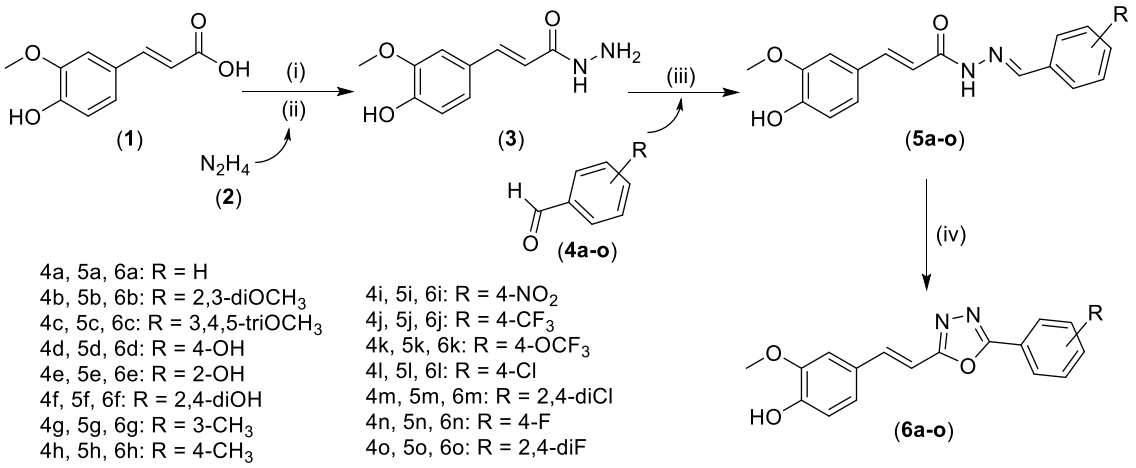
4.1 Synthesis

4.1.1 Chemicals and reagents

All the reagents and chemicals were purchased from commercial suppliers and used without further purification.

4.1.2 Series I: Ferulic acid tethered with benzylidenehydrazine and Series II: Ferulic acid tethered with 2-phenyl-1,3,4-oxadiazole.

The compounds of Series I and II were synthesized as per the reaction sequence outlined in Scheme 1.



Scheme 1. Synthesis of compounds from Series I (**5a–o**) and Series II (**6a–o**). **Reagents and conditions:** (i) Hydroxybenzotriazole, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, acetonitrile, stirring, room temperature, 2 h; (ii) hydrazine hydrate; (2) acetonitrile, stirring, 0–5 °C, 3h; (iii) substituted benzaldehydes (**4a–o**), absolute ethanol, glacial acetic acid, reflux, 8 h; (iv) chloramine T, absolute ethanol, stirring, room temperature, 30 min.

4.1.2.1 General procedure for the synthesis of compound (3)

The ferulic acid (5.1 mmol) was dissolved in solvent acetonitrile (10 ml) and further added with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 6.1 mmol) and hydroxybenzotriazole (HOBT, 6.1 mmol) with continuous stirring at room temperature. The progress of the reaction was monitored by TLC. Immediately after first step, the reaction mixture was added dropwise into the solution of hydrazine hydrate (80% v/v, 10.2 mmol) in acetonitrile (5 ml) under cold condition (0–5 °C) with continuous stirring and completion of reaction was observed by TLC. [Zhang et al. 2002]. The reaction

mixture was extracted with DCM (2 x 10 ml) and washed with water. The organic phase was passed through a saturated solution of NaCl (30 ml) and dried over Na_2SO_4. The organic layer was evaporated under reduced pressure to obtain a crude product, which was purified by column chromatography using DCM/Methanol (90:10) as mobile phase to afford pure compound (**3**).

*4.1.2.2 General procedure for the synthesis of target compounds, Series I (**5a–o**)*

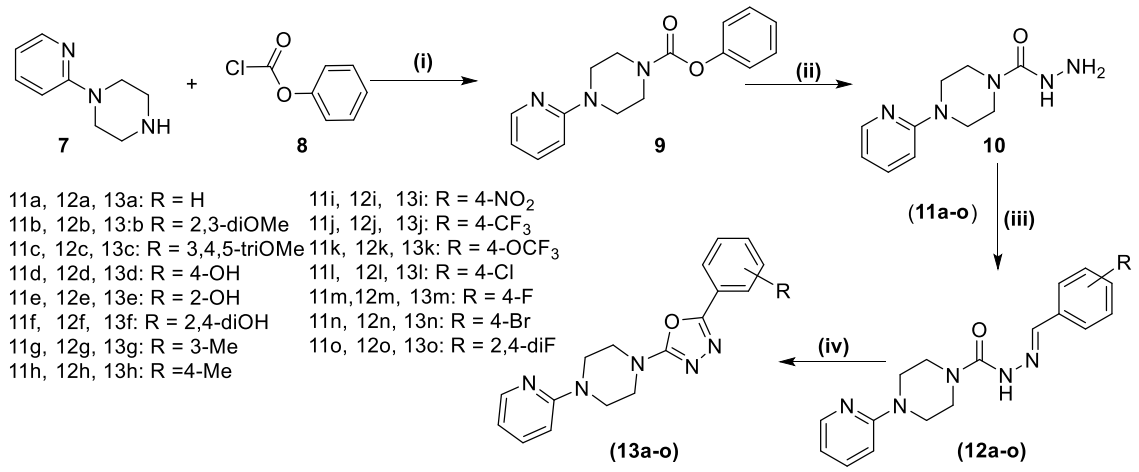
The intermediate **3** (4.8 mmol, 1 equiv) was reacted with respective aromatic aldehydes (**4a–o**, 1.2 equiv) in absolute ethanol (10 mL) with the addition of few drops of glacial acetic acid [Sprung 1940]. The reaction mixture was heated at reflux for 6–8 h. The completion of the reaction was monitored by TLC using DCM:MeOH (90:10 v/v). The reaction mixture was kept overnight and obtained precipitate was filtered, washed, dried, recrystallized in absolute ethanol, and collected as the pure compounds.

*4.1.2.3 General procedure for the synthesis of target compounds, Series II (**6a–o**)*

The mixture of respective imines (**5a-o) (**1 equivalent) and chloramine T (2.2 equivalent) in absolute ethanol was stirred at room temperature for 30 min to obtain a white solid precipitate, which was filtered, and purified by column chromatography using activated silica gel to afford pure oxadiazole hybrids (**6a-o**) [Musad et al. 2011].

4.1.3 Series III: Pyridyl piperazine analogs with substituted N'-benzylideneacetohydrazides (12a–o); and Series IV: Substituted 2-phenyl-1,3,4-oxadiazoles tethered with pyridyl piperazine (13a–o)

The compounds from Series III (**12a–o**) and Series IV (**13a–o**) were synthesized following the sequential reactions mentioned in Scheme 2.



Scheme 2. Synthesis of compounds from Series III (**12a–o**) and Series IV (**13a–o**). **Reagents and conditions**: (**i**) pyridine, DCM, stirring at 0–5 °C to room temperature, 2 h; (**ii**) hydrazine hydrate (80% v/v), absolute ethanol, reflux, 6 h (**iii**) substituted aromatic aldehydes (**11a-o**), absolute ethanol, glacial acetic acid, reflux, 6-8 h (**iv**) chloramine T, absolute ethanol, stirring, room temperature, 30 min.

*4.1.3.1 General procedure for the synthesis of compound (**9**)*

The mixture of 1-(pyridine-2-yl)piperazine **(7,** 6.127 mmol) and pyridine (7.965 mmol) in dry dichloromethane (DCM) was added dropwise with phenyl chloroformate (**8**, 7.352 mmol) at 0-5℃ followed by stirring at room temperature for 2 h [Bunnett and Zahler 1951]. The completion of the reaction was monitored by TLC (DCM:MeOH, 98:02 v/v). The reaction mixture was washed with 5% w/v $NaHCO_3$ solution, brine, and passed over anhydrous Na_2SO_4. The organic layer was concentrated *in vacuo*, and the crude product was further purified by column chromatography using activated silica gel to obtain the pure compound (**9).**

*4.1.3.2 General procedure for the synthesis of synthesis compound (**10**)*

The mixture of intermediate **9** (3.529 mmol) and hydrazine hydrate (80% v/v) was refluxed in absolute ethanol till the completion of the reaction, monitored by TLC (DCM:MeOH, 98:02 v/v) [Alves et al. 2015]. The solvent was evaporated *in vacuo*; the obtained residue was taken in diethyl ether and triturated for 15 min. The solid residue was filtered, dried and collected as the pure compound **10**.

*4.1.3.3 General procedure for the synthesis of target compounds (**12a-o**)*

To the mixture of compound **10** (2.260 mmol, 1 eq) in an absolute ethanol (10 mL), few drops of glacial acetic acid, and respective aromatic aldehydes **11a-o** (1.2 eq) were added, and the reaction mixture was refluxed for 6–8 h. The completion of reactionwasmonitored by TLC using DCM:MeOH (90:10 v/v) as the mobile phase [Sprung 1940]. The reaction mixture was kept overnight, and obtained precipitate was filtered, washed, dried, and collected as the pure compounds (**12a-o**).

*4.1.3.4 General procedure for the synthesis of target compounds **(13a-o)***

The mixture of respective carbohydrazide (**12a-o**, 1 eq) and chloramine T (2.2 eq) in absolute ethanol was stirred at room temperature for 30 min to obtain the white solid precipitate, which was filtered, and purified by column chromatography using activated silica gel to obtain the pure target compounds (**13a-o**) [Musad et al. 2011].

4.2 Characterization of the synthesized compounds

4.2.1 Melting point

The capillary melting point determination is an important parameter to quantify the unpredictable properties of organic compounds along with the purity. Hence, different compounds tend to have different melting points. The melting points were determined on a Stuart Melting Point apparatus (SMP10, Barloworld Scientific Ltd., UK) using capillary tubes and reported as uncorrected.

4.2.2 TLC (R_f value)

The TLC is a commonly used technique in synthetic chemistry to determine the purity of the compounds and qualitatively monitor the progress of a reaction. It also allows the optimization of a solvent system for a particular separation task. The R_f value is a distinctive property of a given compound in a particular solvent system and calculated as follows. Thin-layer chromatography was done on using precoated Merck silica gel

60F254 aluminum sheets (Merck, Germany). The visualization of TLC plates was accomplished using the UV light, iodine vapors, or Dragendorff reagent.

The Rf value was calculated using the following equation: $\mathbf{R_f} = \frac{\textbf{Distance travelled by solute}}{\textbf{Distance travelled by solvent}}$

4.2.3 Log P value determination

The partition coefficient quantifies the partitioning of a neutral (unionized) substance between water and n-octanol. The logarithm of the concentration of the unionized solute in the solvents is called Log P. The Log P value is also known as a measure of lipophilicity. It is an important parameter used in the assessment of environmental fate and transport of organic chemicals because the n-octanol phase is a surrogate for the lipid phase or organic carbon content of the environmental compartments. Partition coefficient (log P) was calculated by the shake flask method using the equation:

$\log P = C_o/C_w$

C_o = Concentration in octanol phases

C_w = Concentration in water phases

4.2.4 FT-IR

FT-IR spectra were recorded on Alpha ECO-ATR Spectrophotometer (Bruker, USA), and were reported as % Transmittance *vs.* Wavenumber (cm^{-1}) at the scanning range of 4000-667 cm^{-1}.

4.2.5 ^{1}H NMR and ^{13}C NMR

NMR spectra (^{1}H NMR: 500 MHz and ^{13}C NMR: 125 MHz) were recorded on Bruker Avance FT-NMR spectrophotometer (Bruker, USA) and chemical shift values (δ) were reported as ppm referenced to tetramethylsilane (TMS). DMSO-d_6 or $CDCl_3$ were used as solvents, and spectra were interpreted using MestReNova 6.0.2. The splitting patterns

were depicted as singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), triplet of doublets (td), doublet of doublets of doublets (ddd), multiplet (m).

4.2.6 Mass spectra

The mass spectra were recorded on LC-Q-TOF mass spectrophotometer with an ESI source (Agilent Infinity II 1290 LC).

4.2.7 Determination of percentage purity by HPLC

The % purity of synthesized compounds was determined by Infinity II 1260 High-Performance Liquid Chromatography (Agilent, USA) with the Eclipse Plus C8 column using Methanol/Water (90:10 v/v) as mobile phase at the flow rate of 1 mL/min.

4.3 Biological Evaluation

4.3.1 In-vitro studies

4.3.1.1 Cholinesterase (AChE and BChE) inhibition by Ellman assay

The assessment of inhibition potential of synthesized compounds on the cholinesterase enzyme was evaluated spectrometrically using the Ellman method with some slight modification. The mechanism behind the cholinesterase activity for quantifying the concentration of 5-thio-2-nitrobenzoic acid (TNB) ion which is formed in the reduction of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) through thiocholine to yield a yellow color product that can be detected colorimetrically at 412 nm. The stock solution of hAChE (EC No. 3.1.1.7, from human erythrocytes) was dissolved in 20 mM HEPES buffer (pH 8) with Triton X-100 (0.1% v/v). The hBChE (EC No. 3.1.1.8, from human serum) stock solution was prepared in aqueous gelatin solution (0.1% w/v). The five increasing concentrations of inhibitors producing inhibition in the range of at least 20–80% were prepared in DMSO (final concentration ≤1% v/v). Briefly, the mixture of 25 μL hAChE or hBChE (0.25 U/mL) and 10 μL of test compound was preincubated (10 min), followed by addition of 340 μM 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), and

550 μM of respective substrate (ATCI for hAChE; and BTCI for hBChE). The blank readings were taken with all the components except enzyme to account for the nonenzymatic hydrolysis of substrates. The increased absorbance was monitored at λ = 412 nm at 37 °C for 6 min on a Multimode Microplate Reader (BioTek Synergy H1M, USA). The reaction rates were compared with or without inhibitors, and the percentage inhibition was calculated by the following expression: $[(V_o - V_i)/V_o] \times 100$. The V_i and V_o are the reaction rates with or without inhibitor, respectively. The IC_{50} value for each test compound was determined using nonlinear variable slopes of log (inhibitor) vs normalized response (Graph Pad Prism 5.01). The assay was conducted for three separate experiments in triplicate [Ellman et al. 1961, Peauger et al. 2017b].

4.3.1.2 Enzyme kinetics study

An enzyme kinetics study determined the nature of the enzyme (hAChE) inhibition. The kinetic parameters K_m and V_{max} were calculated by determining the enzymatic activity at six different concentrations (50–500 μM) of the substrate (acetylthiocholine iodide, ATCI for hAChE and butyrylthiocholine iodide, BTCI for hBChE). Test compounds were used in three different concentration ranges. Each concentration of inhibitor was evaluated with six different concentrations of substrate. The values of V_{max} and K_m were calculated using Michaelis-Menten nonlinear regression kinetics, and the mechanism of enzyme inhibition was determined by Lineweaver-Burk plots [Lineweaver and Burk 1934] using Graph Pad Prism 5.01. Further, the K_i value was determined by Dixon plot as a function of four different concentrations of the inhibitor [Dixon 1972]. The enzyme kinetic assay was performed in three separate experiments.

4.3.1.3 BACE-1 inhibition assay

The BACE-1 inhibitory potential of test compounds was evaluated using the FRET-based BACE-1 activity detection kit (Sigma Aldrich, Catalog No. CS0010). The assay

involved the enhancement of fluorescent signal due to cleavage of the substrate by BACE-1. The kit contains a fluorescent assay buffer, stop solution, assay standard, BACE-1 substrate (7-Methoxycoumarin-4-acetyl-[Asn^{670}, Leu^{671}]- Amyloid β/A4 precursor protein 770 fragment 667-676-(2,4-dinitrophenyl) Lys-Arg-Arg amide trifluoroacetate salt), and BACE-1 enzyme. The inhibitor was used in five different concentrations, to achieve target enzyme inhibition in the range of 20-80%. The fluorescence intensity was monitored immediately after the addition of the BACE-1 enzyme with the wavelength of excitation, and emission was set at 320 nm and 405 nm, respectively. Plates were covered and incubated for 2 h at 37 ºC, and fluorescence intensity was again recorded using Multimode Microplate Reader (BioTek Synergy H1M, USA). All the measurements were performed in triplicate. The percentage inhibition was calculated using the following expression: $[(IF_o–IF_i)/IF_o] \times 100$, where, IF_i and IF_o are the fluorescence intensities obtained in the presence and absence of inhibitor, respectively and the IC_{50} values were calculated using linear regression graph (GraphPad Prism 5.1, GraphPad Software Inc.) [Gurjar et al. 2014, Lisa Prostak et al.].

4.3.1.4 Propidium iodide (PI) displacement assay

Propidium iodide displacement assay is useful for determining the competitive binding capability of test compounds to the PAS of AChE. The assay mixture of hAChE (5U) was incubated with or without test compounds (final concentrations 10 µM and 50 µM, 150 µL) for 6 h at 25 °C. Post incubation, PI (final concentration 1 µM, 50 µL) was added to make the final assay volume of 200 µL. After 10 min, fluorescence intensity was measured at excitation and emission wavelength of λ_{ex} = 535 nm and λ_{em} = 595 nm, respectively, using fluorescence plate reader (BioTek Synergy H1M). The percentage inhibition was calculated by the following expression: $100 – (IF_i/IF_0 \times 100)$, where IF_i

and IF_0 are the fluorescence intensities with and without inhibitor, respectively. Each assay was performed in triplicates, as three separate experiments [Peauger et al. 2017a].

4.3.1.5 Parallel artificial membrane permeation assay (PAMPA-BBB)

The brain permeability was assessed *in-vitro* by PAMPA-BBB assay [Di et al. 2003]. The porcine brain lipid (PBL, 4 µL) (Avanti Polar Lipids, India) with dodecane (20 mg/mL PBL in dodecane) was coated on the bottom porous filter disks of the acceptor microplates. Test compounds were dissolved initially in DMSO, followed by the addition of PBS pH 7.4 to obtain a final dilution of 25 µg/mL. The acceptor and donor plates (Merck Millipore, membrane pore size 0.45 µm) were filled with 200 µL of the final test solution and 200 µL of PBS pH 7.4, respectively. The acceptor plate was further sandwiched over donor plate and incubated for 18 h to allow the diffusion of the test compound from the donor to the acceptor well *via* lipid membrane. Post incubation, the drug concentrations in both the plates were determined spectrophotometrically. The experiment was performed in triplicate. The model was validated using nine commercial drug samples with known permeability, and linear correlation was established using the values of reference [$P_{e(ref)}$] and experimental [$P_{e(exp)}$] permeability [Seth et al. 2018].

4.3.1.6 Aβ aggregation (self- and AChE-induced) inhibition by thioflavin T assay

The Aβ oligomers and aggregates formed with or without AChE leading to neuronal cell death. Thioflavin T assay was performed to investigate the Aβ aggregation inhibition potential of the compounds. $Aβ_{1-42}$ (Sigma, India) was dissolved in 1% v/v ammonium hydroxide solution to get the 2000 µM stock solution and was stored at −80 °C. The test compounds were initially dissolved in DMSO, and final dilutions were made in PBS pH 7.4. Different proportions of $Aβ_{1-42}$: inhibitors were tested (10:5, 10:10; and 10:20 µM) by thioflavin T assay [Bolognesi et al. 2007, Kumar et al. 2018, Zha et al. 2015]. All the experiments were performed in triplicate.

For the $A\beta_{1-42}$ self-induced aggregation inhibition experiment, the mixture of $A\beta_{1-42}$ (final dilution 10 µM, 10 µL) with 50 mM PBS pH 7.4 and incubated at 37 °C for 48 h with or without test compound (5 µM, 10 µM, and 20 µM; 10 µL). Blank assays were performed by using PBS pH 7.4 instead of $A\beta_{1-42}$ with or without inhibitor. The fluorescence intensities were measured with the addition of 50 mM glycine-NaOH buffer (pH 8.0) containing 5 µM thioflavin T at the excitation and emission wavelength of λ_{ex} = 450 nm and λ_{em} = 485 nm, respectively. The percentage inhibition of self-induced aggregation was calculated using the following expression: $100 - (IF_i/IF_0 \times 100)$, where IF_i and IF_0 are the fluorescence intensities with and without inhibitor, respectively after subtracting the values with the blank. The results were also reported as normalized fluorescence intensity with respect to control.

For AChE-induced $A\beta_{1-42}$ aggregation inhibition experiment, the mixture of $A\beta_{1-42}$ (final concentration 10 µM, 2 µL) and AChE from human erythrocytes (final concentration 230 µM, 16 µL) were incubated with or without test compounds (5 µM, 10 µM, and 20 µM; 2 µL) at 37 °C for 48 h. The assay solution was diluted to a final volume of 200 µL with the addition of 50 mM glycine-NaOH buffer (pH 8.0) containing 5 µM thioflavin T. The detection method and calculation were performed similarly to the self-induced aggregation experiment.

4.3.1.7 AFM study

AFM analysis is a simple and convenient method to directly observe the morphological details of Aβ aggregation inhibition. The study was performed using the NT-MDT Ntegra Prima (Russia). Briefly, $A\beta_{1-42}$ (final concentration 10 µM, 20 µL) was incubated in 0.1 M PBS pH 7.4 with or without test compound (final concentration 10 µM, 20 µL) at 37 °C for 7–10 days. 5 µL of the mixture was placed freshly cleaved mica surface using spin coater (302 ×g, 60 s) and dried to remove the excess solvent

(1207 ×g, 30 s). AFM measurements were performed on samples at different time points in ambient condition by keeping the probe spring constant between 3.5 N/m and high resolution acoustically driven cantilevers operating at the resonance frequency of 140 kHz in tapping mode with the scanning rate of 0.5 Hz. The images were visualized at 5 × 5 µm using the Nova Px image analysis software (NT-MDT, Russia) [Harte et al. 2015, Ryu et al. 2008].

4.3.1.8 Neuroprotective MTT assay against SH-SY5Y neuroblastoma cell lines

Neuroprotection was assessed by MTT assay on neuroblastoma SHSY-5Y cell lines. Briefly, the cell lines (density 1×10^5 cells/wells) were seeded in 100 µL of medium followed by incubation in 5% CO_2 environment at 37 °C for 24 h. The test compounds in the different concentration range (10, 20, 40, and 80 µM) were added, and cells were incubated for 48 h. Post incubation, 20 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added, and the cells were incubated for an additional 2 h. The obtained purple-colored formazan was solubilized in 100 µL DMSO. The absorbance was measured at $\lambda = 570$ nm, and % cell viability was calculated [Xu et al. 2018].

4.3.2 In-vivo behavioural studies

4.3.2.1 Animals

The Wistar rats weighing 200-250 g of either sex were procured from the Institute of Medical Sciences, Banaras Hindu University, Varanasi. Animals were maintained in an environmentally controlled temperature (25 ± 2 °C) and humidity (65 ± 5 %RH) with 12 h light/dark cycles. The animals had free access to commercial feed and water *ad libitum*. The investigation protocol for behavioral studies was duly approved by the Institutional Animal Ethical Committee (No. Dean/2018/CAEC/814).

4.3.2.2 Acute oral toxicity study

OECD guidelines were used for testing of compounds to determine acute oral toxicity. The animals were randomly assigned different groups with six animals in each group. The test compounds were suspended in 0.3% w/v Na-CMC and administered orally at the graded doses (677, 1333, 2000 mg/kg) to the corresponding group of animals [Wang et al. 2015]. After dosing, the animals were monitored continuously for the first 6 h, intermittently for next 24 h, and daily up to 14 days for abnormal or toxic reactions such as tremors, convulsions, salivation, diarrhea, sleep, lacrimation, and feeding behavior [Lu et al. 2013].

4.3.2.3 Scopolamine-induced amnesia model: Y-maze test

The scopolamine-induced amnesia model was performed to evaluate the cognition-enhancing activity of test compounds on male Wistar rats. Scopolamine hydrobromide (Sigma Aldrich, India) was dissolved in sterilized normal saline. Test compounds and standard donepezil were suspended in 0.3% w/v Na-CMC solution. The rats were divided into following groups of six animals each: control, scopolamine, donepezil (5 mg/kg, p.o.), Test compounds (2.5, 5, and 10 mg/kg, p.o.). Donepezil and test compounds were orally administered once daily for seven consecutive days to the respective group of animals. Scopolamine-group of rats was administered vehicle (0.3% w/v Na-CMC) only.

On the seventh day of trial 30 min after the treatment, scopolamine hydrobromide (0.5 mg/kg) was injected intraperitoneally to all the animals except the control group. After 5 min of scopolamine injection, immediate spatial working memory was evaluated using three arms Y-maze apparatus. The arms were thoroughly cleaned, wiped with 70% v/v ethanol, and labeled as A, B, and C arms. The rats were placed individually in the center of the maze. The first entry was omitted from the calculation as initially rats were

always entered into the arm of facing. The spontaneous alternation behavior, total arm entries, and the number of entries to each arm were recorded for total of 8 min. The maze was cleaned and wiped between each session. An arm entry was considered when a rat had crossed all four paws into the arm. Increased spontaneous alternations, i.e., three consecutive arm entries were considered as an indicator of improvement in learning and memory. Percentage spontaneous alternations were calculated following the expression: [number of spontaneous alternations/(total arm entries-2)] × 100 [Shidore et al. 2016, Wolf et al. 2016].

4.3.2.4 Ex-vivo studies: AChE estimation and antioxidant activity

After completion of the Y-maze test, rats were sacrificed through cervical dislocation. animals of respective groups were sacrificed, and their hippocampal region was isolated from the brain carefully. The hippocampus was homogenized in ice-cold PBS (0.1 M, pH 7.4), and centrifuged at 4 °C (10062 ×g, 20 min). The collected supernatant was analyzed for biochemical estimations. Initially, the actual protein concentration of each of the collected supernatant was determined using Folin and Ciocalteu's (F&C) reagent as per the Lowry's method [Lowry et al. 1951] or standard Bradford assay [Bradford 1976].

The AChE level in each of the supernatant was determined by modified Ellman's colorimetric assay. The assay mixture consisted of supernatant (25 µL), PBS pH 7.4 (10 mM, 150 µL), and DTNB (1 mM, 100 µL) was preincubated (37 °C, 10 min). The reaction proceeded with the addition of ATCI (7.5 mM, 20 µL), and reaction rates were determined at wavelength λ = 412 nm at 37 °C for 6 min. The rate of ATCI hydrolysis was estimated as µM of substrate hydrolyzed/min/mg of protein [Ellman et al. 1961, Shrivastava et al. 2019].

To estimate the antioxidant potential of the compound, biochemical analyses of oxidative stress biomarkers, i.e., MDA and SOD, were performed. The level of MDA was assessed using the TBARS assay, also termed as lipid peroxidation assay. The assay is based upon the spectrophotometric detection of a red-colored complex of TBARS, formed due to the reaction of MDA and thiobarbituric acid under acidic medium. Briefly, a mixture of each supernatant sample (200 µL) and trichloroacetic acid (10% v/v in 0.1 M HCl, 1 mL) was centrifuged at 4 °C (10 min, 101 ×g). The supernatants were collected and mixed with an equal volume of thiobarbituric acid (0.67%) and warmed in a water bath for 10 min. After cooling, the absorbance of the assay mixture was spectrophotometrically estimated at λ = 532 nm. The results were calculated as a number of moles of MDA/mg protein [Ohkawa et al. 1979].

The SOD is a key enzyme responsible for the dismutation of free radicals. The assay was performed based on spectrophotometric detection of blue formazan product formed by autoxidation of hydroxylamine hydrochloride (pH 10.2) in the presence of EDTA. The assay mixture consisted of EDTA (100 µM), nitro blue tetrazolium (24 µM), and Na_2CO_3 (50 mM). The above mixture (200 µL) was mixed with 50 µL of supernatant and hydroxylamine hydrochloride. The absorbance was measured at λ = 560 nm, and the results were calculated as SOD units (U/mL)/min/mg protein [Kono 1978].

4.3.2.5 Aβ-induced AD phenotypic model: Morris water maze test

In the current study, the cognition-enhancing potential of the compound was assessed by $A\beta_{1-42}$ ICV injection through stereotaxic surgery, which produces the AD like phenotypic condition [Bagheri et al. 2011]. $A\beta_{1-42}$ (4 µM) solution was dissolved in sterilized saline (0.9% NaCl) and incubated (37 °C, 7 days) to form neurotoxic aggregates. Test compounds (10 mg/kg, p.o.) and donepezil hydrochloride (5 mg/kg, p.o.) were suspended in a vehicle (0.3% Na-CMC). The rats were divided into different

groups of six or ten animals each. Group 1: sham (saline); Group 2: model ($A\beta_{1-42}$ + vehicle); Group 3: donepezil + $A\beta_{1-42}$; Group 4: Test compounds + $A\beta_{1-42}$.

Stereotaxic surgery of rats was performed under anesthesia by a combination of intraperitoneal administration of ketamine (90 mg/kg) and xylazine (9 mg/kg). All the rats were individually placed on a stereotaxic instrument with the symmetric mounting of incision bars (–3.3 mm). The scalp was cleaned and wiped with sterilized saline. The burr hole was drilled through the skull, and stereotaxic coordinates were set relative to bregma (–0.5 mm AP, +1.2 mm ML, and –3.2 mm DV) [Colaianna et al. 2010]. The $A\beta_{1-42}$ (4 µM, 5 µL) was injected to all the groups of rats except sham group through Hamilton microsyringe (infusion rate: 2 µL/min). The injection lasted for about 2.5 min, and the syringe was left in place for an additional 5 min to prevent further reflux. The same procedure was performed for the sham group of rats, except they were injected with sterilized saline instead of Aβ. Post-operatively, rats were kept in individual cages, and iodine ointment was applied at the surgical site. The rats were given special care until their normal feeding restored. After 7 days of recovery, donepezil and test compounds were administered to the corresponding group of animals for 9 successive days (from 8^{th} to 16^{th} day), while a model group of animals was given vehicle (0.3 % Na-CMC) only.

The improvement in learning and memory was assessed through the Morris water maze test [Morris 1984] during the last five days of treatment. The apparatus consisted of a circular pool (diameter: 121 cm; height: 62 cm; and depth: 32 cm) filled with opaque water (25 ± 2 °C). The pool was designated in four equally sized imaginary quadrants, which were maintained in the same position throughout the test. The task was based on incremental learning and memory by rats to locate the hidden platform submerged 2 cm under the water surface. Each day of the test, two trials were performed to record time

to reach the hidden platform (escape latency time, ELT), and a total number of platform crossovers by rats, respectively. Each trial was conducted for 90 s with an intertrial difference of at least 3 h, and all the results were monitored in a soundproof room with the help of a tracking camera [Wang et al. 2015].

4.3.3 Pharmacokinetic studies

Pharmacokinetic parameters of test compounds were estimated in healthy male Wistar rats (200-250 g, n = 3). The rats were fasted for 12-16 h and had free access to water. Test compound suspended in 0.3% w/v Na-CMC was administered to animals at a dose of 10 mg/kg, p.o. The animals were anesthetized, and blood samples were collected from retro-orbital plexus at several time points (0, 0.5, 1, 2, 4, 8, 16, 24, 36, 48, 60, and 72 h). All the blood samples were pretreated with sodium heparin to prevent coagulation. Blood samples were centrifuged (6797 ×g, 6 min, 4 °C) to collect the plasma, 100 µL of which was mixed with 200 µL of MeOH to extract the test compound**.** The mixture was further mixed, centrifuged (6797 ×g, 6 min, 4 °C), the organic layer was separated, and evaporated *in vacuo*. The collected residue was dissolved in the mobile phase, and 5 µL of the sample was injected into Infinity II 1260 High-Performance Liquid Chromatography (Agilent, USA) using MeOH: H_2O (85:15) as mobile phase at a flow rate of 1 mL/min. The Eclipse Plus C8 column (Pore size: 95 Å; Particle size: 3.5 µ; Inner diameter: 4.6 mm, Length: 30 mm) was used, and retention time was monitored for 10 min using photodiode array detector at λ = 270 nm. The plasma drug concentration of the test compound was calculated using the standard calibration curve by the extravascular noncompartmental model.

4.4 Computational Studies

4.4.1 In-silico molecular docking study

The 'Glide' module of Schrödinger Maestro 2018.2 was used to perform the molecular docking studies of lead compounds on X-ray crystal structures of hAChE (4EY7) and hBACE-1 (2ZJM). Initially, protein structures were pre-processed, refined, and corrected using 'Protein Preparation Wizard' module. The protein structure was optimized by Propka method (at pH 7), and restrained minimization was performed for heavy atom RMSD to 0.30 Å. The grid box (10 × 10 × 10 Å) was generated surrounding the active site of co-crystallized ligand. To validate the docking protocols and prepared grid, the co-crystallized ligands were extracted and re-docked into the corresponding protein grid structures of AChE or BACE-1. The ligands were prepared by 'LigPrep' module, and docking studies were performed using the 'Glide XP' module. The 'Glide XP visualizer' tool was used to observe the docking score and gain insights into the interactions of ligands with active site residues.

4.4.2 Molecular dynamics simulation study

The stability of docked ligand-hAChE/hBACE-1 complexes was affirmed by molecular dynamics simulation runs using Desmond (D.E. Shaw Research). The systems of docked complexes were built by creating virtual TIP3P water environment, and these systems were neutralized by the addition of counterions. These systems were minimized at a convergence threshold of 1.0 kcal/mol/Å with a maximum of 2000 iterations. A molecular dynamics simulation run for each docked complexes was performed for the total simulation time of 40 ns by keeping the trajectory with recording interval energy of 40 ps and energy of 9.6 to obtain the approximate number of 1000 frames.