d, ArCH), 7.23 (2H, d, ArCH), 7.39 (2H, d, ArCH), 7.56 (1H, s, -NH-CO-NH-), 7.74 (2H, d, ArCH), 9.09 (1H, s, -NH-CO-NH-), 10.50 (1H, s, -SO₂NH-); ^{13}C NMR (125 MHz, DMSO- d_6), δ ppm: 18.37 (1C, CH₃), 21.31-21.39 (2C, -CH₃), 22.12 (1C, -CH₃), 51.74 (1C, pyrimidinone), 107.51 (1C, pyrimidinone), 126.78 (2C, ArCH), 128.32-129.37 (6C, ArCH), 136.41-137.58 (2C, ArCH), 140.34 (1C, ArCH), 141.12 (1C, pyrimidinone), 143.06 (2C, ArCH),

150.26 (1C, >C=O), 155.64 (1C, >C=N-), MS: calculated for $[M^+]$ m/z:412.51, Found: 412.49 $[M^+]$; Elemental Analysis calculated for $C_{21}H_{24}N_4O_3S$: C, 61.15; H, 5.86; N, 13.58; O, 11.64; S, 7.77; Found: C, 61.23; H, 5.82; N, 13.52; O, 11.61, S, 7.69.

N'-(1-(4-(4-isopropylphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidin-5-yl)ethylidene)-4-methylbenzenesulphonohydrazide (3c)

Color: yellow powder; % yield: 93%; m.p.: 193-195°C; IR (KBr, cm^{-1}): 3,417.16 (N-H), 3,073.36 (Ar C-H), 2,889.28 (Al C-H), 1,615.52 (C=O), 1,525.65 (C=N), 1,235.28 (S=O), 1,132.50 (S=O), 1,023.83 (N-N); 1H NMR (400 MHz, DMSO- d_6): δ 1.20 (6H, s, -CH(CH₃)₂), 2.07 (3H, s, -CH₃), 2.19 (3H, s, -CH₃), 2.26 (3H, s, -CH₃) 2.43 (3H, s, Ar-CH₃), 2.86 (1H, s, -CH(CH₃)₂), 5.13 (1H, s, -CH-), 7.19 (2H, d, ArCH), 7.28 (2H, d, ArCH), 7.39 (2H, d, ArCH), 7.56 (1H, s, -NH-CO-NH-), 7.74 (2H, d, ArCH), 9.09 (1H, s, -NH-CO-NH-), 10.50 (1H, s, -SO₂NH-); ^{13}C NMR (125 MHz, DMSO- d_6), δ ppm: 18.37 (1C, CH₃), 21.32-22.13 (2C, -CH₃), 23.34 (2C, -CH(CH₃)₂), 33.28 (1C, -CH(CH₃)₂), 51.75 (1C, pyrimidinone), 107.51 (1C, pyrimidinone), 125.96-126.78 (4C, ArCH), 128.39-129.34 (4C, ArCH), 137.58 (1C, ArCH), 140.52 (1C, ArCH), 141.16 (1C, pyrimidinone), 143.07 (1C, ArCH), 146.51 (1C, ArCH), 150.27 (1C, >C=O), 155.63 (1C, >C=N-), MS: calculated for $[M^+]$ m/z:440.56, found: 440.53 $[M^+]$; Elemental Analysis calculated for $C_{23}H_{28}N_4O_3S$: C, 62.70; H, 6.41; N, 12.72; O, 10.89; S, 7.28; Found: C, 62.63; H, 6.32; N, 12.67; O, 10.76, S, 7.23.

N'-(1-(4-(4-chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidin-5-yl)ethylidene)-4-methylbenzenesulphonohydrazide (3d)

Color: yellowish powder; % yield: 92 %; m.p.: 189-191°C; (KBr, cm^{-1}): 3,357.82 (N-H), 3,145.78 (Ar C-H), 2,943.82 (Ar C-H), 1,616.67 (C=O), 1,527.40 (C=N), 1,445.82 (Ar C-C), 1,233.02 (S=O), 1,133.78 (S=O), 1,024.36 (N-N) 1H NMR (400 MHz, DMSO- d_6): δ 2.07 (3H, s, -CH₃), 2.26 (3H, s, -CH₃) 2.43 (3H, s, Ar-CH₃), 5.13 (1H, s, -CH-), 7.26 (2H, d, ArCH), 7.29 (2H, d, ArCH), 7.39 (2H, d, ArCH), 7.56 (1H, s, -NH-CO-NH-), 7.74 (2H, d, ArCH), 9.09 (1H, s, -NH-CO-NH-), 10.50 (1H, s, -SO₂NH-); ^{13}C NMR (125 MHz, DMSO- d_6), δ ppm: 18.34 (1C, CH₃), 21.33-22.14 (2C, -CH₃), 51.74 (1C, pyrimidinone), 107.51 (1C, pyrimidinone), 126.16 (2C, ArCH), 128.38-128.64 (4C, ArCH), 129.36 (2C, ArCH), 132.3 (1C, ArCH), 137.61 (1C, ArCH), 141.12 (1C, pyrimidinone), 141.46 (2C, ArCH), 143.07 (1C, ArCH), 150.26 (1C, >C=O), 155.62 (1C, >C=N-), MS: calculated

for $[M^+]$ m/z:432.92, found: 432.43 $[M^+]$; Elemental Analysis calculated for $C_{20}H_{21}ClN_4O_3S$: C, 55.49; H, 4.89; Cl, 8.19; N, 12.94; O, 11.09; S, 7.41; Found: C, 55.63; H, 4.82; N, 13.02; O, 11.14, S, 7.47.

4-methyl-N'-(1-(6-methyl-4-(4-nitrophenyl)-2-oxo-1,2,3,4-tetrahydropyrimidin-5-yl)ethylidene)benzenesulphonohydrazide (3e)

Color: pale yellow powder; % yield: 94%; m.p.: 182-183°C; IR (KBr, cm^{-1}): 3,415.65 (N-H), 3,123.64 (Ar C-H), 2,971.12 (Al C-H), 1,618.65 (C=O), 1,523.57 (C=N), 1,505.35 (Ar NO₂), 1,233.83 (S=O), 1,173.36 (S=O), 1,056.75 (N-N); 1H NMR (125 MHz, DMSO- d_6): δ 2.07 (3H, s, -CH₃), 2.26 (3H, s, -CH₃), 2.43 (3H, s, Ar-CH₃), 5.15 (1H, s, -CH-), 7.39 (2H, d, ArCH), 7.51(2H, d, ArCH), 7.56 (1H, s, -NH-CO-NH-), 7.74 (2H, d, ArCH), 8.20 (2H, d, ArCH), 9.09 (1H, s, -NH-CO-NH-), 10.50 (1H, s, -SO₂NH-); ^{13}C NMR (125 MHz, DMSO- d_6), δ ppm: 18.34 (1C, CH₃), 21.33-22.13 (2C, -CH₃), 51.75 (1C, pyrimidinone), 107.52 (1C, pyrimidinone), 125.14 (2C, ArCH), 128.31-129.33 (6C, ArCH), 137.60 (1C, ArCH), 141.16 (1C, pyrimidinone), 143.06 (1C, ArCH), 146.01(1C, ArCH), 149.43(1C, ArCH), 150.28 (1C, >C=O), 155.64 (1C, >C=N-), MS: calculated for $[M^+]$ m/z:443.13, found: 443.34 $[M^+]$; Elemental Analysis calculated for $C_{20}H_{21}N_5O_5S$: C, 54.17; H, 4.77; N, 15.79; O, 18.04; S, 7.23; Found: C, 54.20; H, 4.83; N, 15.84; O, 18.12, S, 7.29.

N'-(1-(4-(4-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidin-5-yl)ethylidene)-4-methylbenzenesulphonohydrazide (3f)

Color: light yellow powder; % yield: 90%; m.p.: 203-205°C; IR (KBr, cm^{-1}): 3,346.79 (O-H), 3,114.89 (N-H), 3,038.16 (Ar C-H), 2,975.93 (Al C-H), 1,624.69 (C=O), 1,493.93 (C=N), 1,446.76 (Ar C=C), 1,308.16 (S=O), 1,157.04 (S=O), 1,072.51 (N-N); 1H NMR (125 MHz, DMSO- d_6): δ 2.07 (3H, s, -CH₃), 2.26 (3H, s, -CH₃), 2.43 (3H, s, Ar-CH₃), 5.13 (1H, s, -CH-), 6.71 (2H, d, ArCH), 6.99 (2H, d, ArCH), 7.39 (2H, d, ArCH), 7.56 (1H, s, -NH-CO-NH-), 7.74 (2H, d, ArCH), 9.06 (1H, s, OH), 9.09 (1H, s, -NH-CO-NH-), 10.50 (1H, s, -SO₂NH-); ^{13}C NMR (125 MHz, DMSO- d_6), δ ppm: 18.37 (1C, CH₃), 21.32-22.14 (2C, -CH₃), 51.73 (1C, pyrimidinone), 107.51 (1C, pyrimidinone), 115.72 (2C, ArCH), 126.12 (2C, ArCH), 128.37-129.32 (4C, ArCH), 135.94 (1C, ArCH), 137.60 (1C, ArCH), 141.15 (1C, pyrimidinone), 143.06 (1C, ArCH), 150.26 (1C, >C=O), 155.64 (1C, >C=N-), 156.66 (1C, ArCH-OH); MS: calculated for $[M^+]$ m/z:414.14, found: 414.01 $[M^+]$; Elemental Analysis calculated for $C_{20}H_{22}N_4O_4S$: C, 57.96; H, 5.35; N, 13.52; O, 15.44; S, 7.73; Found: C, 57.99; H, 5.42; N, 13.54; O, 15.49, S, 7.81.

N'-(1-(4-(4-methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidin-5-yl)ethylidene)-4-methylbenzenesulphonohydrazide (3g)

Color: light yellow powder; % yield: 89%; m.p.: 198–200°C; IR (KBr, cm⁻¹): 3,348.90 (N-H), 3,294.87 (Ar C-H), 2,923.79 (Al C-H), 1,614.85 (C=O), 1,527.20 (C=N), 1,445.49 (Ar C=C), 1,235.05 (S=O), 1,173.21 (S=O), 1,085.98 (N-N); ¹HNMR (400 MHz, DMSO-d₆): δ 2.07 (3H, m, -CH₃), 2.26 (3H, s, CH₃), 2.43 (2H, m, -CH₃), 5.13 (1H, s, -CH-), 6.70 (2C, Ar-CH), 7.16 (2H, d, Ar-CH), 7.39 (2H, d, ArCH), 7.56 (1H, s, -NH-CO-NH-), 7.74 (2H, d, ArCH), 9.09 (1H, s, -NH-CO-NH-), 10.50 (1H, s, -SO₂NH-); ¹³CNMR (125 MHz, DMSO-d₆), δppm: 12.39 (1C, CH₃), 21.32–22.15 (2C, -CH₃), 45.74 (1C, Pyrimidinone), 55.79 (1C, -OCH₃), 107.51 (1C, pyrimidinone), 114.15 (2C, ArCH), 125.73 (2C, ArCH), 128.37–129.32 (4C, ArCH), 135.94 (1C, ArCH), 137.60 (1C, ArCH), 141.15 (1C, pyrimidinone), 143.06 (1C, ArCH), 150.26 (1C, >C=O), 155.64 (1C, >C=N-), 158.61 (1C, Ar-OH), MS: calculated for [M⁺] m/z: 428.51, found: 428.21 [M⁺]; Elemental Analysis calculated for C₂₁H₂₄N₄O₄S: C, 58.86; H, 5.65; N, 13.08; O, 14.93; S, 7.48; Found: C, 58.91; H, 5.74; N, 13.21; O, 15.06; S, 7.56.

***In vitro* hAChE and BuChE inhibition studies**

The *in vitro* anti-cholinesterase activity of the synthesized compound was assessed by employing Ellman's method (Ellman *et al.*, 1961), targeting both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes. The assay was conducted in UV-visible spectroscopy. The assay solution comprising 1 mL of phosphate buffer (0.1 M, pH 8.0), 50 µL of test compound at six different concentrations (25, 50, 75, 100, 125, 150 nM), and 50 µL of enzyme solution (AChE from *Electrophorus electricus* or BChE from equine serum) was pre-incubated for 15 min at 37°C. Following the pre-incubation period, the reaction was begun by adding 50 µL of DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid), 0.01M), and 20 µL of the substrate (acetylcholine chloride for AChE or butyrylthiocholine iodide for BChE, both at 2U/mL). The enzymatic activity was monitored by measuring the absorbance at 412 nm for 5 min using a UV spectrophotometer. A control without the test compound and a blank without the enzyme were run in parallel. Donepezil was used as the standard AChE inhibitor. The percentage inhibition was calculated using absorbance values, and IC₅₀ values were determined from dose-response curves. All measurements were performed in triplicate, and results were expressed as mean ± standard deviation. This assay assessed the potential of the compound to inhibit AChE and BChE, indicating its

therapeutic relevance for neurodegenerative diseases.

Statistical Analysis

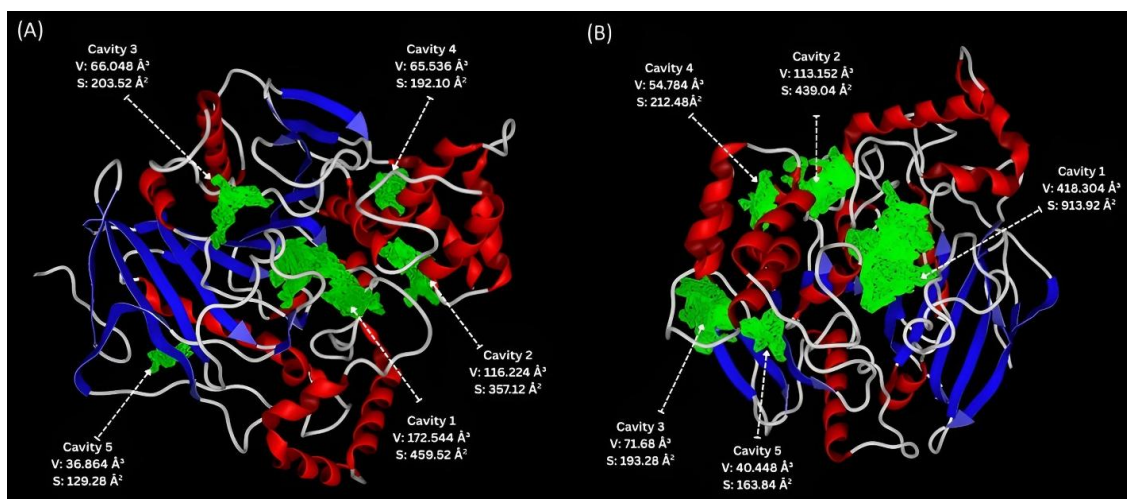
The statistical analysis was conducted using GraphPad Prism version 8.0. Experimental data, obtained in triplicate, are presented as mean ± SEM. One-way ANOVA followed by Dunnett's test and Tukey's post hoc test was used to determine statistical significance, with p-values indicated as *p < 0.05, **p < 0.01, and ***p < 0.001 for *in vitro* acetylcholinesterase, antioxidant activity, and cognitive-enhancing effect, respectively.

Molecular Docking Simulation

Molecular docking is a computational technique in drug discovery that employs algorithms to predict the interactions and binding affinities between ligands and target proteins, thereby aiding in the optimization of molecular structures and the identification of potential ligands. The two-dimensional (2D) structures of the synthesized sulphonyl pyrimidinone (3a-g) and the reference drug Donepezil were obtained using ChemDraw 22.2.0 (64-bit) software. ChemDraw 3D 22.2.0 (64-bit) was utilized to convert the 2D structure of ligands into the 3D structure. The 2D structures were opened in Chem3D, where energy minimization was performed using the MM2 force field. The optimized 3D structures were subsequently saved in .pdb format for further analysis (Jin *et al.*, 2021). The target enzymes were obtained from the RCSB Protein Data Bank (PDB). It can be accessed directly at the following URL (<http://www.rcsb.org/pdb>). The recombinant human acetylcholinesterase in complex with Donepezil (PDB ID: 4EY7, resolution: 2.35 Å, chain A) and human butyrylcholinesterase in complex with N-((1-(2,3-dihydro-1H-inden-2-yl)piperidin-3-yl)methyl)-N-(2-methoxyethyl)-2-naphthamide (PDB ID: 4TPK, resolution: 2.70 Å, chain A) were used for the further computational study (Chhabra *et al.*, 2025). Molecular docking simulations were performed using Molegro Virtual Docker (MVD) version 6.0. The software employs an integrated cavity detection algorithm that automatically identifies potential binding cavities within the 30×30×30 Å³ cube. This cavity identification process instructed the docking algorithm to concentrate on relevant regions of the protein structure during the simulation phase. For each protein structure, five distinct binding sites were identified (as shown in Figure 5). Among these, the site exhibiting the largest volume and surface area was selected as the primary binding site for molecular docking analysis, as detailed in Table 3. Molecular docking simulations were

Table 3. Coordinate values of the Binding site of the target proteins

Protein Target	PDB ID	Volume (Å ³)	Surface Area (Å ²)	X-Center	Y-Center	Z-Center
AChE	4EY7	172.544	459.52	-0.94	-38.30	33.65
BChE	4TPK	418.304	913.92	-2.95	8.79	16.76

**Figure 5. Detected binding cavities in protein targets: (A) 4EY7, and (B) 4TPK (cartoon model of target protein represented in blue and red colors, and cavities represented in green color)**

executed using the MolDock SE search algorithm, with scoring calculated using the MolDock scoring function at a grid resolution of 0.30 Å. A spherical search space with a 15.0 Å radius, centered on specific X, Y, and Z coordinates (Table 1), was defined to enclose the selected binding site. To ensure comprehensive sampling, docking was performed with a population size of 50, over 10 independent runs per ligand, with 1,500 iterations per run executed to cover the entire surface of the identified active binding site of the target protein (Kusumaningrum *et al.*, 2014; Puspaningtyas, 2014).

Conclusion

The successful synthesis of the novel sulphonyl-pyrimidinone derivatives (3a-g) was efficiently achieved using a microwave-assisted method, which outperformed conventional techniques by providing faster reactions, higher yields, minimal waste, and improved overall efficiency, highlighting the advantages of green synthesis via microwave-assisted method over conventional methods. The structure of the synthesized compounds was confirmed through comprehensive spectroscopic

analyses (IR, NMR, Mass, and elemental analysis). Biological evaluation revealed that the nature of the para-substituent on the aromatic ring plays a significant role in cholinesterase inhibition, predominantly the p-methyl substituted compound 3b exhibited the greatest inhibitory potential against both AChE and BChE, outperforming the standard drug Donepezil. Further molecular docking simulations are consistent with these findings by exhibiting that 3b demonstrates strong binding interactions within both the CAS and PAS sites of AChE. The interaction of PAS residues, especially Tyr124, suggests additional potential to modulate AChE-induced β -amyloid aggregation. Overall, the combination of an efficient microwave-assisted synthetic strategy, potent dual cholinergic inhibition, and favorable binding interactions identifies compound 3b as a potentially promising lead molecule. These results support continued exploration of sulphonyl-pyrimidinone derivatives as multifunctional agents for combating Alzheimer's disease and other neurodegenerative disorders. Further studies will focus on investigating their pharmacokinetic and pharmacodynamic properties, and assessing the active compounds in relevant *in vivo* AD model systems and cellular assays to evaluate neuroprotective efficacy, blood-

brain barrier permeability, and toxicity profiles to confirm their efficacy, safety, and mechanistic potential in a physiological context.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Author Contributions

Lokkanya Dewangan: Conceptualization, Methodology, Investigation, Data Collection, Writing – Original Draft.

Alok Singh Thakur: Formal Analysis, Validation, Writing – Review & Editing, Supervision.

Anju Daharia: Writing – Review & Editing, Writing – Original Draft, software.

Ethics Statement

Ethical approval was not required for this study because it did not involve human participants, animals, or biological materials.

Declaration of Generative AI Use

The authors declare that no Generative AI tools were used in the preparation of this manuscript.

Conflict of Interest

All authors confirm that there are no conflicts of interest associated with this manuscript. The authors further declare that no financial or personal relationships could have appeared to influence the work reported in this manuscript.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements

The authors gratefully acknowledge NIT Trichy, IISER Berhampur, and SAIF, Panjab University, Chandigarh, for providing analytical facilities and technical support for this research.

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