3. MATERIALS AND METHODS

3.1. *In vitro* AChE and BuChE inhibition assays

The assays were performed according to the method described by Ellman *et al* [48]. AChE from human erythrocytes and BuChE from equine serum, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB-Ellman's reagent), acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI) were purchased from Sigma. Tacrine and donepezil were used as reference compounds (Sigma). All the experiments were conducted in 50 mM Tris-HCl buffer at *p*H 8. Five different concentrations (0.001–100 µM) of each compound were used to determine the enzyme inhibition activity. Briefly, 50 µl of AChE (0.22 U/ml) or 50 µl of BuChE (0.06 U/ml) and 10 µl of the test or standard compound were incubated in 96-well plates at room temperature for 30 min. Further, 30 µl of the substrate viz. ATCI (15 mM) or BTCI (15 mM) was added and incubated for additional 30 min. Finally, 160 µl of DTNB (1.5 mM) was added and the absorbance was measured at 415 nm wavelength using microplate reader 680 XR (BIO-RAD, India). The IC_{50} values were calculated from the absorbance changes. IC_{50} value depicts the concentration of the drug resulting in 50 % inhibition of the enzyme activity. All the determinations were performed in triplicate and at least in three independent runs.

3.2. Thioflavin T (ThT) assay for determining $A\beta_{1-42}$ aggregation inhibitory effect of the test compounds

Inhibition of AChE-induced $A\beta_{1-42}$ aggregation was evaluated using the thioflavin T (ThT) fluorescence assay as described earlier [49, 50]. $A\beta_{1-42}$ (Sigma) was dissolved in phosphate buffer saline (PBS) and diluted with 0.215 M sodium phosphate buffer (*p*H 8). Test compounds were dissolved in DMSO and were further diluted with 0.215 M sodium phosphate buffer (*p*H 8). Briefly, 2 µl of $A\beta_{1-42}$ was incubated with 16 µl of AChE in the presence of 2 µl of the test compound to obtain final concentration of 50 µM $A\beta_{1-42}$, 230 µM AChE and 10 µM test compound. The mixture was co-incubated at room temperature for 24 hr. After incubation, 180 µl of 20 µM ThT (prepared in 50 mM glycine-NaOH buffer; *p*H 8.5) was added. The fluorescence intensity was read at 442 nm excitation and 490 nm emission wavelengths using Synergy HTX fluorescence microplate reader. The percentage inhibition of the AChE-induced $A\beta_{1-42}$ aggregation was calculated using the following formula: $100-(IF_i/IF_o\times100)$, where IF_i and

IF_o are fluorescence intensities in the presence and absence of the test compound respectively. Each assay was conducted in triplicate and at least three times independently.

3.3. Congo red (CR) binding assay for determining $A\beta_{1-42}$ aggregation inhibitory effect of the test compounds

$A\beta_{1-42}$ aggregation inhibitory potential of the test compounds was assessed using Congo red (CR) binding assay. CR (Hi-Media) solution prepared in PBS (*p*H 7.4) was diluted to get a final concentration of 5 µM. $A\beta_{1-42}$ prepared in PBS was diluted to get a final concentration of 20 µM. Briefly, 20 µM of $A\beta_{1-42}$ was incubated with or without the test compound (10 µM) for 6 hr at 37 ºC. Later on, the mixture was incubated with 5 µM of CR solution for 30 min at room temperature. Following incubation, CR spectra were measured using a UV-spectrophotometer (UV-1700, Shimadzu) at 480 nm and 540 nm wavelengths. CR binding was calculated using the following formula: CB (M) = (OD at 540 nm/25,295) - (OD at 480 nm/46,306); where, CB (M) is the amount of CR bound to β sheets of $A\beta_{1-42}$ and OD is optical density [51, 52].

3.4. Determination of cell viability and neuroprotection against H_2O_2 insult using SH-SY5Y cells

The SH-SY5Y cells (National Centre for Cell Science, Pune) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with (10 % v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37 ºC and 5 % CO_2. The cells, cultured in 75 cm^2 flasks, were seeded in 96 well plates and incubated for 24 hr.

To determine the cytotoxicity of the selected test compounds, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. SH-SY5Y cells were seeded in 96-well plate at a density of 5×10^4 cells per well. After 24 hr, the medium was replaced with two different concentrations of the test compounds (40 µM and 80 µM) for another 24 hr at 37°C. After incubation period, the cell viability was determined using MTT assay. In another set of experiment, the test compounds were assessed for their ability to protect SH-SY5Y cells against oxidative damage induced by H_2O_2 [53, 54]. The cells were exposed to the test compounds at relatively lower concentrations (5 µM, 10 µM and 20 µM) and incubated for 2 hr. After the incubation period, the test compounds were replaced with a media containing the cytotoxic insult, i.e. H_2O_2 (100 µM) [53] which was left for an additional 24 hr period.

Thereafter, cell viability was assessed using MTT assay. Briefly, the medium was replaced with 80 µl of fresh medium and 20 µl of MTT (0.5 mg/ml, final concentration; Sigma) in PBS. After 4 hr, MTT was removed and crystals of the formazan were dissolved in DMSO. Formazan concentrations were quantified at 570 nm with 630 nm reference wavelengths using a microplate reader 680 XR (BIO-RAD, India). Percentage protection against H_2O_2 insult was calculated by considering the absorbance of control cells as 100 % of the cell viability.

3.5. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) assay to determine free radical scavenging ability of the test compounds

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay is based on the reduction of DPPH, a purple colored stable free radical. DPPH gets paired off and reduced to yellow colored diphenylpicrylhydrazine by antioxidants. Thus the assay measures an electron (or hydrogen atom) donating activity and hence provides assessment of antioxidant activity of a compound which may be attributed to its free radical scavenging ability [55, 56]. The spectrophotometric DPPH assay was carried out according to the earlier described reports [57]. Concentrations at which the selected test derivatives showed promising neuroprotective effects against H_2O_2 insult were selected for the DPPH assay. In brief, 10 µl of a test compound (10 and 20 µM, in Tris-HCl buffer-*p*H 7.4) was mixed with 20 µl of DPPH (from 10 mM stock, in methanol) (Hi-Media) in a 96 well plate. Finally, the volume was adjusted to 200 µl using methanol. After 30 sec incubation at room temperature and protection from light, the absorbance was read at 520 nm wavelength using a microplate reader 680 XR (BIO-RAD, India). The free radical scavenging activity was determined as the reduction percentage (RP) of DPPH using the equation: $RP= 100[(A_0 - A_C)/A_o]$, where A_0 is the untreated DPPH absorbance and A_C is the absorbance value for added sample concentration C. Ascorbic acid was used as a standard antioxidant.

3.6. *In vitro* blood-brain barrier permeation assay

Before proceeding for *in vivo* evaluation of neuroprotective potential of the test compounds, it was decided to assess their permeability through blood-brain barrier (BBB). To estimate the possible *in vivo* BBB penetration of the selected test compounds, a parallel artificial membrane permeation assay for blood-brain barrier (PAMPA-BBB) was performed as described by Di. *et al* [58, 59]. The donor microplate (polyvinylidene fluoride PVDF membrane, diameter

25 mm, pore size 0.45 µm) (Millipore) and acceptor microplate were used for the study. The acceptor microplate was filled with 200 µl phosphate buffer saline (PBS)-ethanol (70:30) and the filter surface of the donor microplate was impregnated with 4 µl of porcine brain lipid (Avanti Polar Lipids) in dodecane (Sigma) (20 mg/ml). The test compounds were dissolved in DMSO at 5 mg/ml and diluted in PBS-ethanol (70:30) to get a final concentration of 100 µg/ml. 200 µl of this solution was added to the donor well [60]. The donor plate was carefully placed on the acceptor plate to form a sandwich, which was left undisturbed for 120 min at 25°C. After the incubation period, the donor plate was carefully removed and concentrations of the compounds in the acceptor wells were determine using UV spectrophotometry. Each sample was analysed at five different wavelengths, in four wells, and at least in three independent runs. Assay validation was performed by using nine commercial quality standards of known BBB permeability [58].

3.7. Animals

Male Swiss Albino mice (25-30 gm) and male Wistar rats (200-250 gm) were selected for the studies with free access of food and water *ad libitum*. Animals were maintained on 12 hr light/dark cycle. After 15 days acclimatization, animals were used for the studies. All the protocols were approved by Institutional Animal Ethics Committee (IAEC) and experiments were performed as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines with approval no: MSU/IAEC/2014-15/1401.

3.8. Effect of the test compounds on AD-like phenotype using scopolamine-induced mice model

Scopolamine is commonly used to induce amnesia. Scopolamine rodent model is widely utilized to reproduce AD-like phenotype [61]. Male Swiss Albino mice were divided into different experimental groups of six animals each. Scopolamine (1.4 mg/kg, i,p.) (Sigma) was administered to all the groups except vehicle-treated control group [0.1 % sodium carboxy methyl cellulose (Na-CMC)] after the treatment of standard [donepezil (5 mg/kg, p.o.)] or the test (5 or 10 mg/kg, p.o.) compounds. All the drug solutions were prepared in 0.1 % Na-CMC. Scopolamine was administered 30 min after administration of each compound. All the treatments were continued for nine consecutive days. Doses of the test compounds were decided after dose deciding pilot study (see supplementry information).

3.8.1. Morris water maze test to assess spatial learning ability

The spatial learning ability was assessed using Morris water maze (MWM) test. The test was performed during last five days of the treatment period. Time required to reach the hidden platform (i.e. escape latency time-ELT) and number of crossings over the platform area were recorded during 2 min of training session to determine spatial learning ability of mice [62].

3.8.2. Effect of test compounds on neurochemical levels

After MWM test, animals were sacrificed, whole brains were isolated from the skull and homogenized in glass Teflon homogenizer in 12.5 mM sodium phosphate buffer, *p*H 7. The homogenates were centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was utilized to determine AChE and BuChE activities using Ellman's method [48]. 100 µl of the supernatant was incubated with 2.7 ml of phosphate buffer and 100 µl of freshly prepared ATCI or BTCI (15 mM) for 5 min. Finally, 100 µl of DTNB (1.5 mM) was added and the absorbance was read at 415 nm wavelength spectrophotometrically.

Malondialdehyde (MDA), an indicator of lipid peroxidation, was evaluated using thiobarbituric acid reacting substance (TBARS) method similar to the earlier described reports.[63, 64] MDA reacted with thiobarbituric acid in acidic medium at high temperature and formed a red complex of TBARS which was read spectrophotometrically. Briefly, 200 µl of the supernatant was mixed with 1 ml of 50 % trichloroacetic acid in 0.1 M HCl and 1 ml of 26 mM thiobarbituric acid. After vortex mixing, samples were heated at 95 °C for 20 min. Later on the samples were centrifuged at 15,000 rpm for 10 min and the supernatants were read at 532 nm wavelength.

Catalase (CAT) is an enzyme mediating breakdown of toxic form of oxygen metabolite H_2O_2 into oxygen and water. CAT activity was determined following the method described by Sinha [65]. Briefly, 100 µl of the supernatant was mixed with 150 µl of 0.01 M phosphate buffer (*p*H 7). Reaction was started by addition of 250 µl of H_2O_2 (0.16 M), incubated at 37 °C for one min and the reaction was stopped by addition of 1 ml of dichromate-acetic acid reagent (5 % $K_2Cr_2O_7$:glacial acetic acid; 1:3; v/v). The reaction mixture was immediately kept on a boiling water bath for 15 min that resulted into development of a green color. Finally, the mixture was read at 570 nm wavelength spectrophotometrically.

3.9. Effects of test compounds on hippocampal neurons

3.9.1. Culturing of hippocampal neurons

Primary rat hippocampal neuronal culture was prepared according to the earlier described methods [66]. Briefly, hippocampal tissues were dissected out from 18 days old rat foetuses, washed with cold HBSS, minced and incubated in 0.1 % trypsin for 30 min at 37°C. Trituration was carried out to form a single cell suspension. Cells were plated at a density of 0.53×10^6 viable cells/ml in serum free neurobasal medium (Invitrogen) containing N-2 supplement (1 %) (Invitrogen), B-27 supplement (2 %) (Invitrogen) and antibiotic-antimycotic solution (1 %) (Sigma). The cells were allowed to grow as neurons. Cultures of the neurons were placed in a humidified incubator at 37 °C and 5 % CO_2. Medium was changed every 3 days. *In vitro* formation of small proliferating neurons started after 1 week, and mature neurons were observed on 20 days. The number and size of neurons were monitored.

3.9.2. Effect of test compounds on cell viability against $A\beta_{1-42}$ toxicity

Effect of the selected test compounds on cell viability of hippocampal neurons was assessed using MTT assay according to the earlier described reports [67]. Hippocampal neuronal cells cultured in 96 well plates were incubated with different concentrations of the test and reference compounds (10-40 μM) for 24 hr to determine their cytotoxicity. Another set of experiments was performed to determine the neuroprotective potential of the test compounds against $A\beta_{1-42}$ insult. Cultured neurons were exposed to $A\beta_{1-42}$ (10 μM) (Sigma) for 24 hr with 2 hr pre-incubation of the test and reference (denepezil) compounds (10-40 μM). MTT assay was performed for both the experiments to determine percentage cell viability.

3.9.3. Determination of ROS scavenging ability of test compounds

Intracellular estimation of ROS was performed by 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay [68]. Briefly, primary rat hippocampal neuronal cells were seeded in 96 well plates. Cells were pre-treated with the test compounds (10-40 μM) for 2 hr, followed by $A\beta_{1-42}$ (10 μM) treatment for 24 hr. After the incubation period, the hippocampal cells were loaded with 10 μM DCFH-DA (Sigma) at 37 °C for 15 min. Fluorescence intensity was determined at 492 nm excitation and 495 nm emission wavelengths using a fluorescence plate reader (Proton

Technology International, Birmingham, NJ). The fluorescence intensities in the presence and absence of inhibitors were compared using appropriate controls. Percentage inhibition of ROS generation was determined using the fluorescence intensities of the test compounds/standard and the control.

3.9.4. Effect of test compounds on apoptotic cell death assessed by Hoechst staining

$A\beta_{1-42}$ mediated apoptotic cell death has been extensively studied [69, 70]. To evaluate the *in vitro* antiapoptotic potential of the test compounds, primary rat hippocampal neurons were cultured in the chamber slides and were exposed to the test compounds (20 µM) for 2 hr followed by $A\beta_{1-42}$ (10 µM) treatment for 24 hr. After the incubation period, the cells were fixed with 4 % paraformaldehyde for 20 minutes, stained with 5 µg/ml Hoechst 33258 (Sigma) for 10 min and then the cover slips were applied [71]. Nuclear morphology was checked utilizing Nikon Eclipse Ti-S inverted fluorescence microscope. The number of cells with apoptotic morphology (i.e. appearing condensed or fragmented nuclei) was counted.

3.9.5. Effect of test compounds on rate of apoptosis assessed using flow cytometry

Apoptosis was further determined using Annexin V-FITC and propidium iodide (PI) staining by flow cytometry. The rat hippocampal neuronal cells were seeded in six well plates. The cells were exposed to $A\beta_{1-42}$ (10 µM) for 24 hr. To determine the antiapoptotic potential, cells were pre-treated with the test compounds (20 µM) for 2 hr followed by $A\beta_{1-42}$ treatment. After incubation period, cells were harvested and suspended in 500 µl Annexin V binding buffer. Later, 5 µl Annexin V-FITC (BD Biosciences) and 10 µl PI (Sigma) were added and incubated with cells for 5 min in the dark. Untreated cells were used as the control for double staining. The stained cells were directly analysed using a FACScan flow cytometer.

3.9.6. Immunocytochemistry (ICC) of hippocampal cells exposed to $A\beta_{1-42}$ to evaluate caspase-3 activation

Caspase-3 activation is a key process during apoptosis [72]. Apoptosis was evaluated by determining the expression of cleaved caspase-3 (active) protein in hippocampal neurons as per the previously described methods [73, 74]. Primary hippocampal neurons were cultured in chamber slides. Cells were pre-treated with the test and reference (donepezil) compounds (20 μM) for 2 hr followed by treatment of $A\beta_{1-42}$ (10 μM) for 24 hr. After the incubation period, cells were washed with 1X PBS followed by fixation with 4 % paraformaldehyde for 20 min. Nonspecific binding sites were blocked by incubating the cells in PBS containing 4 % goat serum, 3 % bovine serum albumin (BSA) and 0.1 % Triton X-100 for 1 hr. The cells were incubated with primary rabbit anti-cleaved capase-3 antibody (1:500, Sigma) in blocking solution overnight at 4 °C. After the incubation, cells were rinsed thrice with PBS and incubated with alexafluor 594 linked secondary antibody (Invitrogen) for 2 hr at room temperature. Following incubation, cells were washed with PBS thrice and mounted with Anti-fade mounting medium (Sigma). Slides were covered with cover slips and viewed under fluorescence microscope for cleaved capase-3 and DAPI staining.

3.10. Effect of test compounds on $A\beta_{1-42}$-induced Alzheimer's rat model

Adult male Wister rats (200-250 gm) were divided into different experimental groups of six animals each. Animals were anaesthetized with ketamine (100 mg/kg, i.p.) and xylazine (30 mg/kg, i.p.) and mounted on a stereotaxic apparatus (Stoelting, USA). All the groups (except vehicle-treated control group which received equal volume of normal saline) were injected intracranially with 4 μl of $A\beta_{1-42}$ (2 μM/μl in normal saline) unilaterally at the following co-ordinates: -4.0 mm anteroposterior, -2.5 mm mediolateral and -3.5 mm dorsoventral from Bregma. Donepezil (5 mg/kg, p.o.) and the test compounds (5 or 10 mg/kg, p.o.) were administered (in 0.1 % Na-CMC) to the animals of the respective groups for 15 days after five days of surgical recovery [14, 75].

3.10.1. Y maze test to assess immediate working memory

Immediate working memory was evaluated using Y-maze test [76]. The Y-maze test was carried out during the last five days of the treatment period. 1 Hr after treatment with the test compounds/standard drug, each animal from the treated groups was placed at the end of any one arm of the maze and allowed to explore all the arms. The sequence and the number of arm entries were recorded visually for each rat over a period of 5 min. The arm entry was considered to be completed when all four paws of the rat were placed completely in the arm. An actual "alteration" was defined as entries in all the three arms in consecutive choices (i.e. ABC, BCA or CAB but not BAB). Repeat arm entry was considered as a sign of memory impairment. Maze arms were thoroughly cleaned with water sprays between the tasks to remove residual odour and residues. The number of arm entries was used as an indicator of locomotor activity. The "alteration score" for each rat was calculated using the equation:

$$\% \text{ Alternation} = [(\text{Number of alternations}) / (\text{Total arm entries} - 2)] \times 100.$$

3.10.2. Western blot analysis

Hippocampal regions of rat brains from different experimental groups were homogenized separately in tissue lysis buffer supplemented with protease and phosphatase inhibitors (Sigma). Homogenized samples were sonicated for 5 s, and centrifuged at 4 °C at 15,000 rpm for 30 min. Equal amounts of proteins (100 µg) were loaded on 10 % Tris-glycine gel. Membranes were blocked for 1 hr at room temperature using Tris-buffered saline/Tween-20 (TBST) (50 mM Tris-HCl, 150 mM NaCl, *p*H 7.4, 1 % Tween-20) containing 5 % non-fat-dried milk. Membranes were incubated overnight at 4 °C with rabbit anti-$A\beta_{1-42}$ (1:500, Santa Cruz), goat anti-*p*-tau (1:500, Santa Cruz), rabbit anti-cleaved caspase-3 (active) (1:500, Sigma), rabbit anti-cleaved poly (ADP-ribose) polymerase-1 (PARP) (1:1000, Cell Signalling), rabbit anti-*p*-GSK-3 (1:1000, Cell Signalling), mouse anti-β-catenin (1:1000, Santa Cruz), rabbit anti-neuroD1 (1:1000, Cell Signalling) and mouse anti-β-actin (1:10,000, Sigma) primary antibodies. After incubation, membranes were washed thrice with TBST and incubated for 1 hr with HRP–conjugated secondary antibody (Sigma). Immunoreactive proteins were detected using the ECL Plus chemiluminescent kit (Invitrogen) according to the manufacturer's instructions. Protein bands were quantified using Scion Image for Windows.

3.11. Acute toxicity study

Twenty male Swiss Albino mice (20-25 gm) were used to determine the acute toxicity of the most potent test compound. During the experiment, animals were maintained with free access of food and water *ad libitum*. The test compound was suspended in 0.5 % Na-CMC and given orally to the divided experimental groups (at 0, 677, 1333 and 2000 mg/kg, n = 5 per group). After the test compound administration, the animals were observed continuously for the first 4 hr for any abnormal behaviour and mortality. Later on the animals were observed intermittently for the next 24 hr and occasionally for 14 consecutive days after administration of the test compound. After 14 days mice were sacrificed and macroscopically examined for possible damage to the heart, liver and kidneys.[60, 77]

3.12. Pharmacokinetic study

Healthy male Wistar rats (200-250 gm, n=4) were used for the pharmacokinetic analysis. The animals were fasted overnight and pre-dosed blood samples were withdrawn. The most potent test compound suspended in Na-CMC was administered orally to the animals at a dose of 5 mg/kg. After administration, rats were anesthetized with ether and blood samples were collected from retro-orbital plexus at 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, 60 and 72 hr into the heparinised tubes. The tubes were centrifuged at 15,000 rpm for 10 min at 4 ºC to separate the plasma. Later, 400 µl of acetonitrile was added to 100 µl of the rat plasma to extract the test compound. The mixture was vortex-mixed for 5 min and centrifuged for 10 min at 15,000 rpm. The organic layer was taken in separate Eppendorf tube and evaporated to dryness under a stream of nitrogen gas. The residue was reconstituted with 100 µl of mobile phase, vortexed briefly and transferred to pre-labelled autosampler vials. From these, 20 µl of the sample was injected into high-performance liquid chromatography (HPLC) system with photo-diode array (PDA) detector (Waters Corporation, USA) for analysis. The samples were analysed using the mobile phase consisting of 0.05 M phosphate buffer (*p*H 4.8) (27 %) and acetonitrile (63 %) at a flow rate of 1 ml/min using a Sunfire® C18 column (4.6 mm×150 mm, particle size 5µm). The pharmacokinetic parameters were calculated using the plasma concentration data of the test compound obtained at different time points using extravascular analysis of non-compartmental model.

3.13. Statistical analysis

The observed data were analysed using GraphPad Prizm (version 5). The data were expressed as mean ± SEM. Significant difference between the experimental groups was determined using two way ANOVA (MWM and Y maze test) and one way ANOVA followed by Bonferroni test. Images were visualized and captured using a Carl-Zeiss confocal microscope. While capturing images, all parameters such as gain, contrast, brightness, and the positions and types of filters were set to standard parameters, such that signals were not saturated and all images could be quantified and compared to one another. Captured images were analyzed by

ZEN 2012 imaging software. For statistical analysis, a minimum of 30 randomly chosen cells per condition were analyzed (n=4 independent experiments with 3-4 replicates). A value of $p<0.05$ was considered significant. * and # indicated the level of significance.

3. MATERIALS AND METHODS

3.1. Animals

Male Wistar rats (200-250 gm) and female Swiss Albino mice (25-30 gm) were used for the studies with free access of food and water *ad libitum* during the course of the study. Animals were maintained on 12 hr light/dark cycle. After 15 days of acclimatization, animals were used for the studies. All the protocols were approved by Institutional Animal Ethics Committee (IAEC) and were conducted as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) with approval No: MSU/PHARM/IAEC/2013/02.

3.2. *In vitro* 5-HT_{2A} bioassay using isolated rat thoracic aorta strip

Wister rats (200-250 g) were used for the study. The animals were sacrificed by overdose of sodium pentobarbital (50 mg/kg). After dissecting the thoracic aorta, the helical strips were mounted in the Kreb's solution aerated with 95% O_2 and 5% CO_2 at 37 °C under 1 g of resting tension according to the earlier described reports [47]. For each determination at least six arterial strips were utilized. Isometric contractions were recorded using a force transducer (UGO BASILE, Italy) coupled to a Gemini 7070 recorder (UGO BASILE, Italy). All the test compounds were screened for their 5-HT_{2A} receptor agonist and antagonist actions at a single concentration of 2×10^{-5} M before determining their pA_2 or pD_2 values. 5-HT (10^{-8} to 10^{-5} M) (Sigma) was used as standard agonist to determine 5-HT_{2A} antagonism by the test compounds.

3.3. *In vitro* 5-HT_{2B} bioassay using isolated rat fundus preparation

Wistar rats weighing 200-250 gm, fasted for 48 hr, were sacrificed by overdose of sodium pentobarbital (50 mg/kg). The fundus strips were prepared as per the earlier described methods [48] and mounted in a 25 ml organ tube containing Kreb's solution at 37 °C, well aerated with 95% O_2 and 5% CO_2 with initial resting tension of 2 g. At least 6 longitudinal and 6 horizontal strip preparations were used for each test substance. The contractile responses were recorded on a 2-channel recorder using a force transducer coupled to a Gemini 7070 recorder (UGO Basile, Italy). Initially the test compounds were assessed to check their 5-HT_{2B} receptor responsiveness at 2×10^{-5} M concentration. The compounds showing contractile responses were further evaluated (at 10^{-6} to 10^{-4} M) in presence of SB-206553 (10^{-6} and 10^{-7} M) (Sigma) (5-HT_{2B}

receptor antagonist) to confirm their $5\text{-}HT_{2B}$ agonism which was evident by pD_2 value. Similar to the previous experiment, the antagonistic activity of the selected compounds was determined using 5-HT (10^{-8} to 10^{-5} M) as the agonist.

3.4. *In vivo* rodent models for $5\text{-}HT_{2C}$ receptor agonistic activity

After the *in vitro* screening of the test compounds for evaluating $5\text{-}HT_{2A}$ and $5\text{-}HT_{2B}$ receptor agonist/antagonist actions, the inactive compounds in both the studies were further evaluated for different *in vivo* rodent models as described below for their $5\text{-}HT_{2C}$ receptor agonistic potentials. *m*-CPP (2 mg/kg, p.o.) (Sigma) was used as a standard while the test compounds were administered at a dose of 10 mg/kg, p.o. Doses of the test compounds were decided after dose-deciding pilot study (data not shown). For all the *in vivo* experiments, six animals per group were utilized.

3.4.1. Despair swim test

Swiss Albino mice were forced to swim inside a vertical Plexiglas cylinder (45 cm height × 20 cm diameter) filled with water up to 30 cm height and maintained at 25 °C to assess their depression state. After a 15 min training period, animals were randomly divided into different experimental groups. Next day, 1 hr after the test compound administration, the animals were again placed in the cylinder and the total duration of immobility was measured during a 5 min test period [49].

3.4.2. Elevated plus maze test

Anxiety was assessed on elevated plus maze apparatus using Swiss Albino mice. Animals were acclimatized to the apparatus for 10 min before performing the experiment. One hour after the drug's treatment, animals were individually placed at the centre of the maze facing their heads towards open arm and allowed to explore all the arms for 5 min. Number of entries and time spent in open arms were recorded [50].

3.4.3. Hypophagic response

Food deprived Wistar rats were housed individually for 24 hr. Next day after the treatment, animals were supplemented with weighed amounts of the normal food pellets. Amount of food remaining after 2 hr was measured. $5\text{-}HT_{2C}$ receptor selectivity was also assessed by the administration of RS-10222 (Sigma) (selective 5-HT$_{2C}$ receptor antagonist) (2 mg/kg, i.p.) [51, 52] 30 min prior to the administration of the test compounds [53].

3.4.4. Penile erection model

Immediately after the treatment, rats were placed in Plexiglas observation cages. A mirror was placed behind the cages to observe penile erection over 2 hr period. To assess the 5-HT$_{2C}$ receptor selectivity, the animals were administered with RS-102221 (2 mg/kg, i.p.), 30 min prior to the administration of the test compounds. Number of lickings of the penis was recorded [54].

3.4.5. Estimation of monoamines in rat brains

5-HT$_{2C}$ receptor agonists negatively regulate the monoamines' firing [55-57]. It was confirmed by the estimation of 5-HT and DA levels in the rat brain using HPLC. After demonstrating the hypophagic response of the selected test compounds, their 5-HT$_{2C}$ receptor selectivity was confirmed in presence of a known 5-HT$_{2C}$ antagonist RS-102221. At the end of the study, all the rats were sacrificed by an overdose of sodium pentobarbital (50 mg/kg). The brains were dissected out, weighed and homogenized in 10 % w/v 0.17 M perchloric acid to extract out the monoamines. Homogenates were allowed to stand for 15 min and the supernatants were decanted in separate Eppendorf tubes that were centrifuged at 4 °C for 20 min at 15,000 rpm. After centrifugation, supernatants were separated and either immediately analysed or stored at -70 °C till assayed. Brain concentrations of 5-HT and DA were assessed by RP-HPLC coupled with electrochemical detector (Waters Corporation, USA). The estimation was performed as per the previously described method with some minor modifications [58, 59]. Briefly, a Sunfire® C18 column (4.6 mm×150 mm, particle size 5µm) was used and separation was carried out using mobile phase containing methanol (15% v/v) in a solution (*p*H 4.2) of 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate, 0.5 mM octyl sodium sulphate, 0.5 mM EDTA and 2 mM KCl, at a flow rate of 1.2 ml/min, an operating pressure of 3000 psi and an operating potential of 0.61 V. The standard calibration curves were prepared by spiking known amounts of mixed standards (10, 20, 40, 80 and 160 ng/ml of each 5-HT and DA) in 1 ml of the pooled brain homogenates. The standard curves were used to quantify the amount of 5-HT and DA in each sample by calculating the area under the curve (AUC). The limit of detection (LOD) for 5-HT and DA were found to be 0.83±0.06 ng/ml and 0.11±0.03 ng/ml, respectively while the limit of quantification (LOQ) was found to be 2.53±0.12 ng/ml and 0.34±0.03 ng/ml respectively.

3.5. Statistical analysis

All the observed data were analyzed using GraphPad Prizm-5 software. Comparison within groups was made by repeated measures ANOVA and comparison among groups was made by factorial ANOVA followed by Bonferroni test. Data were expressed as mean ± SEM. A value of $p<0.05$ was considered as significant.

3. MATERIALS AND METHODS

3.1. Cell culturing and MTT assay for determining NMDAR antagonism of test compounds

The SH-SY5Y cells (National Centre for Cell Science, Pune) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with (10 % v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37 ºC and 5 % CO_2. The cells, cultured in 75 cm^2 flasks, were seeded in 96 well plates and incubated for 24 hr. Subsequently, the growth media was replaced with fresh normal media (control cultures) or with media supplemented with NMDA (5 mM) (Sigma) for another 24 hr to induce excitotoxicity. For antagonism studies, SH-SY5Y cells were exposed to a series of benzazepine derivatives (at 10 µM) for 2 hr followed by NMDA treatment for 24 hr. After incubation period, cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, 20 µl of MTT (Sigma) solution (5 mg/ml stock solution) was added into each well and incubated for 4 hr at 37 °C. Later, the culture media was replaced by 200 µl DMSO to dissolve the formazan crystals. The optical density was measured at 570 nm with 630 nm reference wavelength using a micro plate reader 680 XR (BIO-RAD, India). The absorbance of the control cells was considered as 100 % of the cell viability [28]. Percentage protection against NMDA-induced excitotoxic damage was calculated for individual compounds.

3.2. Thioflavin T (ThT) assay for determining $A\beta_{1-42}$ aggregation inhibitory activity of the test compounds

In order to identify multi-target-directed ligands, $A\beta_{1-42}$ aggregation inhibitory activity was determined using the thioflavin T (ThT) fluorescence assay for the most promising test compounds obtained from the *in vitro* MTT assay, as per the earlier described method [29, 30]. $A\beta_{1-42}$ (Sigma) dissolved in phosphate buffer saline (PBS) was further diluted with 0.215 M sodium phosphate buffer (*p*H 8) to get the final concentration of 20 µM. The test compounds were dissolved in DMSO and diluted with 0.215 M sodium phosphate buffer (*p*H 8). To determine $A\beta_{1-42}$ aggregation, the solution containing 20 µM of $A\beta_{1-42}$ or $A\beta_{1-42}$ plus the test compound (10 µM) in 0.215 M sodium phosphate buffer was incubated at room temperature for 24 hr. After incubation, 20 µM ThT (prepared in 50 mM glycine-NaOH buffer; *p*H 8.5) was added to the above solution. Finally, the fluorescence intensity was read at 442 nm excitation and

490 nm emission wavelengths using spectrofluorometer (RF-5301 PC, Shimadzu). The percentage inhibition of $A\beta_{1-42}$ aggregation was calculated using the formula: $100-(IF_i/IF_o\times100)$, where IF_i and IF_o are fluorescence intensities in the presence and absence of the test compound respectively. Each assay was run in triplicate.

3.3. Congo red (CR) binding assay for determining $A\beta_{1-42}$ aggregation inhibitory activity of the test compounds

$A\beta_{1-42}$ Aggregation inhibitory potential of the potent test compounds was assessed using Congo red (CR) binding assay. CR (Hi-Media) solution prepared in PBS (*p*H 7.4) was diluted to get a final concentration of 5 µM. $A\beta_{1-42}$ (Sigma) prepared in PBS was diluted to get a final concentration of 20 µM. Briefly, 20 µM of $A\beta_{1-42}$ was incubated with or without the test compound (10 µM) for 6 hr at 37 ºC. Later on, the mixture was incubated with 5 µM of CR for 30 min at room temperature. Following incubation, CR spectra were measured using a UV-spectrophotometer (UV-1700, Shimadzu) at 480 nm and 540 nm wavelengths. CR binding was calculated using the following formula: CB (M) = (OD at 540 nm/25,295) - (OD at 480 nm/46,306); where, CB (M) is the amount of CR bound with β sheets of $A\beta_{1-42}$ and OD is the optical density [31, 32].

3.4. Effects of test compounds on hippocampal neurons

3.4.1. Culturing of rat hippocampal neurons

Previous studies have demonstrated the neurotoxicity of Aβ in the primary rat hippocampal neuronal culture [33]. Aβ elevates oxidative stress which contributes to apoptotic neuronal cell death in AD [34, 35]. So to determine neuroprotective, free radical scavenging, antioxidant and antiapoptotic effects of potent NMDAR antagonists against $A\beta_{1-42}$ insult, primary rat hippocampal neuronal culture was used. The primary culture was prepared according to the previously described methods [36]. Briefly, hippocampal tissues were dissected out from 18 days old rat foetuses, washed with cold HBSS, minced and incubated in 0.1 % trypsin for 30 min at 37 ºC. Trituration was carried out to form a single cell suspension. Cells were plated at a density of 5×10^4 viable cells/ml in serum-free neurobasal medium (Invitrogen) containing N-2 supplement (1 %) (Invitrogen), B-27 supplement (2 %) (Invitrogen) and antibiotic-antimycotic solution (1 %) (Sigma). The cells were allowed to grow as neurons. Culture of neurons was placed in a humidified incubator at 37 °C and 5 % CO_2. Medium was changed in every 3 days. Formation of small proliferating neurons started after 1 week, and mature neurons were observed on 20 days *in vitro*. The number and size of neurons were measured.

3.4.2. Assessment of the effect of test compounds on cell viability challenged by $A\beta_{1-42}$–induced excitotoxicity

Cell viability of hippocampal neurons was determined using MTT assay as described earlier [37]. Hippocampal neuronal cells, cultured in 96 well plates were exposed to different concentrations of the test and the standard compounds (10-40 µM) for 24 hr to assess their cytotoxicity. Another set of experiments was performed to determine the neuroprotective role of potent NMDAR antagonist test compounds against $A\beta_{1-42}$ toxicity. Cultured neurons were exposed to the test and the standard compounds (10-40 µM) for 2 hr followed by $A\beta_{1-42}$ (10 µM) treatment for 24 hr. MTT assay was performed in both the experiments to determine the cell viability.

3.4.3. ROS scavenging activity of test compounds

The ROS scavenging activity of the potent test compounds was determined using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay [38]. Briefly, primary rat hippocampal neuronal cells were seeded in 96 well plates. Cells were exposed to the test compounds (10-40 µM) for 2 hr, followed by $A\beta_{1-42}$ (10 µM) insult for 24 hr. After the incubation period, the hippocampal cells were loaded with 10 µM DCFH-DA (Sigma) at 37 °C for 30 min. Fluorescence intensity was determined using the Synergy HTX multi-mode microplate reader with excitation wavelength of 492 nm and emission of 495 nm. The fluorescence intensities in the presence and absence of the inhibitors were compared using appropriate control and the percentage inhibition of ROS was calculated for individual inhibitors.

3.4.4. Assessment of apoptotic cell death by Hoechst staining

Antiapoptotic effect of the potent benzazepines was determined using Hoechst staining as described earlier [33, 39]. Briefly, primary rat hippocampal neurons cultured in chamber slides were exposed to the test compounds (20 µM) for 2 hr followed by $A\beta_{1-42}$ (10 µM) intoxication for 24 hr. After the incubation period, the cells were fixed with 4% paraformaldehyde for 20 min, stained with 5 µg/ml Hoechst 33258 (Sigma) for 10 min and then the cover slips were applied [39]. Nuclear morphology was observed using Nikon Eclipse Ti-S inverted fluorescent microscope. The number of cells with apoptotic morphology (i.e. appearing condensed or fragmented nuclei) was counted.

3.4.5. Estimation of the rate of apoptosis by flow cytometry

Effect on apoptosis by the test compounds was further determined using Annexin V-FITC and propidium iodide (PI) staining by flow cytometry. Briefly, the rat hippocampal neuronal cells, seeded in six well plates, were exposed to $A\beta_{1-42}$ (10 µM) for 24 hr. To determine antiapoptotic potential, cells were pre-treated with the test compounds (20 µM) for 2 hr followed by $A\beta_{1-42}$ insult. After the incubation period, cells were harvested and suspended in 500 µl Annexin V binding buffer. 5 µl Annexin V-FITC (BD Biosciences) and 10 µl PI (Sigma) were added and incubated with cells for 5 min in the dark. Untreated cells were used as the control for double staining. The stained cells were directly analysed using a FACScan flow cytometer.

3.4.6. Effect of test compounds on caspase-3 activation in hippocampal cells exposed to $A\beta_{1-42}$

In the present study, effect of the potent test compounds on apoptosis was further verified by determining the expression of cleaved caspase-3 (active) protein in the hippocampal neurons as per the earlier described reports [40, 41]. Primary rat hippocampal neurons cultured in chamber slides were exposed to the test and reference compounds (20 µM) for 2 hr followed by $A\beta_{1-42}$ (10 µM) intoxication for 24 hr. After incubation period, cells were washed with 1X PBS followed by fixation with 4 % paraformaldehyde for 20 min. Nonspecific binding sites were blocked by incubating the cells in PBS containing 4 % goat serum, 3 % bovine serum albumin (BSA) and 0.1 % Triton X-100 for 1 hr. The cells were incubated with primary rabbit anti-cleaved capase-3 antibody (1:500, Sigma) in blocking solution overnight at 4 °C. After incubation, the cells were rinsed with PBS thrice and incubated with alexafluor 594 linked secondary antibody (Invitrogen) for 2 hr at room temperature. Following incubation, the cells were washed with PBS thrice and mounted with Anti-fade mounting medium (Sigma). Slides were covered with cover slips and viewed under fluorescence microscope for cleaved capase-3 and DAPI staining.

3.5. *In vitro* blood-brain barrier permeation assay

Before assessing the *in vivo* neuroprotective potential of the selected test compounds against $A\beta_{1-42}$-induced excitotoxicity, it was decided to determine their permeability through the blood-brain barrier (BBB). The possibility of *in vivo* BBB penetration of the test compounds was evaluated using parallel artificial membrane permeation assay of the blood-brain barrier (PAMPA-BBB) as described by Di. *et al* [42, 43]. The donor microplates (polyvinylidene fluoride PVDF memberane, diameter 25 mm, pore size 0.45 µm) (Millipore) and acceptor microplates were used for the study. The acceptor microplate was filled with 200 µl PBS/ethanol (70:30) and the filter surface of donor microplate was impregnated with 4 µl of porcine brain lipid (Avanti Polar Lipids) in dodecane (Sigma) (20 mg/ml). The test compounds were dissolved in DMSO at 5 mg/ml and diluted with PBS/ethanol (70:30) to get a final concentration of 100 µg/ml. 200 µl of the solution was added to the donor wells and the donor plate was carefully placed on the acceptor plate to form a sandwich. Both the plates were kept aside undisturbed for 120 min at 25 °C. After the incubation period, the donor plate was carefully removed and the concentrations of the test compounds in the acceptor wells were determined using UV spectroscopy. Each sample was analysed at five different wavelengths, in four wells, and at least in three independent runs. Nine quality standards of known BBB permeability [43] were used to validate this assay.

3.6. Effect of tes compounds in $A\beta_{1-42}$-induced excitotoxicity model of rat

Adult male Wister rats (200-250 gm) were divided into different experimental groups of twelve animals each. Animals anaesthetized with ketamine (100 mg/kg, i.p.) and xylazine (30 mg/kg, i.p.) were mounted on a stereotaxic apparatus (Stoelting, USA). Animals from all the experimental groups (except vehicle-treated control group which received equal volume of normal saline) received intracerebroventricular (ICV) injection of 4 µl $A\beta_{1-42}$ (2 µM/µl in normal saline) unilaterally at the following co-ordinates: -4.0 mm anteroposterior, -2.5 mm mediolateral and -3.5 mm dorsoventral from Bregma. Memantine was suspended in 0.1 % sodium carboxy methyl cellulose (Na-CMC) and was administered at a dose of 5 mg/kg orally. Equivalent doses of the test compounds (5 mg/kg p.o. in Na-CMC) were administered to the respective group animals. All the treatments were continued for fifteen consecutive days after five days of surgical recovery [44, 45].

3.6.1. Morris water maze test for assessing spatial learning ability

The spatial learning ability of the animals was assessed using Morris water maze (MWM) test. The test was performed during the last five days of the treatment period. Time required to reach the hidden platform (i.e. escape latency time-ELT) and number of crossings over the platform area were recorded during 2 min of training session to determine spatial learning ability [46].

3.6.2. Y maze test for assessing immediate working memory

Immediate working memory was evaluated using Y-maze test [47]. The Y-maze test was also carried out during the last five days of the treatment period. Each animal was placed at the end of any one arm of the maze and allowed to explore all the three arms. The sequence and the number of arm entries were recorded visually over a period of 5 min. An actual "alteration" was defined as entries in all three arms in consecutive choices (i.e. ABC, BCA or CAB but not BAB). Repeat arm entry was considered as a sign of memory impairment. The number of arm entries indicated locomotor activity. The "alteration score" for each rat was calculated using the equation:

$$\% \text{ Alternation} = [(\text{Number of alternations}) / (\text{Total arm entries} - 2)] \times 100.$$

3.6.3. Effect of test compounds on Neurochemical levles

3.6.3.1. Estimation of MDA and catalase levels in rat brain

After assessing the effects of the test compounds on learning and memory, the treated animals were sacrificed. Whole brains were isolated from the skull and washed with ice cold PBS. The hippocampal regions were isolated and homogenized in glass Teflon homogenizer in 12.5 mM sodium phosphate buffer, *p*H 7. The homogenates were centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatants were utilized to determine malondialdehyde (MDA-a byproduct of lipid peroxidation) and catalase (CAT-which catalyzes hydrogen peroxide decomposition) levels to evaluate antioxidant potential of the test compounds [48].

3.6.3.2. Estimation of glutamate and glycine levels in rat brain

Excitotoxicity correlates with elevated levels of excitatory neurotransmitters viz. glutamate and glycine in the brain [44, 49]. In the current study, levels of glutamate and glycine in the hippocampal region of rat brains, which underwent ICV injection of Aβ_{1-42}, were analysed using HPLC technique. At the end of the behavioural experiments, the hippocampal regions were carefully isolated from the animal brains, weighed and homogenized in 0.17 M perchloric acid in the ratio of 10 % w/v. The homogenates were kept on ice at least for 30 min for complete protein precipitation. The supernatants decanted in separate Eppendorf tubes were centrifuged at 4 °C for 20 min at 15,000 rpm. After centrifugation, supernatants were separated and either immediately analysed or stored at -70 °C until assayed. The amount of glutamate and glycine in the hippocampal region was assessed using RP-HPLC coupled with electrochemical detector (Waters Corporation) as per the previously described method [44, 50] using mobile phase containing a solution of 0.1 M monosodium phosphate, 0.5 mM EDTA, 25% (v/v) methanol; *p*H was adjusted to 4.5 using 0.1 M perchloric acid, at a flow rate of 1.2 ml/min and an operating potential of 0.85 V. The derivatization reagent mixture consisted of 37 mM orthophthalaldehyde (OPA), 50 mM sodium sulfite, 90 mM tetraborate buffer (which was set to *p*H 10.4 with sodium hydroxide, prior to addition of OPA), and 5% methanol. The standard calibration curves prepared by spiking known amount of mixed standards in 1 ml of pooled brain homogenate with 10 min incubation of 20 µl derivatizing reagent at room temperature were used to quantify the amount of glutamate and glycine in each sample by calculating the area under curve (AUC). The limit of detection (LOD) for glutamate and glycine were found to be 0.18±0.04 ng/ml and 0.13±0.02 ng/ml respectively while the limit of quantification (LOQ) were found to be 0.56±0.06 ng/ml and 0.40 ± 0.07 ng/ml respectively.

3.7. Western blot analysis

Hippocampal regions of rat brains were homogenized separately in tissue lysis buffer supplemented with protease and phosphatase inhibitors (Sigma). Homogenized samples were sonicated for 5 sec and centrifuged at 15,000 rpm for 30 min at 4 °C. Equal amount of proteins (100 µg) were loaded on 10 % Tris-glycine gel. Membranes were blocked for 1 hr at room temperature using Tris-buffered saline/Tween-20 (TBST) (50 mM Tris-HCl, 150 mM NaCl, *p*H 7.4, 1 % Tween-20) containing 5 % non-fat-dried milk. Membranes were incubated overnight at

4 °C with rabbit anti-Aβ_{1-42} (1-500, Santa Cruz), goat anti-*p*-tau (1-500, Santa Cruz), rabbit anti-cleaved caspase-3 (active) (1:500, Sigma), rabbit anti-cleaved-poly (ADP-ribose) polymerase-1 (PARP) (1:1000, Cell Signalling), rabbit anti-*p*-GSK-3 (1:1000, Cell Signalling), mouse anti-*p*-ERK (1:1000, Santa Cruz) and mouse anti-β-actin (1:10,000, Sigma) primary antibodies. After incubation, membranes were washed thrice with TBST and incubated for 1 hr with HRP–conjugated secondary antibody (Sigma). Immunoreactive proteins were detected using the ECL Plus chemiluminescent kit (Invitrogen) according to the manufacturer's instructions. Protein bands were quantified using Image J software.

3.8. Molecular Dynamics and Relative Binding Affinity Studies

The docking conformations of the ligand (**10**) with their respective targets generated from docking studies on Glide were subjected to molecular dynamics using AMBER 10. Protons were added to the protein using the Leap module of AMBER 10. The ligand was prepared by using generalized amber forcefield (GAFF). To neutralize the charge of the system, an appropriate number of chloride counter ions were placed in the grid with the largest positive Coulombic potential around the complex. The solute was surrounded by a truncated, cubic periodic box of TIP3P water molecules at a distance of 10 Å from the solute atoms. The system was minimized with the SANDER module in a constant volume by 1000 cycles of steepest descent minimization followed by 1000 cycles of conjugated gradient minimization under harmonic restraints with force constant 10 kcal ⁄ (mol $Å^2$) to all solute atoms. This procedure ensured that the initial structures were maintained while the solvent was allowed to relax. After energy minimization and applying the harmonic restraints with force constants of 2 kcal ⁄ (mol $Å^2$) to all solute atoms, canonical ensemble (NVT)-MD was then carried out for 70 ps, during which the system was gradually heated upto 300 K. The subsequent isothermal isobaric ensemble (NPT)-MD was used for 500 ps to adjust the solvent density. Finally, the 5-ns isothermal isobaric ensemble (NPT)-MD was applied to both simulations without any restraints. The temperature was regulated at 300 K using the Langevin thermostat, and the pressure was kept at 1.0 atm using isotropic positional scaling. Trajectories were analysed at every 1 ps using the PTRAJ module.

3.9. Statistical analysis

The observed data were analysed using GraphPad Prism (version 5) software. The data are expressed as mean ± SEM. Significant difference between the experimental groups was determined using two way ANOVA (MWM and Y maze test) and one way ANOVA followed by Bonferroni test. Images were visualized and captured using a Carl-Zeiss confocal microscope. While capturing images, all parameters such as gain, contrast, brightness, the positions, and types of filters were set to standard parameters, such that the signals were not saturated and all images could be quantified and compared to one another. Captured images were analysed using ZEN 2012 imaging software. For statistical analysis, a minimum of 30 randomly chosen cells per condition were analysed (n=4 independent experiments with 3-4 replicates). A value of $p<0.05$ was considered significant. * and # indicated the level of significance.

3. MATERIALS AND METHODS

3.1. Animals

Male Wistar rats (200–250 gm) were housed in a temperature and humidity controlled facility with a 12 hr light/dark cycle and free access to food and water *ad libitum* throughout the study. After 15 days acclimatization, animals were used for the studies. All the protocols were approved by Institutional Animal Ethics Committee (IAEC) and the experiments were performed as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines with approval No: MSU/IAEC/2014-15/1401.

3.2. Effects of test compounds on rat superior mesenteric artery strip

Wistar rats weighing 200-250 gm were sacrificed by overdose of sodium pentobarbital (50 mg/kg). After dissecting the superior mesenteric artery, the helical strips were mounted as described earlier in Kreb's solution aerated with 95 % O_2 and 5 % CO_2 at 37 °C under 1 g of resting tension. Isometric contractions were recorded using a force transducer (UGO BASILE, Italy) coupled to a Gemini 7070 recorder (UGO BASILE, Italy) [22].

Contractile effects of noradrenaline (10^{-5} M) on the rat superior mesenteric artery strips were recorded. After reaching the plateau, endothelium denudement was checked by assessing the vasorelaxant effect of acetylcholine (10^{-5} M). DA receptor agonist mediated vasorelaxant effect was determined by cumulative addition of the selective DA receptor agonists (D_1 agonist-A-77636, D_2 agonist- bromocriptine and D_3 agonist- 8-OH-PBZI) on the arterial strips, pre-constricted submaximally (80%) by noradrenaline.

Test compounds were investigated to assess their DA receptor modulatory potential. For that, different concentrations (10^{-9} M to 10^{-6} M) of the test compounds were added cumulatively to the endothelium-denuded arterial strips which were pre-constricted by noradrenaline and their pD_2 values were determined (which is the negative logarithm to the base 10 of the EC_{50} of an agonist). For the test compounds showing DA receptor agonist activity, receptor specificity was determined by pre-incubation of the strips for 30 min with a D_1 receptor antagonist R-SCH-23390 (10^{-6} M), a D_2 receptor antagonist L-741626 (10^{-6} M) and a D_3 receptor antagonist SB-277011A (10^{-6} M), and the vasorelaxant effect of the test compounds was determined thereafter .

3.3. MTT assay to determine D_1 receptoir agonism by test compound

The neuroprotective effect of the potent test compound was determined using human neuroblastoma SH-SY5Y cell line (National Center for Cancer Research, Pune). The SH-SY5Y cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with (10% v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37 ºC with 5% CO_2. The cells, cultured in 75 cm^2 flasks, were seeded in 96 well plates followed by incubation for 24 hr. Next day, the growth medium was replaced with fresh normal medium (control cultures) or with a medium supplemented with 6-OHDA (100 µM) [23] for 24 hr. The cells were pre-treated with the test compound (5 µM, 10 µM and 20 µM) or A-77636 (5 µM, 10 µM and 20 µM) for 3 h before 6-OHDA treatment. In another set of experiments, cells were pre-treated with R-SCH-23390 (10 µM) for 2 hr followed by treatment with the test compound or A-77636 at the same concentrations (5 µM, 10 µM and 20 µM) for 3 h before 6-OHDA treatment. After incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess the cell viability [24, 25]. 20 µl of MTT (Sigma-Aldrich) solution (5 mg/ml stock solution) was added into each well and the contents incubated for 4 hr at 37 °C. Later, the culture medium was replaced by 200 µL of DMSO to dissolve the formazan. The optical density was measured at 570 nm with 630 nm reference wavelength using a micro plate reader 680 XR (BIO-RAD, India). The absorbance of the control was considered as 100% of the cell viability.

3.4. Effect of test compound in 6-OHDA-lesioned rat model of Parkinson's disease

Adult male Wistar rats (200–250 gm) were anaesthetized with 100 mg/kg, i.p. ketamine and 30 mg/kg, i.p. xylazine. Animals were mounted on a stereotaxic frame, the skin overlying the skull was cut to expose it, and the coordinates for the striatum were precised correctly as lateral 2.5 mm, anterio-posteror 0.5 mm, dorso-ventral 4.5 mm, with reference to bragma. Subsequently, the animals from the different experimental groups (n=8) were lesioned by injecting 10 µg of 6-OHDA/2 µL in 0.1% ascorbic acid-saline into the right striatum, except the sham operated group which received 2.0 µL of the vehicle. The injection rate was maintained at 1.0 µL/min by an injecting pump [26]. After one week of surgical recovery, the test (5 mg/kg, p.o.) or standard (A-77636, 1 mg/kg, s.c.) [27] compound was administered for seven consecutive days to the respective group animals. Dose of the test compound was decided after dose deciding pilot study.

3.4.1. Drug-induced rotational behaviour

One week after the surgical recovery, rats were placed in a cylindrical container (diameter of 33 cm and a height of 35 cm) and allowed to acclimatize for 15 min. The total number of contralateral rotations were counted over 60 min period following treatment with the test/standard compound for seven consecutive days.

3.4.2. Effect of test compound in neurochemical levels

After behavioural studies, animals were sacrificed, skull cut open and the whole brain dissected out. Striatum of the injected side of the brain was isolated and stored at -70 °C for further use. The striatum was homogenized in 12.5 mM sodium phosphate buffer *p*H 7.0 using glass Teflon homogenizer. The obtained homogenate was centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatant was collected and used as a source of enzyme for the following assays.

3.4.2.1. Reduced glutathione (GSH)

GSH was measured in ipsilateral striatal region. In brief, equal volumes of tissue homogenate (supernatant) and 20 % trichloroacetic acid (TCA) were mixed. The precipitated fraction was centrifuged and to 0.25 ml of the supernatant, 2.0 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent (0.6 mM) was added. The final volume was made up to 3.0 ml with phosphate buffer (0.2 M, *p*H 8.0). The colour developed was read at 412 nm against the reagent blank [28].

3.4.2.2. Lipid peroxidation potential (LPO)

LPO in ipsilateral striatal region was measured by estimating MDA levels. In brief, 2.0 ml of the tissue homogenate (supernatant) was added to 2.0 ml of freshly prepared 10 % v/v TCA and the mixture was allowed to stand in ice bath for 15 min. After 15 minutes, the precipitate was separated by centrifugation and 2.0 ml of the clear supernatant solution was mixed with 2.0 ml of freshly prepared thiobarbituric acid (TBA) (0.67 % v/v). The resulting solution was heated in boiling water bath for 10 min and then immediately cooled in ice bath for 5 min. The colour so developed was measured at 532 nm against the reagent blank [29].

3.4.2.3. Superoxide dismutase (SOD)

SOD in the ipsilateral striatal region was measured as described earlier [30]. Briefly, 0.5 ml of the tissue homogenate was diluted with 0.5 ml of distilled water, to which 0.25 ml of ice-cold ethanol and 0.15 ml of ice-cold chloroform was added. The mixture was mixed well using cyclomixer for 5 min and centrifuged at 15,000 rpm. To 0.5 ml of the supernatant, 1.5 ml of carbonate buffer (0.05 M, *p*H 10.2) and 0.5 ml of ethylenediaminetetraacetic acid (EDTA) (0.49 M) solution were added. The reaction was initiated by the addition of 0.4 ml of epinephrine (3 mM) and the change in optical density/minute was measured at 480 nm against the reagent blank.

3.4.2.4. Catalase activity

Catalase activity in the ipsilateral striatal region was measured. Briefly, 1.0 ml of hydrogen peroxide (30 mM) was added in 2.0 ml of the diluted sample to initiate the reaction. The blank was prepared by mixing 2.0 ml of the diluted sample (similar dilution) with 1.0 ml of phosphate buffer (50 mM; pH 7.0). The decrease in absorbance was measured at 240 nm [31].

3.4.2.5. Estimation of DA level using HPLC technique

Levels of DA were estimated using high performance liquid chromatography (HPLC) with electrochemical detector (Waters Corporation, USA) [32-34]. Rats from all the groups were used for preparing the brain tissue samples. Ipsilateral striatal (which was given 6-OHDA or saline treatment) region was used for preparing the tissue homogenate. Homogenates were then centrifuged at 15,000 rpm at 4 °C for 20 min. Supernatants were filtered with 0.22 µm membrane filter and 20 µl volume was injected via HPLC pump into a column (RP C18, 5 µm particle size, 4.6 mm i.d x 250 mm at 30°C) connected to an electrochemical detector kept at a potential of +0.61 V with glassy carbon working electrode vs. Ag/AgCl reference electrode. The mobile phase consisted of 32 mM citric acid, 12.5 mM disodium hydrogen phosphate, 1.4 mM sodium octylsulfonate, 0.05 mM EDTA and 16% (v/v) methanol (*p*H 4.2) at a flow rate of 1.2 ml/min. The standard calibration curves were prepared by spiking known amounts of the standard (10, 20, 40, 80 and 160 ng/ml of DA) in 1 ml of pooled brain homogenates. The standard curves were used to quantify the amount of DA in each sample by calculating the area under the curve (AUC). The limit of detection (LOD) for DA was found to be 0.11±0.03 ng/ml, while the limit of quantification (LOQ) was found to be 0.34±0.03 ng/ml.

3.4.3. Immunohistochemical analysis of substantia nigra neurons to determine cleaved caspase-3 and tyrosine hydroxylase (TH) levels

Animals were sacrificed after behavioural study by transcardial perfusion under ketamine and xylazine (1:3) deep anaesthesia. Perfusion was made with PBS (0.1 M, *p*H 7.2) followed by 4 % ice-cold paraformaldehyde. Following perfusion, brains were removed and stored overnight at 4 $^{\circ}$C in the same fixative. Cryopreservation of the removed brains was finished with 10, 20 and 30 % (w/v) sucrose solution in PBS. Brain sections of 15 µm thickness were cut coronally, surrounding substantia nigra region using a freezing microtome (Slee Mainz Co., Germany). Immunohistochemical analysis of the proliferating cells was done with every sixth section so that every section was 90 µm apart from each other. PBS containing 0.5% BSA, 3% NGS and 0.1% Triton X-100 was used to block non–specific-binding sites. Then, tissue sections were incubated with rabbit anti-caspase-3 (cleaved) antibody (1:500; Sigma Aldrich) and mouse anti-tyrosine hydroxylase (TH) antibody (1:500; Sigma Aldrich) for 24 h at 4 $^{\circ}$C for immunofluorescence analysis. The sections were incubated with a cocktail of secondary antibodies conjugated with Alexa Fluor 488 (1:200; Molecular Probes, Invitrogen) and Alexa Fluor 594 (1:200; Molecular Probes, Invitrogen) for 2 hr at room temperature. During each step the sections were washed thrice with PBST. To mount the sections gelatin-coated slides were used, and then cover slipped with mounting medium (Vectashield, Vector Laboratories, CA). The slides were stored in dark at 4 $^{\circ}$C and analyzed for fluorescence labeling under Nikon Eclipse Ti-S inverted fluorescent microscope equipped with Nikon Digital Sight Ds-Ri1 Charged Coupled Device camera and NIS Elements Basic Research (BR) imaging software (Nikon, Japan) and Carl Zeiss LSM710. While capturing images, all parameters such as gain, contrast, brightness, and the positions and types of filters were set to standard parameters, so that signals were not saturated and all images could be quantified and compared to one another. Captured images were analysed by ZEN 2012 imaging software. For statistical analysis, a minimum of 30 randomly chosen cells per condition were analyzed (n=4 independent experiments with 3-4 replicates).

3.5. Statistical analysis

Data was statistically analysed using GraphPad InStat software (version 5.00, San Diego, CA). All data was presented as mean±SEM. The mean significant difference in the experimental groups was determined using two way ANOVA (Contralateral rotations) & one way ANOVA followed by Bonferroni test. Values of $p<0.05$ were considered statistically significant.

3. MATERIALS AND METHODS

3.1. Animals

Male Wistar rats (200-250 gm) and male Swiss Albino mice (20-30 gm) were used in different experiments with free access to food and water *ad libitum*. Animals were maintained on 12 hr light/dark cycle. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Pharmacy Department, The M. S. University of Baroda and the experiments were conducted in accordance to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) (Approval No. MSU/IAEC/2014-15/1401). All efforts were made to reduce the number of animals and their suffering.

3.2. Effect of test compounds on different behavioural rodent models

Among the series of test compounds, two compounds (**8** and **15**) were found to be the most potent and relatively more selective D_3 antagonists in the *in vitro* assessment. These compounds were further evaluated in different *in vivo* experimental rodent models for antipsychotic activity. All the behavioural experiments were carried out in a sound proof dark room and supervised by a blind observer.

3.2.1. Apomorphine-induced stereotype behaviour

Rats were administered with apomorphine (1mg/kg, s.c.) (Sigma) and placed individually in polycarbonate cages. Treatment of the animals with the test compounds (5, 10 and 20 mg/kg, p.o.) was given 30 min prior to the apomorphine administration. The animals were observed for a minute after every 15 min interval up to 2 hr period. Following scoring system was adopted to evaluate the stereotype behaviour. **1** = compulsive sniffing and exploratory activity; **2** = licking the floor or walls of the cage at least once during the observation period; **3** = biting the cage wires at least once during the observation period; **4** = compulsive continuous biting. Score of the animals treated with the test compounds was compared with the animals receiving apomorphine alone. Each group comprised of six animals. Clozapine (Sigma) was used as a standard drug for comparison (5 and 10 mg/kg, p.o.) [19].

3.2.2. Spontaneous locomotor activity

On the test day, rats were placed in a transparent polycarbonate cage located in the photoactometer for 30 min to habituate. To assess the spontaneous locomotor activity, test compounds (5, 10 and 20 mg/kg, p.o.) or clozapine (5 and 10 mg/kg, p.o.) were administered to separate group of animals. After 30 min of drug treatment, the activity counts were recorded after every 15 min interval up to 1 hr time period [15].

3.2.3. 7-OH-DPAT-induced hypothermia

Before administration of the test compounds and the standard drug, rectal temperature of the animals was recorded using digital thermometer. Rats were treated with the test compounds (5, 10 and 20 mg/kg, p.o.) or clozapine (5 and 10 mg/kg, p.o.). 30 Min after the drug treatment, rats were administered with 7-OH-DPAT (0.2 mg/kg, s.c.) and the rectal temperature was recorded finally after another 30 min [20].

3.2.4. Induction of catalepsy

Catalepsy is defined as the inability of the animals to correct the unusual posture or reduced ability to initiate the movement. Rats were administered with the test compounds (5, 10 and 20 mg/kg, p.o.) or clozapine (5 and 10 mg/kg, p.o.). One hour after the treatment, the catalepsy score was recorded as follows: the front paws of the rats were placed on a 9 cm elevated bar, and the time period for which the paws remained on the bar was determined. The mean value of three independent tests separated by an interval of 1 min was recorded. The animal who maintained the imposed posture for more than 10 sec was considered cataleptic. Catalepsy scoring was modified from that of Costall and Naylor protocol as follows: 0-10 sec **(0)**; 10-30 sec **(1)**; 30-60 sec **(2)**; 1-2 min **(3)** and 2 min or more **(4)** [21].

3.2.5. Rota rod test for ataxia

Rota rod test was used to evaluate the ataxia. All the rats were trained on the rota rod apparatus, adjusted at a speed of 8 rpm, till they were able to survive up to 60 sec on the spinning rod. On the day of the test to be performed, animals were treated with the test compounds (5, 10 and 20 mg/kg, p.o.) or the standard drug clozapine (5 and 10 mg/kg, p.o.). After 1 hr, animals were placed on the rotating rod for a 3 min session in two successive trials. The time period for which each animal remained on the spinning bar was recorded [22].

3.3. Effect of test compounds on DA level in rat brain striatum

For estimation of the striatal DA level, all the rats, except for the vehicle-treated control group received apomorphine (1mg/kg, s.c.). Treatment of the test compounds (5, 10 and 20 mg/kg, p.o.) or clozapine (5 and 10 mg/kg, p.o.) to the animals was given 30 min prior to the apomorphine administration. After another 30 min of apomorphine intoxication, the animals were sacrificed to dissect the striatum. Striatum was weighed and homogenized in 0.17 M perchloric acid in ratio of 10 % w/v for 30 sec using electric homogenizer to extract the DA from the tissue. The homogenate was allowed to stand for 15 min for complete protein precipitation. The resulting supernatant was decanted in a separate Eppendorf tube and centrifuged at 4 °C for 20 min at 15,000 rpm. After centrifugation, the supernatant was separated and either immediately analysed or stored at -70 °C till assayed. DA concentration was estimated using RP-HPLC coupled with electrochemical detector (Waters Corporation, USA). Estimation of DA was performed as per the previously described method with some modifications [23-25]. Briefly, a Sunfire® C18 column (4.6mm×150mm, particle size 5µm) was used and separation was carried out using a mobile phase containing methanol (15% V/V) in a solution (*p*H 4.2) of 32 mM citric acid, 12.5 mM disodium hydrogen orthophosphate 0.5 mM octyl sodium sulphate, 0.5 mM EDTA and 2 mM KCl at a flow rate of 1.2 ml/min and at an operating potential of 0.61 V. The standard calibration curves were prepared by spiking known amounts of standard (10, 20, 40, 80 and 160 ng/ml of DA) in 1 ml of pooled brain homogenates. The standard curves were used to quantify the amount of DA in each sample by calculating the area under the curve (AUC). The limit of detection (LOD) for DA was found to be 0.11±0.03 ng/ml, while the limit of quantification (LOQ) was found to be 0.34±0.03 ng/ml.

3.4. Lethality in mice

Mice were treated either with the test compounds or clozapine. Lethality was scored 14 days after administration of a single dose of each compound by determining the median lethal dose (LD_{50}). LD_{50} is the dose which causes death of 50% of the test animals. The lethality study was performed in accordance with the OECD 423 guidelines.

3.5. Statistical analysis

The data obtained from the above described experiments were analysed using GraphPad Prizm (version 5) software. The values are expressed as mean±SEM. The significant difference between different experimental groups was calculated by repeated measures of one way ANOVA while comparison with the corresponding control group was made by repeated measures of two way ANOVA followed by Bonferroni test. A value of $p<0.05$ was considered significant.