Chapter 5
Material and Methods

5 Materials and Methods

Materials

Materials required for the preparation of nanoscaffolds were poly (lactic-co-glycolic acid) (PLGA), and Pluronics F127, which were provided as a gift sample by Evonik Pvt Ltd. Mumbai, India. Memantine hydrochloride was purchased from Sigma Aldrich, and 1,4 Dioxane (solvent) was purchased from Merck, India. Acetylcholine Iodide, Butyrylcholine iodide MCA-EVKMDAEFK-(DNP)-NH2 (synthetic APP derivative), Porcine brain lipid (PBL) and Ellman's reagent 5, 5'-dithio-bis-[2-nitrobenzoic acid (DTNB) were purchased from Sigma Aldrich. IL-1β, IL-10, IL-6 and TNF α GENLISATM Mouse Kit were purchased from KRISHGEN Biosystems which consists of Lyophilized reconstitutable standards of (IL-1β, IL-10, IL-6 and TNF α), Biotin Conjugated Detection Antibody (BIO-CONJ), Streptavidin Horseradish Peroxidase (STRP-HRP), TMB (3,3',5,5'-Tetramethylbenzidine) substrate Microtiter coated Plate (12x8), wash buffer and assay diluent. PVDF membrane acceptor microplates (0.45 m pore size) and donor microplates were purchased from Merck Millipore. HighQC™ Human Bone Marrow-Derived Stem Cell (BMSc) was obtained from ACCEGEN BIOTECHNOLOGY.

Methods

5.1 Preparation of nanoscaffolds

In brief, nanoscaffolds were formulated by non-solvent-induced phase separation (N-TIPS) method with modification followed by freeze-drying [194]. According to this method, PLGA (polymer) solutions of 75/25 and 50/50 in 1:1 proportion were dissolved in Dioxane (solvent) with continuous stirring at 50-55°C. Separately, a solution of 10 mg Memantine HCl (drug) and Pluronic F127 (pore-forming agent) was prepared in water (non-solvent) with constant stirring. The drug solution was added to PLGA solution with constant stirring at 500 rpm until non-solvent induced phase separation occurred and resulted in the formation of PLGA nanoscaffolds, which were then upon pre-freezing, lyophilized at -40°C for 24 hours and then vacuum drying at room temperature (25±5°C).

5.2 Methods for determination of response variables

5.2.1 Optimization of nanoscaffolds

Optimization of the formulation was performed by using Box Behnken design as mentioned in table 1; PLGA concentration (5-20%w/v), Pluronics F127 concentration (1-5%w/v) and rotation

speed (500-1500 RPM) were used as independent variables to study the effect on formulation characteristics i.e., % porosity and drug loading (response variable). The design space consisted of a total of 15 batches.

Table 1: Box Behnken design of PLGA nanoscaffolds

Independent variables	Levels		
	-1	0	1
X_1= Concentration of PLGA (% w/v)	5	12.5	20
X_2= Concentration of Pluronics- F12 (% w/v)	1	3	5
X_3= Rotation speed (RPM)	500	1000	1500
Dependent variables (Response)		Constraints	
Y_1= Porosity (%P)		Maximize	
Y_2= Drug loading (% DL)		Maximize	

5.2.2 Determination of Drug Loading (%)

The nanoscaffold suspension was ultra-centrifuged at 12000 rpm for 10 min at 4°C in a cooling centrifuge (REMI). After centrifugation, the supernatant containing the free drug was separated. The supernatant was centrifuged again at 12000 rpm at 4°C for 5 minutes. The drug content in the supernatant layer was estimated by the UV-visible spectrophotometric method at 218 nm [195]. The drug loading was then calculated using equation 1:

$$DL(\%) = (Total\ Drug\ Content - Free\ Drug\ content)/Total\ weight\ of\ Nanoscaffolds \times 100$$

……eq 1

5.3 Characterization of Optimised Memantine loaded Nanoscaffolds

5.3.1 Surface morphology (SEM)

The Scanning electron microscope (SEM) is the most widely used technique for the characterization of nanomaterials and nanostructures. A sample solution of memantine loaded nanoscaffolds was placed on an aluminum holder precoated with aluminum glue and dried under vacuum and then observed under varying resolutions of 1000, 2000 and 5000 on a scale between 500 nm and 1μm. The size of the PLGA nanoscaffolds was observed on Zetasizer (Malvern Instruments Korea, Seoul, South Korea) at a wavelength of 633 nm and laser scattering angle of 90°he signals derived from electron-sample interactions reveal information about the sample; surface morphology of prepared memantine-loaded nanoscaffolds was assessed using scanning electron microscopy (SEM) (FEI QuantaTM 200, USA) possessing a secondary electron detector at an accelerated voltage of 10 kV.

5.3.2 Entrapment Efficiency and Drug Loading

The entrapment efficiency is the amount of drug entrapped within nanoscaffolds. The suspension was ultra-centrifuged at 12000 rpm for 10 min at 4°C in a cooling centrifuge (REMI). After centrifugation, the supernatant containing the free drug was separated. The supernatant was centrifuged again at 12000 rpm at 4°C for 5 minutes. The supernatant drug content was estimated using a UV-visible spectrophotometric method at 218 nm [196,197]. The entrapment efficiency was calculated using the equation 2:

$$DL(\%) = (Total\ Drug\ Content - Free\ Drug\ content) \,/\, (Total\ Drug\ content) \times 100 \ldots..\text{eq 2}$$

5.3.3 Biodegradability study

The biodegradability of PLGA nanoscaffold was evaluated by placing PLGA nanoscaffold (1 × 1 cm^2) in 4 mg lysozyme/mL solution in PBS (pH = 7.4) incubated at 37 °C[31]. The hydrolysis solution was refreshed by replacement of the lysozyme solution once a week. At pre-determined time intervals, samples were withdrawn from the degradation solution, rinsed, and vacuum dried. The weight loss was measured as the ratio of the weight after enzymatic hydrolysis to the PLGA nanoscaffold by using equation 3 [198].

$$Degradation\ \% = (W_i - W_f)/W_i \times 100 \ldots\ldots\ldots\ldots\ldots\ldots..\text{eq 3}$$

Where, W_i is initial weight of nanoscaffolds, W_f is final weight of nanoscaffolds.

5.3.4 Preparation of Pegylated Memantine loaded self-assembled PLGA Nanoscaffolds (PEG-MEM-PLGA) SANs

(PEG-MEM-PLGA) SANs were formulated by a simple non-solvent-induced phase separation (N-TIPS) method with modification followed by the freeze-drying technique [199]. According to this method, polymeric mixture containing 19.18% w/v PLGA with copolymer of lactide and Glycolide in molar ratio of (75:25) and (50:50) in equal proportion. This polymeric mixture of PLGA (75:25) and PLGA (50:50) was dissolved in dioxane (solvent) with continuous stirring at 50-55°C. Another solution of each of 5 mg Memantine HCl (drug) and 4.98% w/v Pluronic F127 (pore-forming agent) were prepared in water (non-solvent) with constant stirring. The drug solution was added to PLGA solution with constant stirring at 500 rpm until non-solvent induced phase separation occurred, resulting in the formation of Memantine loaded PLGA nanoscaffolds (MEM-PLGA) SANs. Further, (MEM-PLGA) SANs were coated by Polyethylene glycol (PEG) by incubating 100mg/ml aqueous solution of PEG 35,000 with constant stirring at 500 rpm for 30 min at room temperature. The resulting solution was further lyophilized by pre-freezing at - 40°C for 24 hours and vacuum drying started from - 40°C (sample temperature) and allowed to reach room temperature (25±5°C) (Figure 10) [200]. The dried (PEG-MEM-PLGA) SANs were carefully extracted and stored at cool and dry place.

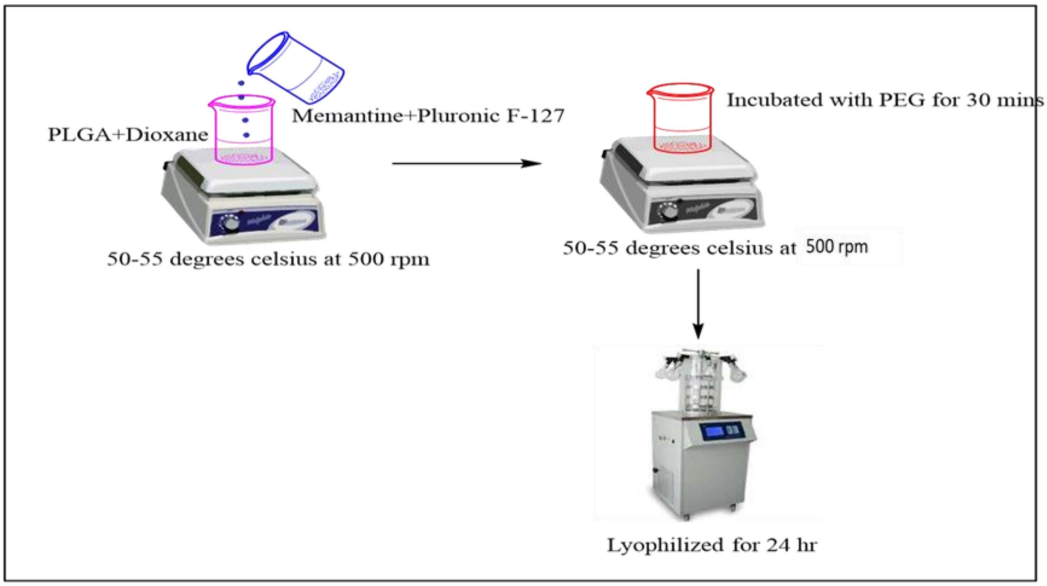

Figure 10: Preparation of (MEM-PLGA) SANs and (PEG-MEM-PLGA) SANs.

PEG-coated Memantine loaded self-assembled PLGA Nanoscaffolds were prepared by non-solvent-induced phase separation followed by desolvation procedure, and drying in a freeze drier [200]. This preparative process is simple and reproducible, without employing reactive reagents. In this process PEG layer is non-covalently positioned (though hydrophillic-hydrophobic interactions) on the surface of PLGA nanoscaffolds. PEG coating enhances the storage stability of the PLGA nanoscaffolds because PLGA being prone to hydrolytic degradation is biodegraded hence PEG coating improves its stability against hydrolytic and enzymatic degradation. Moreover, with precautions taken during storage will enhnace the shelf-life and upon lyophilization reversible shrinkage of pores and polymeric network occurs that resist hydrolytic degradation during storage.

The hydrophilic-hydrophobic interactions reduce drug crystallinity which in turn improve the rate of dissolution of the drug and polymer due to formation of hydrogen bond [201]. PEG coating on nanoparticles shields the surface from aggregation, opsonization, and phagocytosis, prolonging systemic circulation time. Moreover, studies have shown alteration in the pharmacokinetic profile of the PEGylated PLGA nanoscaffolds by delaying renal excretion, leading to a longer half-life ($t_{1/2}$) of the drug.

5.3.5 Characterization of (PEG-MEM-PLGA) SANs

5.3.5.1 Drug and polymer compatibility study (FTIR) and Thermal Characteristics (TGA and DSC)

Drug and polymer compatibility was evaluated by Fourier transform infrared spectroscopy (FTIR). FTIR spectra of PLGA 50:50/75:25, PEG, Memantine, Pluronics F127 and lyophilized (PEG-MEM-PLGA) SANs, at the same loading ratios, were collected using KBr pellet method by FT-IR spectrometer (SHIMADZU, Model 8400S, Japan). The spectra were further analysed for any change in their characteristic functional groups upon interaction (if any) with any of the ingredients present in the formulation. The thermal properties of, PLGA 50: 50/75: 25, PEG, Pluronic F127 and (PEG-MEM-PLGA) SANs were assessed by thermogravimetric analysis (TGA). Samples were sealed in an aluminium pan and heated at a rate of 10°C/min in the temperature range of 30°C to 600° C under a constant nitrogen flow of 20 mL/min through the sample chamber. All the samples were analyzed in an Automated Thermogravimetry / Differential Thermal Analysis (TG / DTA) system (Diamond TG / DTA 8.0, Perkin-Elmer, Perkin Elmer India Ltd.), in the Department of Chemistry (Banaras Hindu University), Varanasi. The thermogram and DTG plot of PLGA 50: 50/75: 25, PEG, Pluronic F127 and (PEG-MEM-PLGA) SANs were generated using the Origin software Version 19, 2022.

5.3.5.2 Surface morphology (SEM, Particle size and Zeta potential)

The surface morphology of (PEG-MEM-PLGA) SANs was examined using scanning electron microscopy (SEM) (FEI Quanta™ 200, USA) at 10 kV of accelerated voltage. The sample solution (pH 6.8) was placed on an aluminum holder precoated with aluminum glue under vacuum and then observed under varying resolutions of 1000, 2000 and 5000 on a scale of 200-500 nm.

Particle size, particle size distribution (PDI), and zeta potential of (PEG-MEM-PLGA) SANs were evaluated using Zetasizer (Malvern Instruments Korea, Seoul, South Korea) at a wavelength of 633 nm and laser scattering angle of 90°.

5.3.5.3 Determination of Porosity (%)

The Porosity (P) of the (PEG-MEM-PLGA) SANs was determined by ethanol displacement method with modification [202]. Initially, a known weight of nanoscaffold was taken and immersed in a known volume (V_1) of ethanol in a graduated test tube for 5 min to allow efficient penetration of ethanol within the pores of nanoscaffolds (until no air bubbles were released). Then the total volume of ethanol (V_2) was recorded. The final residual volume of ethanol was calculated after removal of impregnated nanoscaffolds (V_3). The volume of ethanol impregnated

into nanoscaffold is represented by (V_1-V_3), and the (V_2-V_3) is the total volume of the scaffold. The porosity of nanoscaffolds (%) was estimated using the following equation 4 [203,204].

$$P = \frac{(W-W_0)V}{q} \; P = \frac{V_1-V_3}{V_2-V_3} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{eq 4}$$

The weight and volume of the nanoscaffold are W_0 and V respectively, W and q represent the weight of ethanol after immersion of nanoscaffolds and density of ethanol (0.789 g/mL), respectively.

V_1 represents the volume of liquid impregnated into nanoscaffold, V_2 is liquid-impregnated nanoscaffold volume and V_3 is the remaining fluid volume after removal of liquid-impregnated nanoscaffold.

5.3.5.4 Entrapment Efficiency

The entrapment efficacy is the amount of drug encapsulated in the nanostructure. The free drug was estimated from the supernatant after ultracentrifugation of the suspension at 12,000 rpm for 10 min at 4 °C in a centrifuge (REMI). The sample was again centrifuged for 5 min at 12,000 rpm at 4 °C. The free drug content was determined by UV-Visible spectrophotometry at 218 nm [196,197].

The entrapment efficiency (EE%) was calculated using equation 5:

$$EE\ (\%) = \frac{Total\ drug\ content - Free\ drug\ content}{Total\ drug\ content} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{..eq 5}$$

5.3.5.5 Biodegradability study and Swelling index study

The biodegradability of (PEG-MEM-PLGA) SANs was studied by placing PLGA nanoscaffold in 4 mg lysozyme/mL solution in acidic and phosphate buffers (pH 1.2, 5.7, 6.8, 7.4) incubated at 37 °C [198]. The hydrolysis solution was regenerated by addition of fresh lysozyme solution at the end of every week. The solution was periodically sampled, rinsed and dried in vacuum, following which the weight loss was measured in reference to weight before enzymatic hydrolysis. W_i is the initial weight before degradation and W_f is final weight observed after biodegradation in the equation 6.

$$Degradation\ \% = W_i - \frac{W_f}{W_i} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{eq 6}$$

Swelling index study was determined by immersing 1g weight (*Wd*) of (PEG-MEM-PLGA) SANs in 15 ml of buffer solution (acidic and phosphate-buffered saline) at four pH 1.2, 5.7, 6.8, 7.4 at 37 °C and then weight (*Ww*) of swollen (PEG-MEM-PLGA) SANs was taken. The swelling index (S) was calculated using the following equation 7 [205].

$$S = [(W_w - W_d)/W_d] \times 100 \ \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{eq 7}$$

Where S, *Ww* and *Wd* indicate degree of swelling, wet and dry weight of the nanoscaffolds

respectively. The swelling index of (PEG-MEM-PLGA) SANs was plotted vs pH to understand the effect of pH on the swelling behavior of (PEG-MEM-PLGA) SANs.

5.3.5.6 Stability Studies

According to ICH Q1A (R2) Stability Testing of New Drug Substances and Products guidelines recommended by the International Conference of Harmonization, accelerated Studies are designed to understand the stability conditions in short-term excursion conditions. The exaggerated storage conditions such as temperature and relative humidity (RH) lead to an increase in the rate of chemical degradation and physical change of a drug and excipients of the formulation. To understand stability conditions of (PEG-MEM-PLGA) SANs, an stability study was performed by placing the sample in undisturbed conditions for three months under different environmental conditions (temperature and relative humidity): 25 °C ± 2°C, 60±5% RH, and other at 40°C ± 2°C/75 ± 5%RH (accelerated stability conditions) in stability chambers. After three months, (PEG-MEM-PLGA) SANs samples were analysed for changes in entrapment efficiency and % porosity [206].

5.4 In-vitro studies

5.4.1 Drug Release study and Kinetic model

In-vitro drug release was estimated by using dialysis bag diffusion method in acidic buffer (pH 1.2 and 5.7) and phosphate buffer (pH 6.8 and 7.4) [207–209]. The dialysis bag (cellulose membrane mol. wt. cut-off 12-14000 Da, HIMEDIA, India) hydrated overnight in dissolution media was used to hold 10mg of (PEG-MEM-PLGA) SANs. (PEG-MEM-PLGA) SANs containing bag was placed in a release chamber containing 100 ml of dissolution media under continuous stirring at 75 rpm at 37±0.5 ºC. At fixed time intervals (0, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 48 and 72 h) 5 ml aliquots of media were withdrawn with replacement of equal volume of fresh media to maintain sink conditions. The drug content was analysed at 218 nm by UV-Vis spectroscopic method and cumulative percent drug release (CPR) was determined after filtration with a 0.2 µm syringe filter and graph was plotted between CPR and time. The experimental data was used to determine the best fit kinetic model (zero-order, first-order, Higuchi and Peppas – Korsemeyer) and drug release pattern based on correlation coefficient (R^2).

The dialysis bag diffusion method was used for estimation of *in vitro* release pattern of memantine in phosphate buffer pH 6.8 from PLGA nanoscaffolds and PEG coated PLGA nanoscaffolds and was compared with memantine HCL (Admenta 10 mg) tablet and pure memantine drug suspension [210]. As the pH at neurodegenerated site in brain is 6.8, and also, because at this pH self-assembly of nanoparticles is facilitated within the brain which will act

as extracellular matrix for growth and regeneration of neurons, pH 6.8 was selected for the release studies [211].

5.4.2 Blood-brain barrier permeation assay (PAMPA-BBB)

The penetration of Memantine HCL (MEM-PLGA) SANs, PEG-MEM-PLGA SANs and market memantine HCL tablet (Admenta 5 mg) across blood-brain barrier was determined by parallel artificial membrane permeation assay (PAMPA- BBB) as reported previously with modifications (Figure 11) [212].

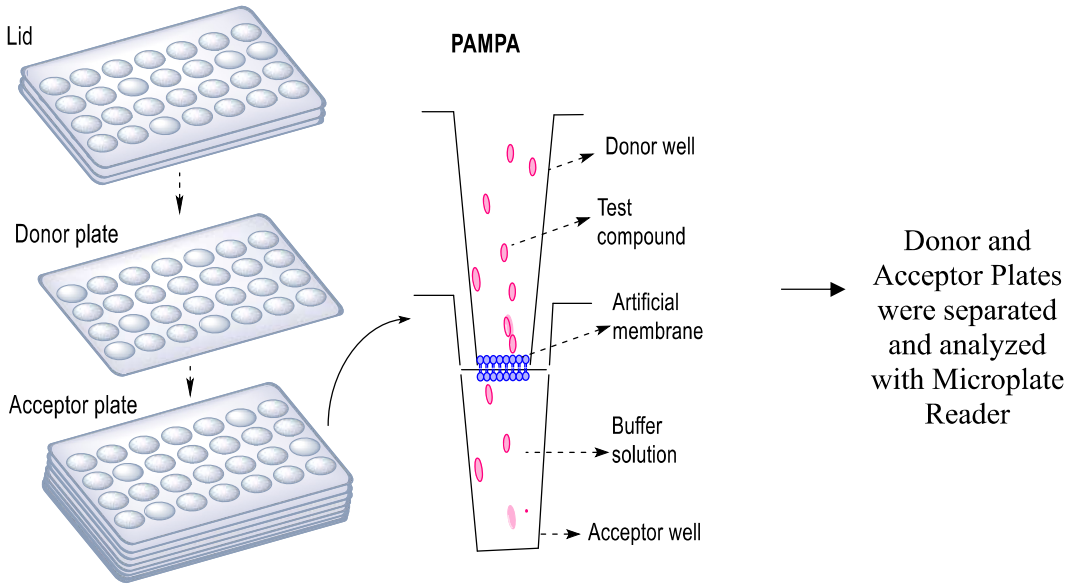

Figure 11: Blood-brain barrier permeation assay of MEMp, (MEM-PLGA) SANs and (PEG-MEM-PLGA) SANs.

Briefly, the acceptor plates were soaked in 4 µL of 20 mg/mL Porcine Brain Lipid solution prepared in dodecane and then filled with 300 µL of buffer (pH 6.8). The acceptor plate was left overnight for saturation. The drug and nanoscaffolds (10 mg each) were dissolved in 1 mL DMSO and 5 µL of drug and nanoscaffolds solution were further diluted with 70% combination of DMSO and pH 6.8 in 1:6 ratio to achieve final concentration of 25 µg/mL. 300 µL of the drug and nanoscaffolds in DMSO at a drug concentration of 25 µg/mL was added in triplicate to the donor well plates. The acceptor plate (200 µL) was carefully placed on the donor plate and incubated for 18 hours. Then acceptor and donor plates were carefully separated and absorbance of blank (buffer, pH 6.8), donor, acceptor and reference wells were measured in triplicate with a Microplate reader to calculate effective permeability (Pe*)* value using the following equation 8:

$$P_e = [(V_D \times V_A)/(V_D + V_A) \times A \times T] \times \ln [\frac{Drug\ acceeptor}{Drug\ equilibrium}]]\ldots\ldots\ldots\ldots\ldots\text{eq 8}$$

Where V_D is volume of donor compartment, V_A is volume of acceptor compartment, A is surface

area at defined porosity. V_A and V_D are the acceptor well volume (0.2 ml) and donor well volume (0.3 ml). A is 0.2826 cm^2 filter area and T (64800 s) is time. Drug acceptor and drug equilibrium are the concentration of drug in acceptor wells and the equilibrium drug concentration is the concentration of drug and nanoscaffolds in the total volume of the donor and acceptor compartments.

As higher DMSO concentration may alter the membrane permeability andcould induce the penetration of hydrophilic compound in a concentration-dependent manner therefore, overnight pretreatment of the porcine brain lipid membrane with DMSO were initially performed to saturate the membrane which will not affect the permeability of the membrane in accountable manner [213].

5.5 Ex-vivo Study

5.5.1 Animal experiments

The research protocol for behavioural studies has been approved by the Animal Ethical Committee of the Institute of Medicine of the Banaras Hindu University (Varanasi, India) (CPCSEA / 2019 / iAEC). Swiss albino mice weighing 40±5 g were housed in conditioned animal cages for 12 h with alternating light and dark cycles at 20±2 °C and 50–60 % relative humidity. Free access to pelleted food and distilled water *ad libitum* was provided to the animals.

5.5.2 Enzyme Kinetic study against Acetylcholinesterase (AChE), Butyrylcholinesterase (BUChE and β-Secretase in Cortex and Hippocampus

The three-month-old mice were sacrificed and their brains were isolated; cortex and hippocampi were separately dissected, weighed and homogenized in 10 mM cold Tris-HCl buffer (pH 6.8) containing 160 mM sucrose. The homogenate was centrifuged at 10,000 rpm at 4°C for 10 minutes and the resulting clear supernatant was thawed and stored at -20°C for further use. The acetylcholinesterase, butyrylcholinesterase and secretase enzymes were derived from the supernatant of brain tissue homogenate [214].

Briefly, enzyme samples were incubated in 20 mM phosphate buffer (pH 6.8) in the presence of 10 mM 5,5'-dithio-bis-[2-nitrobenzoic acid (DTNB with various concentrations of (PEG-MEM-PLGA) SANs (0, 2.5, 5 and 20 μM). The initiation of enzymatic reaction occurs after the addition of different concentrations of acetylthiocholine iodide and butyrylthiocholine iodide as substrate (10, 20, 30, 40 and 50 μM) followed by the formation of the yellow anion of the 5-thio-2-nitrobenzoic acid. Acetylcholinesterase and butyrylcholinesterase respectively catalyse

the conversion of acetylthiocholine to acetyl and thiocholine, and butyrylthiocholine to butyric and thiocholine. Then, thiocholine binds to Ellman's reagent (DTNB) to give a yellow colour due to formation of the 5-thio-2-nitrobenzoic acid which is measured by colorimetric assay. Absorbance was measured every 30 s for 3.5 minutes at 412 nm with a SpectraMax 190 microplate reader.

BACE1 (β-secretase or APP β−Site Cleaving Enzyme) is a transmembrane protease responsible for the production of amyloid β-peptide (Aβ) by cleaving amyloid precursor protein (APP). The accumulation of Aβ in the brain is a major cause of the development of Alzheimer's disease. The β-secretase inhibitory activity of (PEG-MEM-PLGA) SANs was determined by an earlier reported method with modifications [215]. The β secretase enzymatic reaction was initiated by the addition of different concentrations of MCA-EVKMDAEFK-(DNP)-NH2 (synthetic APP derivatives) (75, 150, 300 and 450 μM) which were incubated with 10 mM DTNB at different concentrations of (PEG-MEM-PLGA) SANs (0, 4, 8 and 16 μM) in phosphate buffer (pH 6.8) for studying the inhibition of β secretase. The hydrolysis of substrate was monitored by the formation of 7-methoxycoumarin- 4-yl acetyl (MCA) at 520 nm every 30 seconds for 3.5 minutes in SpectraMax 190, 96-well plate reader. In brain regions (cortex and hippocampus), inhibition levels were assessed after 60 minutes of incubation, with each test performed in triplicate. The Inhibition (%) was calculated using the following equation [216]:

$$Inhibition(\%) = [1 - \{\frac{S-S_0}{C-C_0}\}] \times 100 \text{.....eq 9}$$

where C and C_0 represents fluorescence of control (in enzyme and substrate buffer) after 60 min incubation and time zero respectively. S and S_0 is the fluorescence of the test sample (enzyme, sample solution and substrate) after 60 minutes of incubation and at time zero respectively. All experiments were repeated in triplicate and presented as mean and SD.

Lineweaver-Burk plots of reciprocal of reaction velocity (1/v) plotted with reciprocal substrate concentration (1/[S])) were constructed. Dixon plots (1/v curve as a function of inhibitor concentration for each substrate concentration) were used to determine the inhibitor Ki constant. The kinetic parameters Km (Michael-Menten constant) and Vmax (maximum velocity) were determined from the Lineweaver-Burk curve for four different doses of (PEG-MEM-PLGA) SANs. The half-maximal inhibitory concentration (IC_{50}) was calculated from the prism curve (Graph Pad 5.0, Dose Response - Inhibition), which was obtained by plotting percent inhibition as a function of concentration.

5.5.3 Hemocompatibility study

The hemocompatibility assay was performed in anticoagulant tubes on blood collected freshly

from healthy mice. Five different concentrations (2, 4, 6, 8, 10 and 12 mg/ml) of Memantine pure drug (MEMp), Memantine loaded PLGA self-assembled nanoscaffolds (MEM-PLGA) SANs, PEG coated memantine loaded PLGA self-assembled nanoscaffolds (PEG-MEM-PLGA) SANs were prepared in sterile saline water. 0.02 ml of blood was added to the six prepared concentrations of samples and incubated for 60 min in a water bath shaker at 37°C. Blood in distilled water and blood in sterile saline water were considered as positive and negative control respectively. Each incubated blood sample was then centrifuged at 1000 rpm for 10 minutes. The amount of free haemoglobin (haemolysis resulting in red blood cell breakdown and haemoglobin release) was measured by the absorbance of the supernatant at 540 nm with a UV spectrophotometer using equation 10. In this study 0.02 ml of blood was added to saline water for negative control (0% haemolysis) and Triton X-100(1%v/v) for positive control (100% haemolysis).

$$Percent\ Hemolysis\ (\%H) = \frac{At-Anc}{Apc-Anc}\ X\ 100 \quad \text{.....eq 10}$$

where At is the absorbance of (PEG-MEM-PLGA) SANs, Anc is the absorbance of negative control and Apc is the absorbance of the positive control. The experiment was performed in triplicate. 5% haemolysis is indicative of strong hemocompatibility behaviour, while 10% shows compatible and > 20% is indicator of incompatibility with blood.

5.6 Stem cell grafting

HighQC™ Human Bone Marrow-Derived Stem Cell (BMSc) was obtained from ACCEGEN BIOTECHNOLOGY. Briefly, BMSc cells were grown in Minimum Essential Media (MEM), 5×10^4 cells/well incubated with tetracycline positive and tetracycline negative conditions in CO_2 incubator in a well plate. BMSc were trypsinized, dispersed in 20% Foetal Bovine Serum (FBS) and incubated at 37 ℃ and 5% CO_2 with (PEG-MEM-PLGA) SANs to allow the cells to adhere and develop monolayer cells for 1-3 days on the surface of nanoscaffolds up to confluence, cells were then fixed by 4% paraformaldehyde with aspiration of media for 10 min to obtain PEG coated memantine loaded PLGA self-assembled nanoscaffolds grafted with Bone Marrow-Derived Stem Cell (BMSc) [(PEG-MEM-PLGA) SANs-BMSc (Figure 12) [217]. All the experiments were performed in UV sterilized biosafety cabinet.

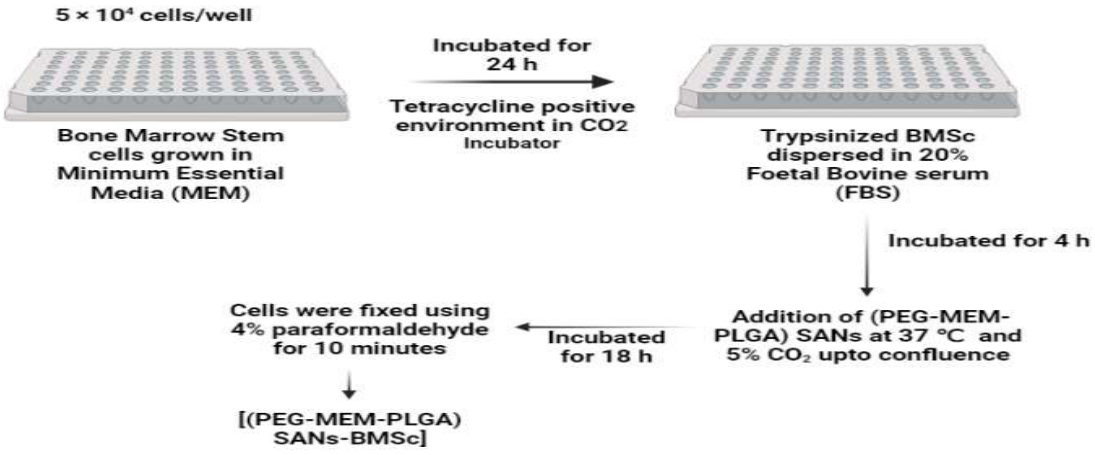

Figure 12: Preparation of (PEG-MEM-PLGA) SANs-BMSc under Biosafety cabinets.

5.6.1 Neurobehavioral studies

5.6.2 Scopolamine induced amnesia model and treatment groups

Scopolamine induced amnesia model was used for *in vivo* evaluation of behavioural activity in animals. The animals were categorized into 7 groups (6 animals /group): Group I was administered with normal saline solution 0.1 ml/10g (Control group); Group II was treated with PEG coated memantine loaded self-assembled nanoscaffolds (PEG-MEM-PLGA) SANs (10 mg/kg) and; Group III was treated with PEG treated memantine loaded self-assembled nanoscaffolds grafted with bone marrow stem cells [(PEG-MEM-PLGA) SANs-BMSc] (10 mg/kg) dispersed in water injected via intrathecal administration. Group IV was administered with memantine HCL tablets (Admenta 10 mg/kg) (marketed formulation) dispersed in water via intra-oral gavaging. Group V was administered Memantine (pure drug) as a standard dose equivalent to 10 mg/kg body weight via intra-oral gavaging. Group VI was administered (MEM-PLGA) SANs at a dose equivalent to 10 mg/kg body weight intrathecally. Group VII was diseased control group administered with normal saline.

The neurobehavioral and pharmacokinetic studies were carried out at same dose to evaluate the effectiveness of intrathecal route over oral therapeutics. We have used a dose 10 mg/kg by using dose calculation method [218]. Equal dose of test, standard and marketed drug of memantine has been used in the study to negatethe probability of additional variability that can be induced due to varying dosage. Admenta is marketed oral dosage form used for the treatment of Alzheimer's disease, and was used to evaluate whether the proposed intrathecal route exhibits improvement in the therapeutic properties of the drug.

5.6.3 Y-Maze Test

Y maze test was performed by following the earlier reported protocol with modifications

[219].Y maze consists of three-arms named A, B and C with each arm being 8 cm high. Initially (during training), the animals were trained to explore the arms of the maze by placing them on one arm of the maze (start arm C). The free exploration activity of mice in arms was observed for 90s in the test trial. The number of entries and time spent in each arm and the first choice of entry were recorded by an observer. These animals were further examined for spontaneous alteration test and the % alteration was determined for each animal. The reduced % alteration represents the impairments in cognitive function of animals exhibiting Alzheimer's behaviour. Other test-related parameters were estimated using Any-maze software and then analysed by applying one-way ANOVA followed by a Tukey post-hoc comparison test. The number and the sequence of arms entered were estimated and percent spontaneous alternation was evaluated by the following equation 11 [219].

$$\%\ Alteration = \frac{No.of\ alterations}{Total\ No.of\ arm\ enteries-2} * 100 \quad \text{.....eq 11}$$

5.6.4 Morris Water Maze Test

The Morris Water Maze test was performed according to the earlier reported method with modifications [220]. The animals were placed in a circular pond of 124 cm diameter filled with opaque water (milky water). During the visual test of the platform, the platform was raised by 0.5 cm above the water level on the first training day, the mice were placed in the pond 30 seconds before the first exercise and were allowed to practice swimming and climbing on the platform for 30 seconds. The mice were then placed on the pool wall facing the water and allowed to search the platform with a rest period of 60 seconds at the end of each experiment. Mice were then immediately returned to the water and allowed to swim and find a platform from a different starting point. The animals were trained every day for 3 days. After the training period, the test was performed on the 4th day. In the test condition mice were placed in pond filled with opaque water and the platform was hidden by raising the opaque water level by 1cm. Then mice were allowed to search platform by placing in the quadrant opposite the location of the training platform for 90 sec. After that mice were taken out and dried with clean towel and placed in the cage. This test was performed for each animal in each group. In each experiment, the delay in reaching the previous position on the platform is measured. The time spent on each northwest (NW), northeast (NE), southwest (SW), southeast (SE), and central platform for looking platforms and the number of times the mice search the quadrant are recorded and evaluated by Any-Maze software. All the estimated parameters were further analysed by one-way ANOVA followed by Tukey's post hoc test.

5.7 Estimation of pro-inflammatory cytokines

5.7.1 Animal model and treatment groups

The research protocol was approved by the Animal Ethical Committee of the Institute of Medicine of the Banaras Hindu University (Varanasi, India) (CPCSEA / 2019 / iAEC). Swiss albino mice weighing 40±5 g was housed in conditioned animal cages for 12 hours with alternating light and dark cycles at 20±2ºC and 50–60% relative humidity. Free access to pelleted food and distilled water ad libitum were provided to the animals. Animals were sacrificed after 60 min of administration of doses. Subsequently, brain from each animal was isolated, weighed and homogenized in cold 10 mM Tris–HCl buffer, pH 7.4, containing 160 mM sucrose. The homogenates were centrifuged at 15,000 rpm for 10 min at 4 ºC, and the resulting clear supernatants were used as enzyme sources that were divided into aliquots and stored at −20 ºC.

Three-month old Swiss albino mice model has been used in study as young mice exhibit higher tolerance to external aversive stimuli used during neurobehavioral, in-vivo and pharmacokinetic studies as compared to 18-20-month-old mice (which represent older life stage). As, old age mice might already have memory impairment, severity and complexity of which might not be known, so use of 3-4 months old mice may ensure absence of the former. In addition, when memory impairment is induced in the young mice model, it is expected that the model exhibits similar pathophysiology with little variation. Hence it becomes easier to classify the animals in different group based on the appropriate parameters with minimum variability [221].

Memory impairment was induced in mice by scopolamine HBr at a dose of 3 mg/kg (administered Intraperitoneally (IP)) once a week up to 21 days [222]. The animals were divided into seven groups (6 animals /group). T1 group was administered with a dose of 10 mg/kg memantine loaded PLGA self-assembled nanoscaffolds (MEM-PLGA) SANs intrathecally, T2 group received 10 mg/kg PEGylated memantine loaded PLGA self-assembled nanoscaffolds (PEG-MEM-PLGA) SANs intrathecally, T3 group was treated with 10 mg/kg PEGylated memantine loaded PLGA self-assembled nanoscaffolds grafted with bone marrow stem cells [(PEG-MEM-PLGA) SANs-BMSc] intrathecally, group T4 was treated with 10 mg/kg memantine HCl tablets (Admenta) by dispersing in water via intra-oral gavaging, group T5 was administered with 10 mg/kg memantine (pure drug) (MEMp) intrathecally, and T6 control group and T7 diseased control group were administered normal saline solution (0.1 mL/10g).

The pro-inflammatory cytokines such as IL-1 β, IL-6, IL-10 and TNF-α were determined in serum, cerebral tissue, hepatic tissue and renal tissue of scopolamine induced amnesia model of

mice (AD mice) following instructions provided in the ELISA Kit manual. This was also used for the determination of effect of (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs, [(PEG-MEM-PLGA) SANs-BMSc], Memantine pure drug (MEMp) and Memantine HCL tablet (Admenta) on secretion, distribution and cellular uptake of pro-inflammatory cytokines in serum, cerebral, hepatic and renal tissues in AD mice. The origin and effect of pro-inflammatory cytokines on neurons and Aβ plaque have been shown in table 2.

Table 2: Effect of pro-inflammatory cytokines on neurons and Aβ plaque

Pro-inflammatory cytokines	Origin in CNS	Effects on neurons	Effect on Aβ	References
IL-1β	Microglia, Astrocytes	Neurodegeneration and synaptic loss	Aβ synthesis increases	[223]
IL-6	Microglia, Astrocytes, endothelial cells and hepatocytes cells	Rescue of damaged neurons and prevent synaptic loss	Decrease Aβ deposition	[224]
IL-10	Microglia	Pro-apoptotic	Increases Aβ synthesis	[225]
TNF-α	Astrocytes, Microglial neurons	Pro-apoptotic, prevents apoptosis and synaptic excitotoxicity	Increases Aβ synthesis but decrease Aβ clearance	[226]

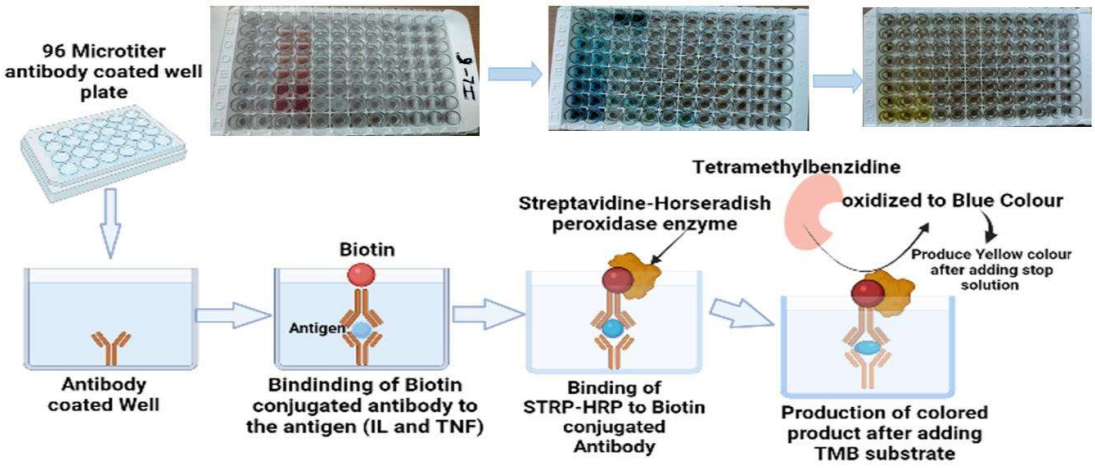

Figure 13: Overview of Enzyme linked immunosorbent assay (ELISA) and quantitative estimation of Biomarkers including pro-inflammatory cytokines.

The lyophilized recombinant mouse IL-1 β, IL-6, IL-10 and TNFα was suspended in 600 µL (only for IL-1 β) and 20 µL of water and allowed to rest for 15 min after gentle agitation to prepare 1000 pg/mL, 1µg/mL, and 0.5 µg/mL of stock solution respectively. Then serial dilutions of stock solution were done to obtain standard concentrations: IL-1 β – 500, 250, 125,

62.5, 31.3 and 15.63 pg/mL from 1000 pg/ml stock solution; IL-6 -2000, 1000, 500, 250, 125, 62.5 and 31.3 pg/mL; IL-10-1000, 500, 250, 125, 62.5, 31.3 and 15.6 pg/mL; TNFα-1000, 450, 225, 112.5, 56.25, 28.13, 14.06 and 3.5 pg/mL. 100 µL of each standard dilution and test samples (serum, cerebral tissue, hepatic tissue and renal tissue homogenates of mice from treatment, control and diseased control group) were added to the Microtiter 96 well plate, sealed and incubated for 2 h at room temperature at 18-25 °C to facilitate the immobilization of IL-1β, IL-6, IL-10 and TNF-α. The immobilization is achieved due to the binding of IL-1β or IL-6 or IL-10 or TNF-α present in sample and standard with antibody (specific to IL-1β or IL-6 or IL-10 or TNF-α) adsorbed on the surface of 96 well plates. The well plates were washed and blotted four times with wash buffer to remove non-specifically bound protein and antibody. Then, each well was filled with Biotin-conjugated detection antibody 100 µL (BIO-CONJ) and incubated for 1 hour between temperature 18-25 ºC. After 1 hour incubation, biotin-conjugated antibody gets bound to immobilized IL-1β or IL-6 or IL-10 or TNF-α present in the wells. Further, 96 well plate was washed 4 times, streptavidin-s (100 µL) enzyme was added and incubated for 15-60 minutes at 18-25 ºC. Streptavidin-HRP enzymes get covalently linked with (IL1β or IL-6 or IL-10 or TNF-α) -conjugated BIO-CONJ antibody complex, to form streptavidin HRP- (IL1β or IL-6 or IL-10 or TNF-α)-BIO-CONJ enzyme-antibody complex. Thereafter, 96 well plate was washed again and TMB (3,3',5,5'-Tetramethylbenzidine) substrate (100 µL) was added and incubated in dark for 15-30 minutes at 18-25 ºC. The incubation resulted in binding of TMB substrate to the streptavidin HRP-IL1β-BIO-CONJ enzyme-antibody complex with production of blue colour indicating the presence of IL-1 β or IL-6 or IL-10 or TNF-α in serum and tissue homogenates, which becomes yellow after addition of 100 µL stop solution. Finally, the absorbance was recorded in Synergy H^1 microplate reader at 450 nm for the quantitative estimation of IL-1β (Figure 13).

5.8 Pharmacokinetic and Biodistribution study

5.8.1 Animal Experiments

Swiss albino mice, weighing 40±5 g, were housed in conditioned animal cages for 12 h with alternating light and dark cycles at 20±2 °C, 50–60% relative humidity, and free access to pelleted food and distilled water ad libitum. All the experimental protocols of animal followed the guidelines and approved from Animal Ethical Committee of the Institute of Medicine of the Banaras Hindu University (Varanasi, India) (CPCSEA / 2019 / iAEC). Animals were selected randomly from the animal house of the Department of Pharmaceutical Engineering and Technology, Indian Institute of Technology (Banaras Hindu University), Varanasi, India and

acclimatized before the experiment for 7 days under standard in-house conditions. Biodistribution, pharmacokinetics and pharmacodynamics of the drug and formulation were investigated in mice. Mice were divided into seven groups (each n = 6) namely, Group I was administered with normal saline solution 0.1 ml/10g (Control group); Group II was treated with PEG coated memantine loaded self-assembled nanoscaffolds (PEG-MEM-PLGA) SANs (10 mg/kg) and; Group III was treated with PEG treated memantine loaded self-assembled nanoscaffolds grafted with bone marrow stem cells [(PEG-MEM-PLGA) SANs-BMSc] (10 mg/kg) dispersed in water injected intrathecally. Group IV was administered with memantine HCL tablets (Admenta 10 mg/kg) (marketed formulation) dispersed in water via intra-oral gavaging. Group V was administered Memantine (pure drug) as a standard dose equivalent to 10 mg/kg body weight intrathecally. Group VI was administered (MEM-PLGA) SANs at a dose equivalent to 10 mg/kg body weight intrathecally. Group VII was a diseased control group administered with normal saline. Serial sampling was performed in which blood was collected from the intraocular vein from the eye at 5 min, 15 min, 30 min, 45 min, 60 min, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h, and 5day post-dose and kept in heparinized microcentrifuge tubes. About 0.3 mL of normal saline was administered after every blood sample to compensate for blood loss. The plasma was separated by centrifuging the heparinized blood at 12000 rpm for 10 min and stored at -80 °C before analysis.

The organs including brain, liver, and kidney were dissected post-dose at 5 min, 15 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, and 5 days from chloroform anesthetized decapitated mice. The organs were rinsed with 0.9% NaCl and blotted using tissue paper to remove adherent blood and fatty matter. Dissected brain, liver, and kidney were homogenized in phosphate buffer saline (pH 7.4 PBS) using high-speed homogenizer and filtered to obtain tissue homogenate. The tissue homogenates were further centrifuged at 12000 rpm for 15 min and stored at -80 °C before analysis. The drug concentration in brain, liver, and kidney tissue homogenate was estimated at the specified time interval post-dosing using High-performance liquid chromatograph (HPLC) method.

5.8.2 High Performance Liquid Chromatography analysis of the pharmacokinetic and Biodistribution study

Apparatus: HPLC analysis was conducted using the earlier reported method with slight modifications [223]. RP-HPLC instrument equipped with the following modules was used for the study: Degasser, quaternary pump (LC 20AD), manual sampler, and UV-Visible detector (Shimadzu, Columbia, MD, USA). The column (Nucleosil® C18 Macherey-Nagel 150 mm × 4.6 mm, 3 µm particle size 100 Å) was thermostated at 40°C.

Chromatographic conditions

The mixture of acetonitrile and water (80:20 v/v) was used as the mobile phase at a flow rate of 1.0 mL/min. The column temperature was kept at 25°C, the injection volume of 20µL was used with gradient elution mode and detection was carried out at 240 nm using a UV detector. Data acquisition was performed and analyzed using Shimadzu Lab Solution software [227].

Stock solution preparation

Stock solution (1 mg/mL) of Memantine was prepared in acetonitrile, and the dilutions were prepared in acetonitrile to obtain the working stock solutions (5, 10, 50, 100, 500, 1000 µg/mL). After that, the prepared solution was subjected to ultracentrifugation at 15000 rpm, 4 °C for 15 min to get the clear supernatant solution and filtered through 0.22 mm Millipore membrane filter, and 20 µL of the sample was injected into HPLC system. Calibration sample solutions of memantine in mice plasma were prepared by spiking 1 mL of each working solution in 5mL of plasma separately and calibration plot was prepared. Stored plasma samples for the pharmacokinetic study, were vortexed for 1 min then centrifuged at 15000 rpm for 15 min at 4 °C to get a clear supernatant solution and filtered through 0.22 mm millipore membrane filters, and 20 µL of the sample was injected in HPLC system. Similarly, for chromatogram of blank, plasma was precipitated by acetonitrile, filtered through 0.22 mm millipore membrane filter and injected into HPLC system.

The pharmacokinetic parameters such as peak serum concentration (Cmax), time for peak serum concentration (tmax), AUCtotal, biological half-life(t1/2), elimination rate constant (Ke), clearance, volume of distribution and mean residence time (MRT) were calculated by using the Kinetica software (version 5.0, Innaphase Corporation, Philadelphia, PA). The values have been expressed as mean ± SD. The statistical comparison of data of two samples was performed by one-way ANOVA followed by Tukey post hoc test using Graph pad prism software (version 5.02.2013, GraphPad Software, San Diego, CA) and $p<0.05$ was considered as statistically significant.

5.9 Pharmacodynamic study

The pharmacodynamic study is primarily characterized by the molecular and functional effects produced by an intervention of treatment that may or may not correlate with biological and clinical effects including molecular, cellular, histopathological, and imaging parameters. These cellular and biological effects usually reflect the altered activities in response to a mechanism-based therapy [228]. The objectives of pharmacodynamic studies are generally based on the principle of selection of the optimal dose and schedule of a drug in conjunction with factors

such as pharmacokinetics and drug toxicity.

5.10 Biochemical parameters

Biochemical parameters :Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Bilirubin total, Bilirubin direct, Serum potassium, Serum calcium, Serum phosphorus, Serum uric acid, Serum sodium, Serum cholesterol and Blood urea of animals from each group were estimated using diagnostic kit (Labtest Diagnostica SA, Lagoa Santa, MG, Brazil). Blood was collected in heparinized tubes and then centrifuged at 12000rpm for 15min and serum were separated, stored at -20 °C, and used for biochemical analysis [229].

5.11 Histological analysis

At the end of the experiment, mice were weighed, sacrificed and blood samples were collected by cardiac puncture. Mice were anesthetized and the brain was carefully separated from body and weighed. Brain was separated from sacrificed anesthetized mice, rinsed in cold saline, dried and weighed, and fixed in buffered formalin 10% and stored for histological study. The tissues were embedded in paraffin wax, sectioned in 5 μm slices, and then mounted on glass slides. The slides were deparaffinised and stained with haematoxylin and eosin [230]. The sections were examined under microscope and visualized through the Color-View Camera Nikon OptiPhot microscope and Ni-kon Digital Sight camera using NIS-Elements software. The histological analyses were based on the following parameters: atrophy, accumulation of amyloid β plaque, neurodegeneration, and cerebral necrosis. For each parameter a score grade has been used to determine the severity of disease: grade zero (absent), grade 01 (+/mild), grade 02 (++/moderate) and grade03 (+++/intense).

5.12 Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey test as post-test. The results were expressed as mean ± standard error of the mean (SEM), using software GraphPad Prism® version 5.00.P <0.05 was considered to be statistically significant.