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Formulation preparation, characterization, optimization, behavior and histological evaluation of brain hippocampus for brain targeted PLGA-Soya lecithin-Tween 80 nanoparticles in an Alzheimer's disease model

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ABSTRACT

In present study, we formulated RT loaded PLGA-Soya lecithin-Tween 80 NPs, for the treatment of Alzheimer's disease (AD). After formulation we optimized formulation by Response Surface Methodology (RSM) using 3^2 factorial design. PLGA-Soya lecithin-Tween 80 nanoparticles were synthesized by modified nanoprecipitation technique combined with self assembly. Influence of important factors on the particle size, polydispersity, entrapment efficiency and in vitro drug release were studied. FTIR and DSC studies demonstrated that there was no interaction between drug, polymers and lipid and they were compatible with each other. Prepared nanoparticle of optimized formulation (D10) showed particle size 171.74 nm, polydispersity 0.154, entrapment efficiency 66.171 % and in vitro drug release 67.336 ± 0.254% (60h). Zeta Potential and stability study for six months demonstrated that the formulations were stable at refrigerator (3-5°C) condition is most suitable for storage of nanoparticles. SEM studies results indicated that the NPs were spherical in shape and smooth at surface. In vivo behavioral studies, AchE activity analysis and histological study of hippocampus demonstrated that, the rats treated with NPs showed markedly better memory retention and better brain condition compare to pure drug treated. The study demonstrated the successful attempt to target brain with RT loaded PLGA-Soya lecithin-Tween 80 NPs, with considerable therapeutic prospective to treat AD and potential carrier for providing sustained brain delivery of RT.

Key words: PLGA-Soya lecithin-Tween 80 nanoparticles, Alzheimer's disease, Brain targeting, Rivastigmine Tartrate, Morris water maze

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INTRODUCTION

Alzheimer's disease (AD) is a fast growing disease; with a population of about 18 million people over the world is suffering from Alzheimer's disease. This number is projected to add up to 34 million by 2025 and expected to comprise majority of the aging population. Developing countries are the unfortunate abode of 50% of the affected population, and estimated to reach 70% by 2025[1]. Moreover, Alzheimer's disease has a tremendous negative economic impact amounting to over $ 100 billion a year[2].

This progressive neurodegenerative disease, characterized by a global cognitive decline, behavioral and functional changes has a great impact on the ability of individuals to perform basic activities of daily living[3, 4]. Although many intellectual functions are impaired (attention, orientation, language, judgmental) the most prominent symptom of AD is represented by a progressive memory loss[5].

The recent therapeutic mechanisms for AD are based on increasing the Acetylcholine (Ach) concentration or increasing the antioxidant activity in the brain[6]. By inhibiting cholinesterase activity, we can prevent Ach breakdown and increasing Ach concentration resulting in memory and behavioral improvements. Rivastigmine Tartrate (RT) is a reversible cholinesterase inhibitor used for the treatment of AD. RT has been shown to improve or

maintain patient's performance in three major domains: cognitive function, global function and behavior. However, limitations with its oral therapy include restricted entry into brain due to its hydrophilicity, necessitating frequent dosing and cholinergic side effects like sever bradycardia, nausea, dyspepsia, vomiting and anorexia[7]. RT was approved by the US Food and Drug Administration (USFDA) for the treatment of AD.

The BBB represents an effective obstacle for the delivery of neuro-active agents to the central nervous system (CNS) and it makes the treatment of many CNS diseases difficult to achieve[8]. Targeting of drugs to the brain is one of the most challenging issues for pharmaceutical research, as many hydrophilic drugs and neuropeptides are unable to cross the blood brain barrier (BBB)[9]. Polymeric nanoparticles have recently attracted great attention as potential drug delivery systems. Due to their small size, NPs penetrate into even small capillaries and are taken up within cells, allowing an efficient drug accumulation at the targeted site over a period of days or even weeks after injection[10]. The use of biodegradable polymeric NPs for drug delivery has been gaining momentum and shown significant therapeutic potential[11, 12]. Biodegradable polymer such as poly(D,L-lactic-co-glycolide) and their co polymers diblocked or multiblocked with PEG have been commonly used for form core-shell structure NPs to encapsulate a variety of therapeutic compounds[13, 14]. These NPs have a number of appealing features: their core is capable of carrying hydrophilic or lipophilic drug with high loading capacity, while coating with Tween 80 will provides hydrophilic shell which provides steric protection and high NPs location near to blood brain barrier. Drug release can be manipulated by choosing biodegradable polymers with different surface or bulk erosion rates, and external conditions such as pH and temperature change may function as a switch to trigger drug release[15]. Polymeric NPs have shown moderate circulation half-lives compared to their liposomal counterparts, despite also being coated with inert and biocompatible polymers[16].

Based on the literature, we tried to develop the self-assemble NPs that combine the properties of liposomes and polymeric NPs. The NPs are formed from three biomaterials: (i) PLGA was selected for core due to its biodegradable nature and ability to encapsulate high amount of hydrophilic and hydrophobic drug (ii) Soya lecithin was chosen for a monolayer around the core (iii) Tween 80 was selected to locate more amount of NPs near to BBB.

MATERIALS AND METHODS

Materials

Rivastigmine Tartrate (RT) was received as a gift sample from Sparc (Vadodara, India). Poly (D,L-Lactide-co Glycolide) (PLGA) (50:50) was purchased from Durate Corporation (Birmingham AL, USA) and was used without further purification. The Polyvinyl alcohol (PVA), Tween 80, Soya lecithin (S D Fine Chemicals, Mumbai, India); Aluminium chloride ($AlCl_3$), sodium chloride (NaCl) (Karnataka Fine Chemicals, Bangalore, India) and all other chemicals, solvents and reagents used were of analytical grade and used as received.

Methodology

Drug-polymer compatibility study

Compatibility studies were carried out to know the possible interactions between RT and polymers used in the formulation. The application of infrared spectroscopy lies more in the qualitative identification of substances either in pure form or in the mixtures and as a tool in establishment of the structure. IR is related to covalent bonds, the spectra can provide detailed information about the structure of molecular compound. In order to establish this point, FT-IR spectroscopy was carried out to confirm the identity of the drug and to detect the compatibility between drug, polymer (PLGA) and lipid (Soya lecithin). The FTIR spectra of drug with polymers were compared with standard FTIR spectrum of the drug. IR spectra of drug and along with polymers were seen in between 600-4000 cm^{-1}.

PLGA-Soya lecithin-Tween 80 NPs preparation method

PLGA-Soya lecithin-Tween 80 nanoparticles were synthesized by modified nanoprecipitation technique combined with self assembly. The polymer PLGA (85 mg) was first dissolved with magnetic stirrer (By Remi Equipment Pvt Ltd, Bangalore, India) in 3 ml of organic solvent (acetonitrile) to form a primary emulsion. In above organic solution, RT (4.3 mg) was added and allowed to dissolve and combined solution was heated up to 65 °C. The PLGA-RT solution was then added into the preheated lipid aqueous (Soya lecithin, Tween 80 and PVA) drop wise under stirring followed by vortexing for 3 mins. The contents were allowed to mix for 20 mins with homogenizer (T25 digital Ultra turax by IKA, Germany) at 18000 RPM. The resulting suspension was sonicated for 10 mins at 45% amplitude with an ultra sonic probe (By Dakshin, Bombay). The nanoparticles were allowed to self-assemble for 2 h with continuous stirring while above solution was allowed to stir over night to evaporate organic solvent. Then, the nanoparticles were collected by centrifugation and washed 3 times with distilled water. Finally, they were resuspended into 2 ml of cryoprotectant solution (Sucrose (2% w/w), dried on lyophilizer (Eqsquire Biotech, Germany) and stored at 4°C[17].

Experimental design for optimization of formulation

Preliminary experiments indicated that variables such as Soya-lecithin and Tween 80 concentrations were the main factors that influence the particle size, polydispersity, and percentage drug release and encapsulation efficiency of the PLGA-Soya lecithin-Tween 80 NPs. 3^2 factorial design has been used to optimized the formulation parameters and examine the main effects statistically, on particle size (Y_1), polydispersity (Y_2), encapsulation efficiency (Y_3) and percentage drug release (Y_4) of NPs. Details of the design are listed in Table 1. For each factor, the experimental range has been selected on the basis of the results of preliminary experiments and the feasibility of preparing the PLGA-Soya lecithin-Tween 80 NPs at the extreme values. The value range of the variables was: Concentration of Soya lecithin (X1): 10–20% (W/V), Tween 80 concentration (X2): 0.5–1.5%

Table 1: Experimental design table for 3^2 factorial design (PLGA-Soya lecithin-Tween 80 NPs)

Formulation No.	Polymer (PLGA) conc. (mg)	Soya lecithin conc. (%W/V)	Tween 80 conc. (%V/V)	PVA conc. (%W/V)	Organic solvent conc. (ml)
D1	85	10	0.5	1	3
D2	85	10	1.0	1	3
D3	85	10	1.5	1	3
D4	85	15	0.5	1	3
D5	85	15	1.0	1	3
D6	85	15	1.5	1	3
D7	85	20	0.5	1	3
D8	85	20	1.0	1	3
D9	85	20	1.5	1	3

Determination of encapsulation efficiency

The entrapment efficiency (EE) of RT was assayed by UV-Visible spectrophotometer. The amount of 10 ml of RT loaded PLGA-Soya lecithin-Tween 80 NPs suspension was carefully transferred to centrifugation tube. The nanoparticles in the form of sediment were separated from the solution by ultracentrifugation at 15,000 rpm at 4°C for 40 mins. The supernatant was carefully decanted and analyzed by UV spectrophotometer for RT at 264 nm. The % entrapment efficiency was calculated using equation as given below[18]:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} * 100 \quad (1)$$

Particle size analysis and polydispersity

Particle size analysis and polydispersity of nanoparticles were determined by particle size analyzer (Brookhaven Instrument Corporation, NY). Lyophilized nanoparticles were dispersed in double distilled water and analyzed in three readings per nanoparticles sample. The polydispersity was calculated based on the volumetric distribution of particles[19].

Zeta Potential determination

Zeta Potential was measured by using zeta size analyzer (Brookhaven instrument Corporation, NY). About 1.5 ml of the sample was placed in the cuvette and electrode was inserted carefully and click “track” button and the Zeta PALS meter instantly calculates and displays the colloidal’s Zeta potential (or electrophoretic mobility)[20].

***In vitro* drug release**

The *in vitro* drug release profile of PLGA-Soya lecithin-Tween 80 NPs formulation has been studied using a dialysis bag. Approximately 1 ml of nanoparticle suspension (Corresponding 6 mg of RT) were taken into a dialysis bag (molecular weight cut-off, 12 KDa, Himedia, India) and placed in a beaker containing 150 ml of phosphate buffer saline (PBS) (pH 7.4). Then the beaker was placed over a magnetic stirrer and the temperature of the assembly was maintained at 37±1°C throughout the study. Samples (5ml) were withdrawn at definite time intervals (1, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48 and 60h) and replaced with equal amounts of fresh buffer. The samples were analyzed for drug concentration by UV-Visible spectrophotometer at 264 nm[21].

Stability studies

A stability study was carried out to assess the stability of PLGA-Soya lecithin-Tween 80 NPs. The samples were stored in room temperature (15-20°C), refrigerator (3-5°C) and 37°C (RH=75%) over a period of 6 months. Samples were periodically withdrawn at monthly intervals for six months and examined for their drug release as well as any changes in physical appearance[22].

Scanning electron microscopy (SEM) study:
The surface morphology of PLGA-Soya lecithin-Tween 80 NPs was measured by scanning electron microscopy (SEM) (EM-LEO 435VP, Carl Zeiss SMT Inc., NY) equipped with 15 kv, SE detector with a collector bias of 300 V. The lyophilized sample was carefully mounted on an aluminum stub using a double stick carbon tape. Sample was then introduced into an automated sputter coated and coated with a very thin film of gold before scanning the sample under SEM[19].

Differential Scanning calorimeter (DSC) study:
The physical state of RT entrapped in the PLGA-Soya lecithin-Tween 80 NPs was characterized by Differential Scanning Calorimetry (DSC - 60, Shimadzu, Japan). Each sample was sealed in standard aluminium pans with lids and purged with air at a flow rate of 40 ml/min. A temperature ramp speed was set at 20 °C/min, and the heat flow was recorded in the range 30-300°C under inert nitrogen atmosphere. Thermograms were taken for RT and PLGA-Soya lecithin-Tween 80 NPs[23].

Experimental animals
The subjects used in this research work were 40 male and/or female adult Wistar rats. Wister rats, 200-220 g, procured from, Bioneeds, Bangalore, were used for investigation. The Institutional Animal Ethical Committee approved the protocol. They were kept in the animal house of Department of Pharmacology, Acharya & B M Reddy college of pharmacy (Bangalore, India) for seven weeks (normal) standard environmental condition (relative humidity of 60%, 12h-12h light-dark cycle) with sufficient food, water and under a good ventilation in order for the animals (Wistar rats) to acclimatized. (Registration number for Institutional Animal Ethics Committee (IAEC)-997/c/06/CPCSEA)

Experimental design for animal study
Drug and treatment schedule
Aluminium chloride solution and the optimized formulation (PLGA-Soya lecithin-Tween 80 Nps) were freshly prepared at the beginning of each experiment. For per oral (p.o) administration, Aluminium chloride was dissolved in distilled water and for intraperitoneal (IP) administration, prepared nanoparticles were dispersed in normal saline solution (0.9 %w/v). Dose calculated equivalent to 1.5 mg/kg of RT for standard as well as for nanoparticles formulations. Animals were divided into four groups:

Group 1: Normal control
Group 2: Positive control (Aluminum chloride 100 mg/kg/day p.o.)
Group 3: Standard (RT 1.5 mg/kg IP in saline + Aluminum chloride 100 mg/kg/day p.o.)
Group 4: PLGA-Soya lecithin-Tween 80 NPs treated (NPs IP in saline + Aluminum chloride 100 mg/kg/day p.o.)

Spatial navigation task (Morris water maze)
The acquisition and retention of a spatial navigation task was evaluated using Morris water maze[24]. Rats were trained to swim to a visible platform in a circular water pool (100cm in diameter and 45cm deep) located in test room. A hidden circular platform (20cm height, 12cm in diameter and 2cm below the water surface, fixed position). The pool was conceptually divided into four equal quadrants. The rats received a training session considering of two trials per day at 1 min interval for 5 days prior to starting dose regimen. In the first test, rats were placed on the platform for 20s, then the rats were placed in the water facing the pool wall at one of the 4 quadrants at a different place every day, and allowed to swim for a maximum of 90s to find the hidden platform were it was allowed to stay for 10s. If rat did not find the platform in 90s, it was placed on the platform by hand and remained there for 10s. The time to reach the platform (escape latency) was measured with a stopwatch. The escape latency was studied on the day 0, 7, 14, 21, 28, 35 and 42^{th}[25].

Elevated plus maze paradigm study
The elevated plus maze considered of two opposite black open arms (50cm × 10cm), crossed with two closed walls of the same dimensions with 40cm high walls[26]. Acquisition of memory by the rats was tested on the day 0, 7, 14, 21, 28, 35 and 42^{th}. Time taken by the rat to move from the open arm to the closed arm was recorded as Retention Transfer Latency (RTL). Rats were allowed to explore the maze for 20s after recording the reading and were made to return to the home cages. If the rat did not enter the enclosed arm within 90s, it was pushed back into one of the enclosed arm and the reading was recorded as 90s, placing the rat in an open arm assessed its retention of memory. The RTL was noted on day 0, 7, 14, 21, 28, 35 and 42^{th}[24].

Experimental procedure for histopathology
After oral administration of Aluminium chloride to each group with different formulations except normal control group (1); the animals were sacrificed after the 42th day morris water maze and elevated plus maze studies, with the

use of chloroform in a closed tight box. Section of the brain was dissected and then fixed in Bouin's solution immediately in order to prevent enzymatic and other postmortem changes that could degrade tissue and also to harden the brain so that it can be sectioned (cut into thin sliced) without tearing. The tissue was processed and stained with Haematoxylin and eosin (H&E). The stained sections of the hippocampus were examined under the light microscope[27].

Activity of AchE

After the last morris water maze and elevated plus maze studies, rats were sacrificed by decapitation, the frontal cortex and hippocampus were removed and homogenized in 5% of sodium phosphate buffer (75 mM, pH 7.4, 4°C), respectively. For the assay of AchE activity, a 4ml reaction mixture that contained acetylthiocholine iodide (0.3 mM), sodium phosphate buffer (0.1 mM Ph 7.4) 1 ml and homogenate 0.1-0.2 ml was incubated at 37°C for 8 min. The reaction was terminated by adding 1 ml of 3% sodium lauryl sulfate, then 1 ml of 0.2% 5, 5'-dithiobis (2-nitrobenzoic acid) to produce the yellow anion of 5-thio-2 nitrobenzoic acid. The color intensity was measured spectrophotometrically at 440 nm. All samples were assayed in duplicate. AchE activity was calculated as optical density (OD) value/mg protein for AchE[28]. Protein concentrations were determined with the Coomassie blue protein-binding method using bovine serum albumin as standard[29].

RESULTS AND DISCUSSION

Drug polymer compatibility studies

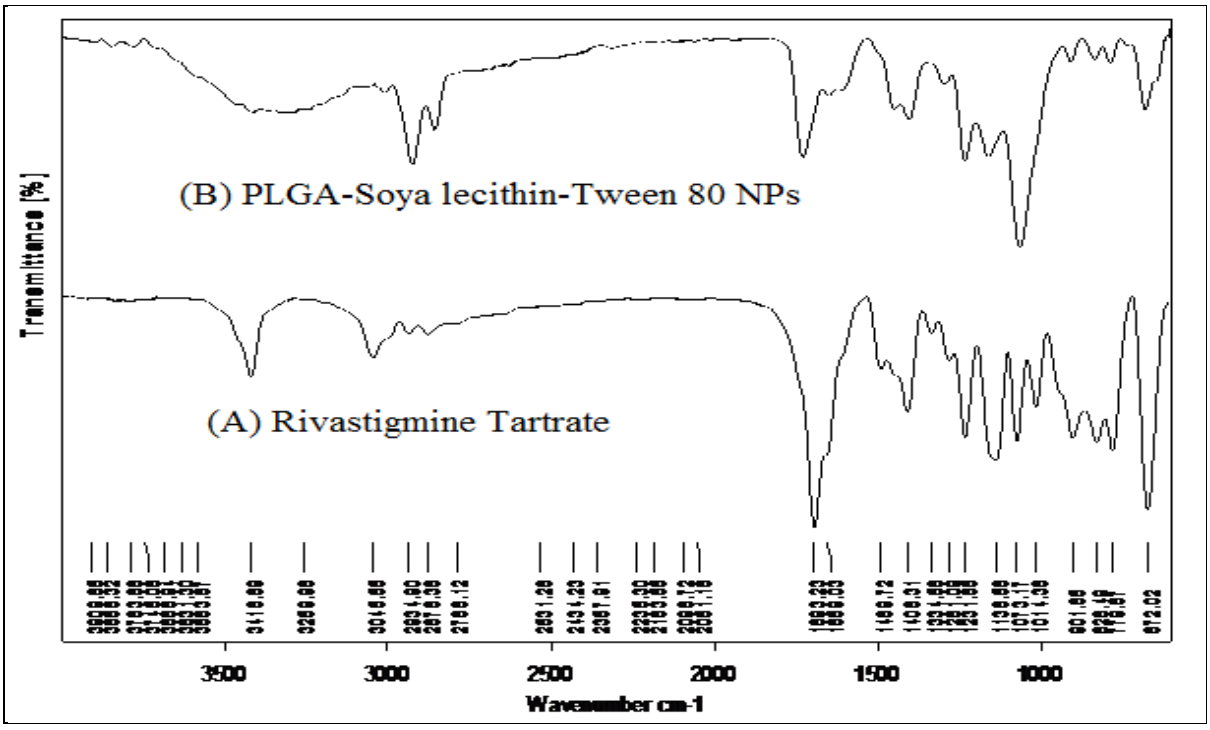


Fig. 1: FTIR spectra of A) Rivastigmine Tartrate and B) PLGA-Soya lecithin-Tween 80 NPs

The FTIR (Fourier Transform Infrared) spectra of the RT and PLGA-Soya lecithin-Tween 80 NPs are given in Fig. 1A) and B), respectively. Fig 1A) shows C-N (str) at 2788 cm^{-1}, CH_3 (str) at 2876 cm^{-1}, = C-H (str) at 3045 cm^{-1}, -C=C- (str) at 1500 cm^{-1}, -C=O (str) at 1693 cm^{-1} and -C-O (str) at 1231 cm^{-1}. The Fig. 1B) shows the FTIR spectra of PLGA-Soya lecithin-Tween 80 NPs and it shows all the characteristic peaks C-N (str) at 2855 cm^{-1}, CH_3 (str) at 2924 cm^{-1}, = C-H (str) at 3009 cm^{-1}, -C=C- (str) at 1447 cm^{-1}, -C=O (str) at 1728 cm^{-1} and -C-O (str) at 1232 cm^{-1}, which signified that there was no interaction between drug and polymers and they are compatible with each other.

Table 2: Results of % entrapment efficiency, particle size, polydispersity, zeta potential and *in vitro* drug release of PLGA-Soya lecithin-Tween 80 NPs formulations prepared as per the experimental design

Formulation	Entrapment efficiency(%)	Particle Size(nm)	Polydispersity	Zeta potential (mV)	Drug release (%)
D1	63.655	119.86	0.124	-25.02	69.647±0.254
D2	63.774	105.98	0.11	-26.87	70.447±0.679
D3	61.963	98.79	0.099	-27.58	72.439±0.384
D4	66.43	169.58	0.178	-29.3	65.283±0.246
D5	66.511	145.07	0.162	-31.69	66.658±0.137
D6	65.621	132.51	0.146	-32.43	67.947±0.383
D7	67.732	205.34	0.22	-36.58	60.269±0.588
D8	67.851	198.49	0.202	-38.71	62.269±0.142
D9	66.324	187.17	0.191	-39.23	64.164±0.149

Examination of key factors effects using response surface methodology (RSM)

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2$

Where, β_0 is the intercept representing the arithmetic averages of all the quantitative outcomes of 9 runs; β_1, β_2, β_{12}, β_{11} and β_{22} are the coefficients computed from the observed experimental values of Y; and X_1 and X_2 stand for the main effects. The terms X_1X_2 and Xi^2 (i = 1 and 2) represent the interaction and quadratic terms, respectively used to simulate the curve of the designed sample space.

A positive value in regression equation for a response represents an effect that favors the optimization (synergistic effect), while a negative value indicates an contrary relationship (antagonist effect) between the factors and responses. The coefficient with one factor represents the influence of that specific factor whereas the coefficients with more than one factor indicate the interaction between those factors and the quadratic nature of the phenomenon, respectively.

Effect of variables on particle size

Particle size is a critical factor for nanoparticles based drug delivery system. It is one of the factors, which controls the kinetics of drug release.

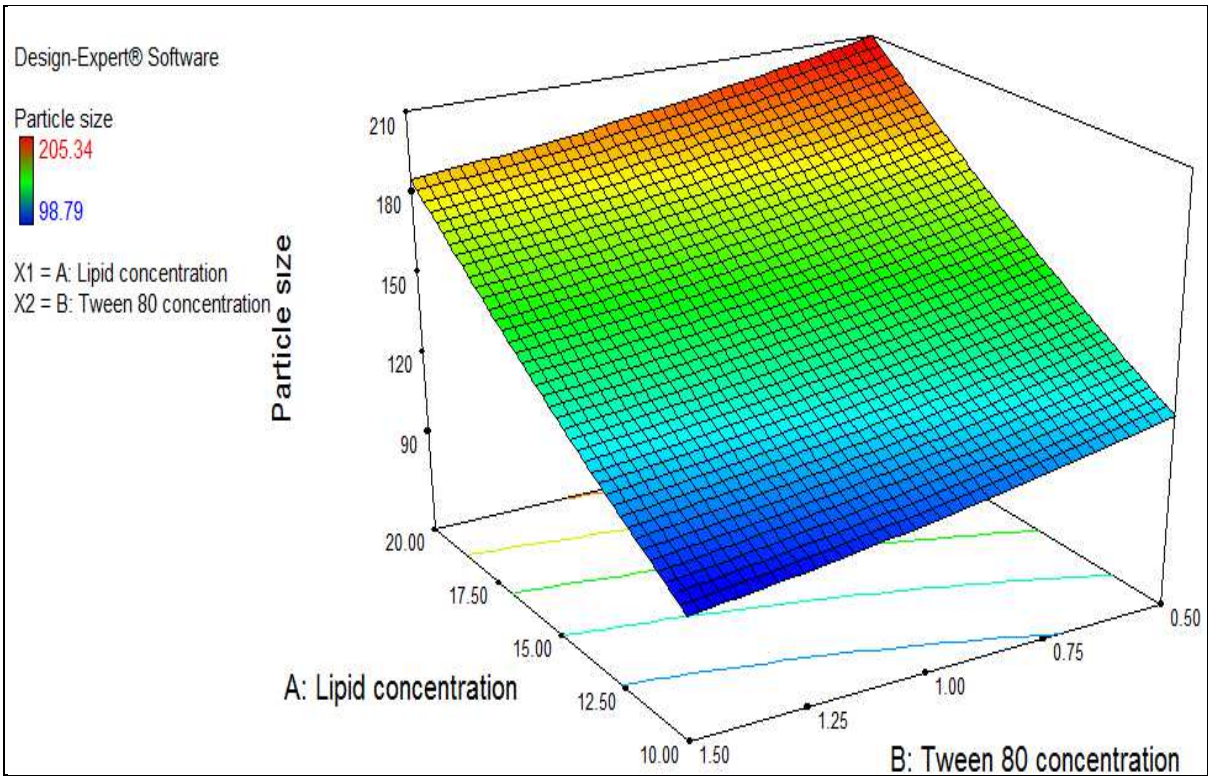


Fig. 2: Response surface plot showing the effect of lipid concentration (X_1), tween 80 concentration (X_2) on particle size of nanoparticles Y_1 (nm)

Particle size (Y_1) = + 147.48 + 44.40 * lipid concentration (X_1) -12.72 * tween 80 concentration (X_2) + 0.72 * lipid concentration (X_1) * tween 80 concentration (X_2) + 3.55 * lipid concentration $(X_1)^2$ + 2.36 * tween 80 concentration $(X_2)^2$

The particle size values for D1-D9 formulations showed a wide variation in response with the response range from a minimum 98.79 to a maximum 205.34 nm. The Fig. 2 shows that the response surface plot obtained for the interaction between the lipid concentration and tween 80 concentrations at constant PLGA value on particle size. The positive sign for the coefficient of lipid concentration shows that particle size increases with increase in the concentration of lipid. The negative sign for the coefficient of tween 80 concentration shows that particle size decreases with increase in the concentration of tween 80.

By using lipid in different concentration with fixed concentration of polymer, it has been observed that particle size increases with increase in concentration of lipid. The increase in particle size of nanoparticles might be due to multilayer depositions of lipid. The particle size study also revealed another trend with tween 80 that with increase in concentration of tween 80 with constant polymer concentration, particle size decreases due to its surfactant effect.

Effect of variables on polydispersity

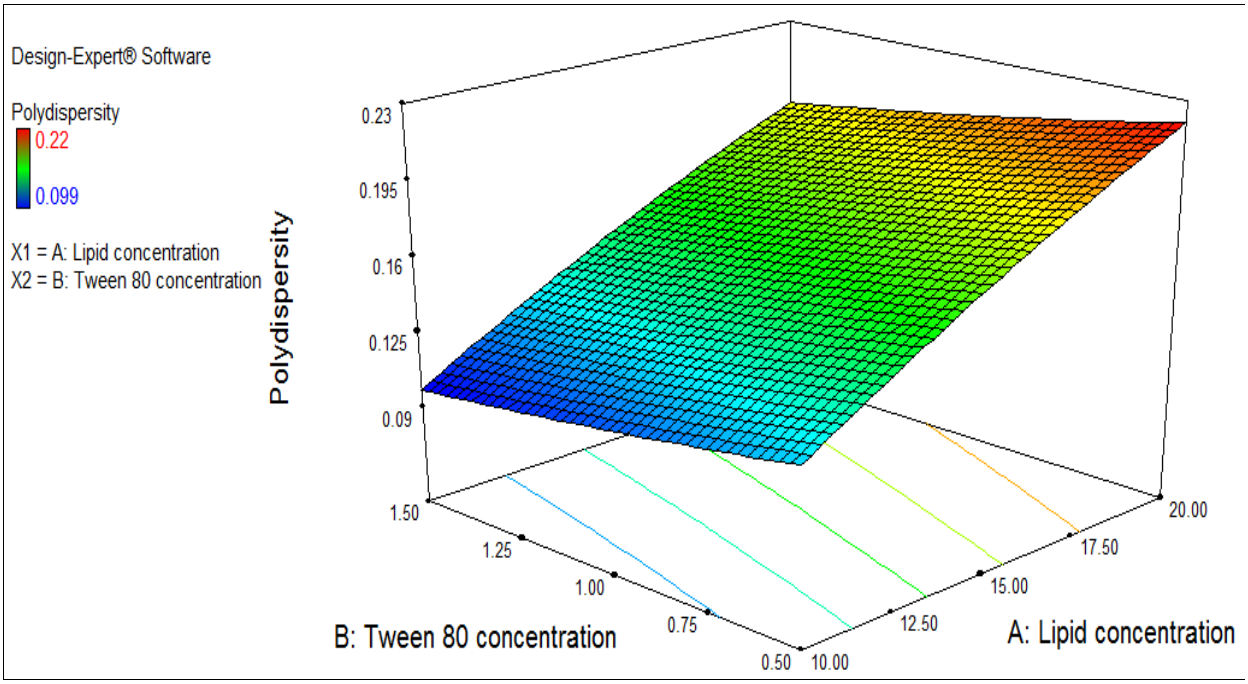


Fig. 3: Response surface plot showing the effect of lipid concentration (X_1), tween 80 concentration (X_2) on Polydispersity of nanoparticles Y_2

Polydispersity (Y_2) = + 0.16 + 0.047 * lipid concentration (X_1) - 0.014 * tween 80 concentration (X_2) - 1.00 * lipid concentration (X_1) * tween 80 concentration (X_1) – 4.33 * lipid concentration $(X_1)^2$ + 1.66 * tween 80 concentration $(X_2)^2$

The polydispersity values for D1-D9 formulation showed a wide variation in response with the range from a minimum 0.099 to a maximum 0.220. The Fig**.** 3 shows the response surface plot obtained from the interaction between lipid concentration and tween 80 concentrations at constant PLGA value on poly dispersity. The positive sign for the coefficient of lipid concentration shows that poly dispersity increases with increase in the concentration of lipid. The negative sign for the coefficient of tween 80 concentration shows that polydispersity decreases with increase in the concentration of tween 80.

Polydispersity results indicated that polydispersity increases due to multilayer deposition of lipid on PLGA particles and decreases due to surfactant like effect of tween 80.

Effect of variables on % entrapment efficiency

To transport the drug to the specific site and increase its resident time, there is a need of high entrapment efficiency. PLGA-soya lecithin molecules have ability to entrap both hydrophilic and lipophilic drug and target them to specific targeted site.

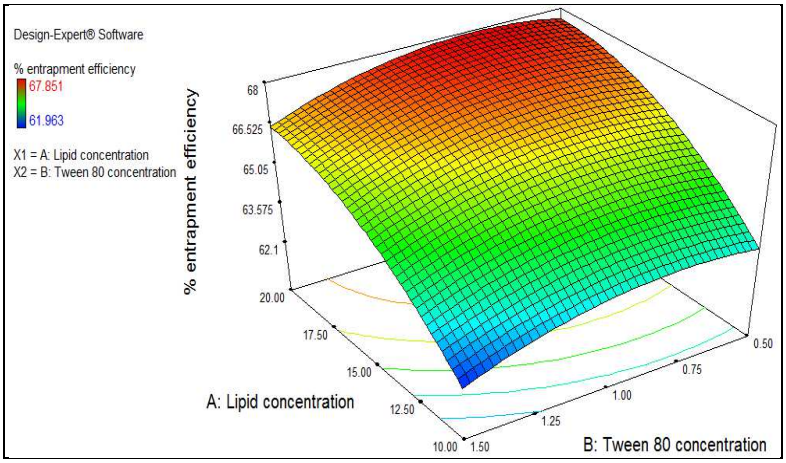


Fig. 4: Response surface plot showing the influence of lipid concentration (X_1), tween 80 concentration (X_2) on % entrapment efficiency of nanoparticles Y_3 (%)

% Encapsulation efficiency (Y_3) = + 66.69 + 2.09 * lipid concentration (X_1) – 0.65 * tween 80 concentration (X_2) + 0.071 * lipid concentration (X_1) * tween 80 concentration (X_2) – 0.97 * lipid concentration $(X_1)^2$ – 0.076 * tween 80 concentration $(X_2)^2$

The % entrapment efficiency values for D1-D9 formulation showed a wide variation in response with the range from a minimum to 61.963 % to a maximum 67.851 %. The Fig**.** 4 shows the response surface plot obtained for the interaction between the lipid concentration and tween 80 concentrations at constant PLGA value on % entrapment efficiency. The positive sign for the coefficient of lipid concentration shows that % entrapment efficiency increases with increase in the concentration of lipid. The negative sign for the coefficient of tween 80 concentration shows that % entrapment efficiency decreases with increase in the concentration of tween 80.

The PLGA nanoparticles coated with soya lecithin initially showed no significant change in the drug entrapment efficiency. Later on the entrapment efficiency started decreasing with increase in the concentration of soya lecithin. Soya lecithin layer is acting as a molecular fence and contributes to keep the drug molecules in polymeric core, which prevents diffusion of hydrophilic drug out of the polymeric core during formulation preparation.

Effect of variables on % cumulative drug release

The *in vitro* drug release study has been carried out for 60 h for formulations of PLGA-Soya lecithin-Tween 80 NPs. From the drug release study; we observed that drug release was biphasic. The initial drug release was burst release, due to surface bound and poorly encapsulated drug of NPs, which was associated with smaller diffusion path. Thereafter drug release was decreased, which reflects the drug release from the core of nanoparticles. The release rate in the second phase was assumed to be controlled by diffusion rate of drug across the polymer matrix. In this model the lipid layer is acting as molecular fence and contributes to keep the drug molecules in the core, as well as keep water out of the core, which hydrolyzed the PLGA polymer and increase erosion and drug release.

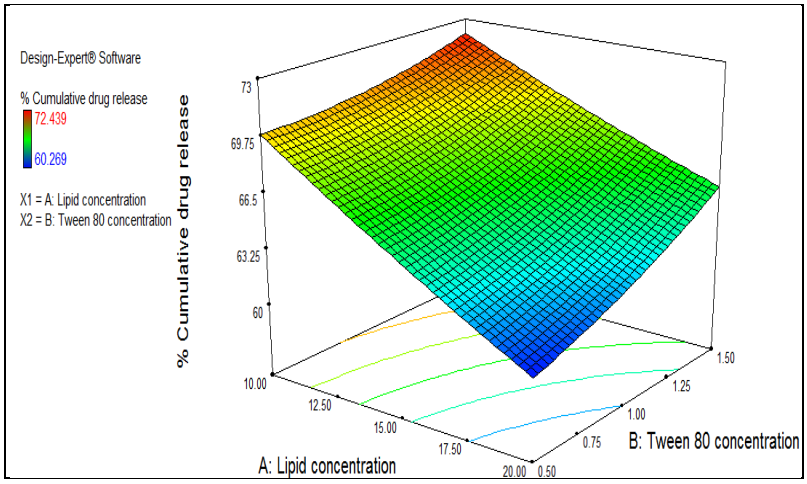


Fig. 5: Response surface plot showing the influence of lipid concentration (X_1), Tween 80 concentration (X_2) on % cumulative drug release of nanoparticles Y_4 (%)

% Cumulative drug release (Y_4) = + 66.29 – 3.97 * lipid concentration (X_1) + 1.89 * tween 80 concentration (X_2) + 0.78 * lipid concentration (X_1) * tween 80 concentration (X_2) + 0.24 * lipid concentration $(X_1)^2$ + 0.50 * tween 80 concentration $(X_2)^2$

The % cumulative drug release values for D1-D9 formulation showed a wide variation in response with the range from a minimum to 60.269 ± 0.588 % to a maximum 72.438 ± 0.384 %. The Fig**.** 5 shows the response surface plot obtained for the interaction between the lipid concentration and tween 80 concentrations at constant PLGA value on % cumulative drug release. The negative sign for the coefficient of lipid concentration shows that % cumulative drug release decreases with increase in the concentration of lipid. The positive sign for the coefficient of tween 80 concentration showed that % cumulative drug release increases with increase in the concentration of tween 80.

The lipid monolayer found to be a limited factor in controlled drug release. The drug release was observed to be increasing with increase in concentration of Tween 80. This is because of the hydrophilicity properties of the tween 80, which hydrolyze the PLGA polymer and results in increase in erosion and drug release

Effect of variables on zeta potential

For any liquid dosage form, surface charge is essential for its stability. SLN were reported to have greater stability when compared to other colloidal dosage forms. The zeta potential of PLGA-Soya lecithin-Tween 80 NPs decreases from -25.02 mV to -39.23 mV. Zeta potential was observed to be increasing with increase in Tween 80 concentration with constant lipid concentration. The same trend was observed with constant tween 80 concentration and varied lipid concentration.

Optimization of formulation

Optimized formulation was selected based on following criteria:

Particle size < 200, Polydispersity- minimum, % entrapment efficiency- maximum and *in vitro* drug release- maximum.Based on this research, the formulation containing 85 mg of PLGA, 15.02% of Soya lecithin and 1.25% of Tween 80 concentrations were selected for optimized formulation. The selection of the optimized formulation was based on minimization of particle size below 200 nm to facilitate brain targeting[30], minimization of polydispersity, maximization of entrapment efficiency and maximization of *in vitro* drug release. The optimized formulation exhibited results were given into below table:

Table 3: Different evaluations and their results of optimized formulation (D10)

No.	Evaluation	Results
1	Particle size	141.74 nm
2	Polydispersity	0.154
3	Zeta potential	-35.79 mV
4	% Entrapment efficiency	66.171%
5	% Cumulative drug release	67.336 ± 0.254%

Stability study

The optimized formulation (D10) PLGA-Soya lecithin-Tween 80 nanoparticles was kept for stability studies at room temperature (15-20°C), refrigerator (3-5 °C) and 37°C (RH = 75 %) over a period of 6 months. Samples were

evaluated at 0, 1, 2, 3, 4, 5 and 6 months for their drug release as well as any changes in physical appearance. The results of the stability studies for 0 to 6 months showed that there was no significant change in the drug release study and appearance of the optimized formulation D10, stored at refrigerator (3-5 °C). While at room temperature (15-20°C) and 37°C (RH = 75 %) showed that there was agglomeration of particles present. Thus, it can be concluded that refrigerator (3-5 °C) condition and ambient temperature and humidity are the most suitable for storage of optimized PLGA-Soya lecithin-Tween 80 nanoparticles.

Differential Scanning Calorimetry (DSC)

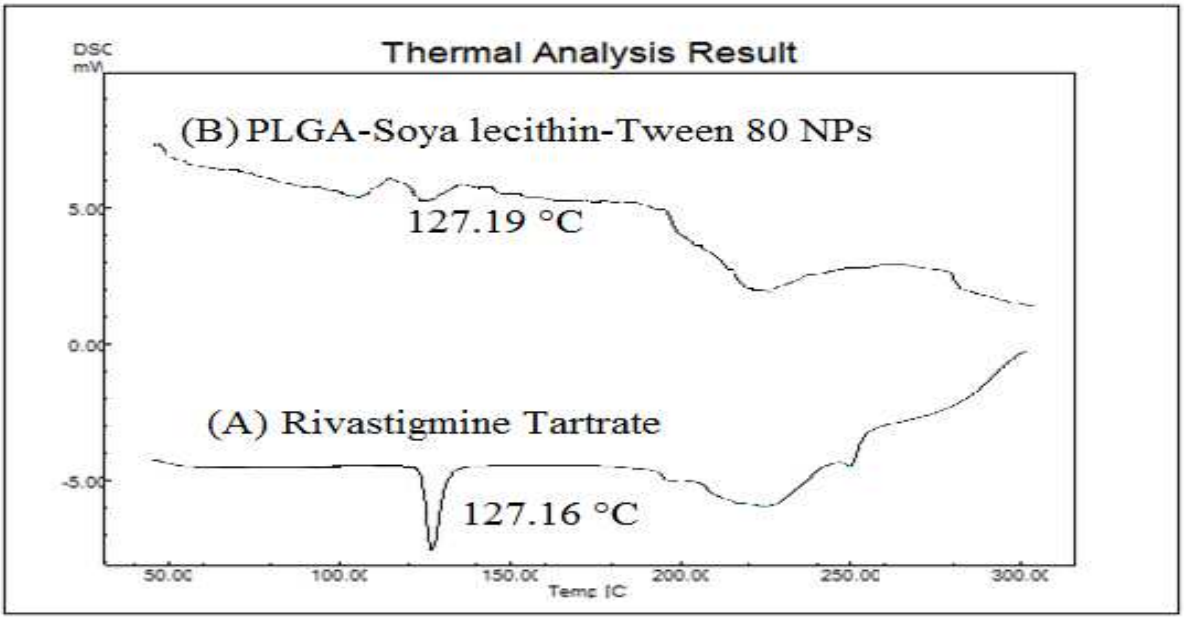


Fig. 6: DSC thermograms of (A) Rivastigmine Tartrate and (B) PLGA-Soya lecithin-Tween 80 NPs

Differential scanning calorimetry study gives information regarding the physical properties like crystalline or amorphous nature of the sample. The DSC thermogram of RT in Fig. 6(A) shows an exothermic peak at 127.16°C corresponding to its melting temperature. However, no sharp endotherm was seen at 127.19°C in Fig. 6(B). This shows that crystallinity of the drug has been reduced significantly in nanoparticles. Hence, it could be concluded that the drug was present in the optimized formulation as amorphous phase and may have been homogeneously dispersed in the PLGA matrix.

Scanning electron microscopy (SEM)

Surface morphology of the specimens was determined by using SEM (EM-LEO 435VP, Carl Zeiss SMT Inc., NY). SEM photograph of optimized formulation were shown in Fig. 7.The optimized nanoparticles of PLGA-Soya lecithin-Tween 80 have smooth surface and spherical morphology.

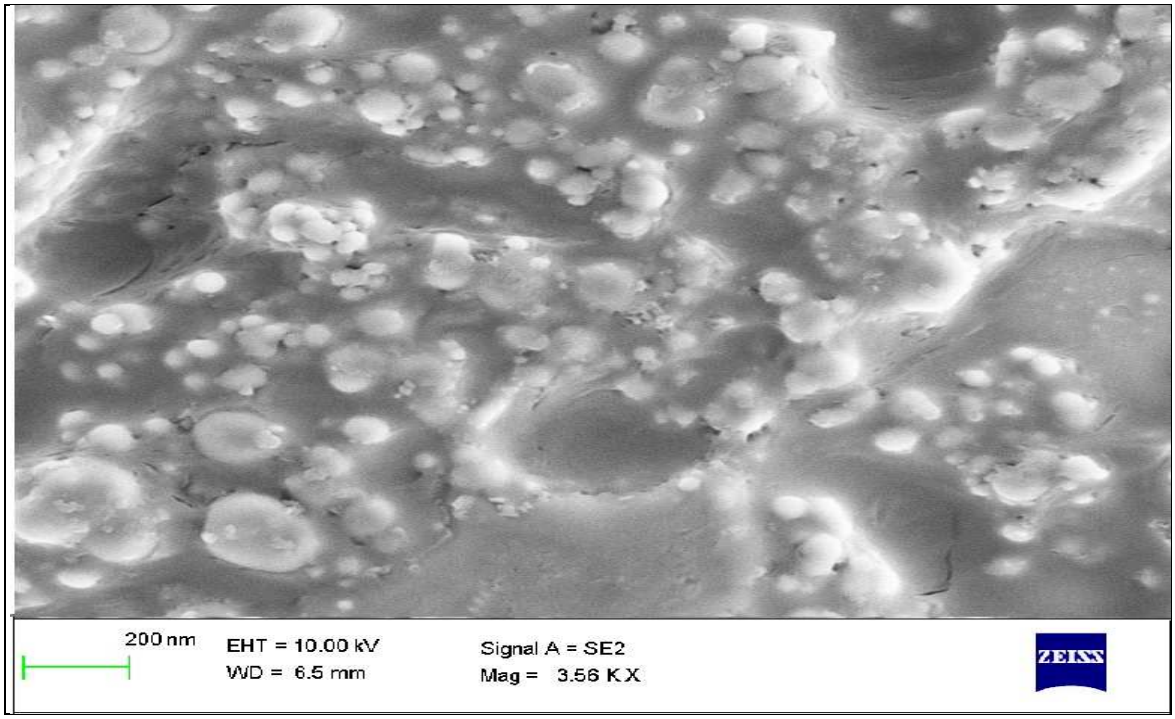


Fig. 7: Nanoparticle morphology of freeze dried PLGA-Soya lecithin-Tween 80 nanoparticles as studied by scanning electron microscopy (SEM)

Spatial navigation task (Morris water maze)

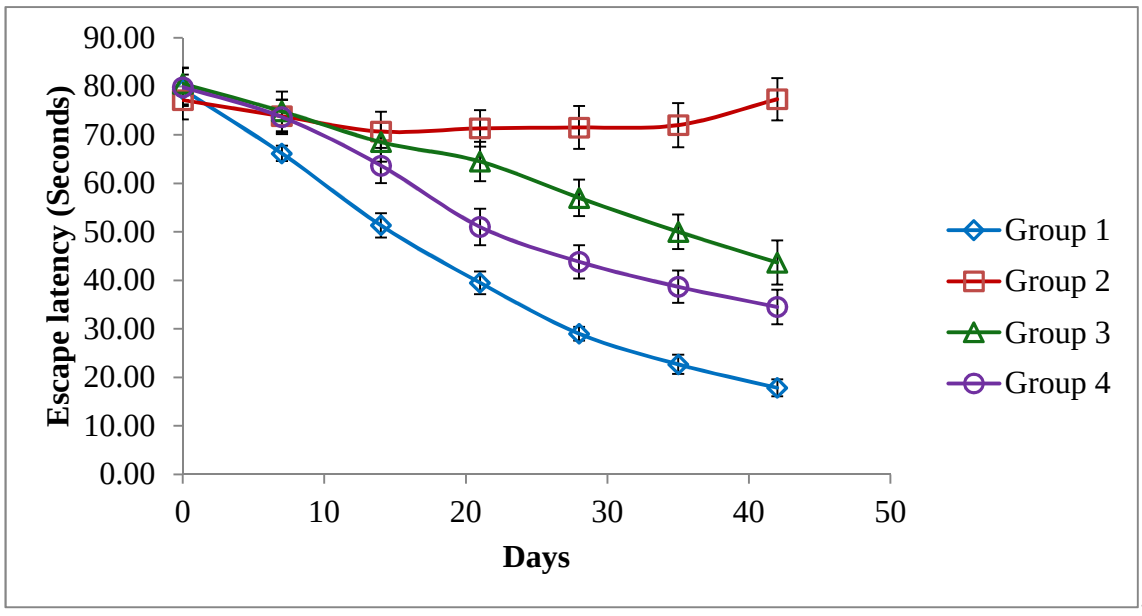


Fig. 8: Comparison of escape latency in various groups of rats using spatial navigation task. (Morris water maze) The values are depicted as mean ± SD (*n* = 6)

In spatial navigation task, the normal (group 1), standard (group 3) and PLGA-Soya lecithin-Tween 80 NPs (group 4) treated groups rats quickly learned to swim directly to the platform in the Morris water maze. Aluminum chloride treated rats (group 2) showed an initial increase in escape latency, which declined during following weeks of Morris water maze test. The rats that received pure drug along with aluminum chloride showed slight improvement in their behavior. In contrast, the rats treat with drug and formulation (D10) with aluminum chloride, showed significantly decrease in time taken to reach platform as compared with aluminum chloride treated rats. Groups arranged according to significance in time taken to reach platform: Group 1< Group 4< Group 3< Group 2

Elevated plus maze paradigm study

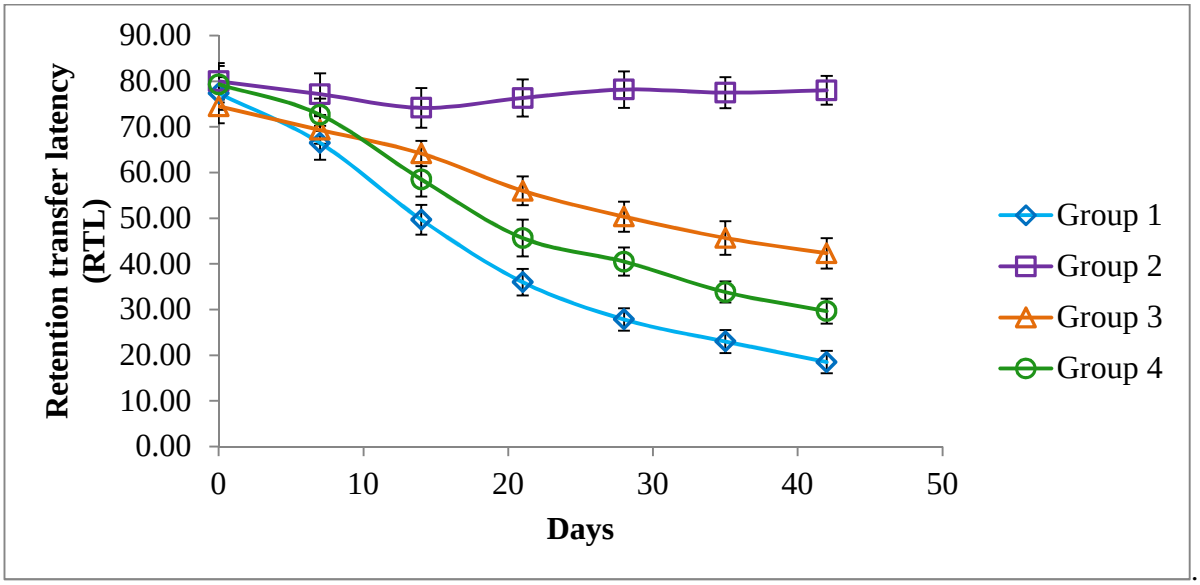


Fig. 9: Comparison of memory retention in various groups of rats using Elevated plus maze paradigm. The values are depicted as mean ± SD (*n* = 6)

In the Elevated plus maze task, we evaluated time taken (Retention Transfer Latency - RTL) by rats to reach from open arm to close arm of maze. The rats from groups 1, 3 and 4 entered the closed arm quickly and RTL found to be decreased. In contrast, group 2 (Aluminium chloride treated rats) carried out initially well followed by poorly trough out the experiments. It demonstrates that the chronic administration of aluminum chloride induced marked memory impairment. Regular administration of standard drug and PLGA-Soya lecithin-Tween 80 NPs with Aluminum chloride decreases the RTL compared to positive control group. Groups arranged according to RTL: Group 1< Group 4< Group 3< Group 2

Histopathology study

The hippocampus is a major component of the brains of humans and other mammals. It belongs to the limbic system and plays important roles in the consolidation of information from short-term memory to long-term memory and spatial navigation. There is now almost universal agreement that the hippocampus plays some sort of important role in memory; however, the precise nature of this role remains widely debated[31-33].

Manuela *et al.*, studied on the quantification of the neuronal density in the four specific areas of the hippocampus (CA1-CA4) of AD brains, stated that in the Alzheimer's disease the decrease of hippocampal neuronal density was more prominent especially at the CA1 and CA3 hippocampal areas[5].

As mention in above reference, in AD most affected brain regions are CA1 and CA3 of hippocampus, we studied only these regions of hippocampus for comparative study between different groups.

Group 1: Normal control

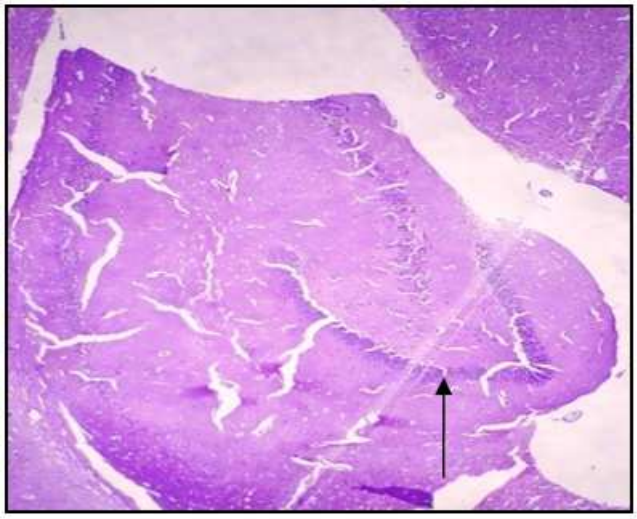


Fig. 10: Section studied from hippocampus shows densely packed pyramidal cells in both CA1 and CA3 layers (H&E; mag. x 50)

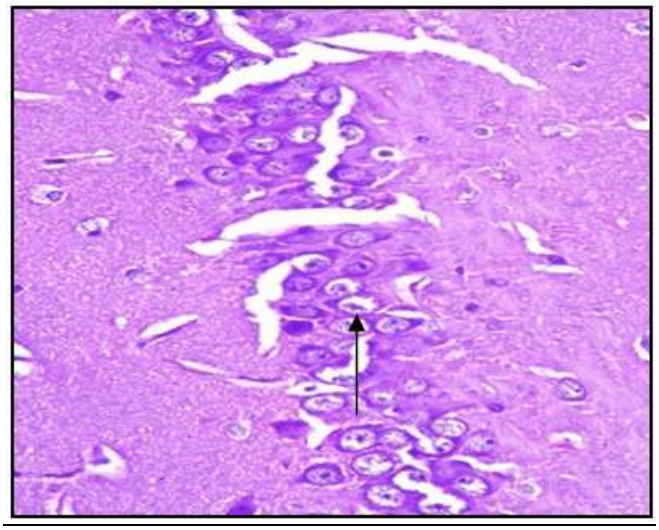


Fig. 11: The CA3 region shows intact pyramidal cell (arrow) in tight clusters (H&E; mag. x 400)

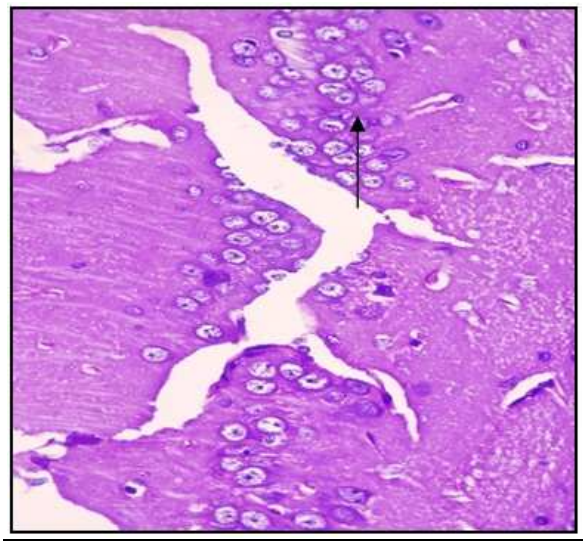


Fig. 12: The CA1 region shows intact pyramidal cells (Arrow) along with intact neurophil fiber. (H&E; mag. x 400)

Group 2: Positive control

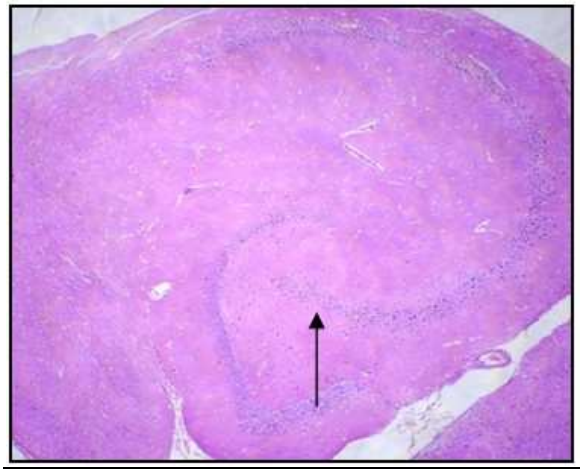
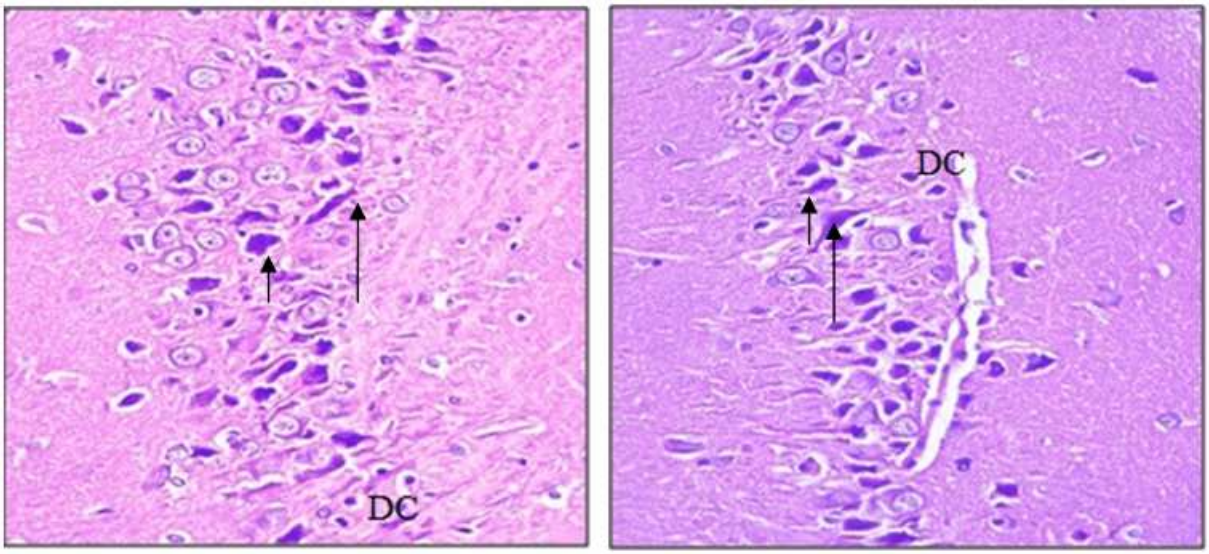


Fig. 13: Section studied from the hippocampus shows (arrow) loosely packed pyramidal cells in both CA1 and CA3 layers. (H&E; mag. x 50)



(A) The CA3 region (H&E; mag. x 400) (B) The CA1 region (H&E; mag. x400)

Fig. 14: The CA3 region (Fig. 14A, Arrow) and CA1 region (Fig. 14B, Arrow) shows loss of both pyramidal cells and neurophil fibers along with neuritic plaques (Short arrow) and neurofibrillary tangles (Long arrow). Some of the pyramidal cells show degenerative changes. (DC – Distorted cells)

Group 3: Standard control

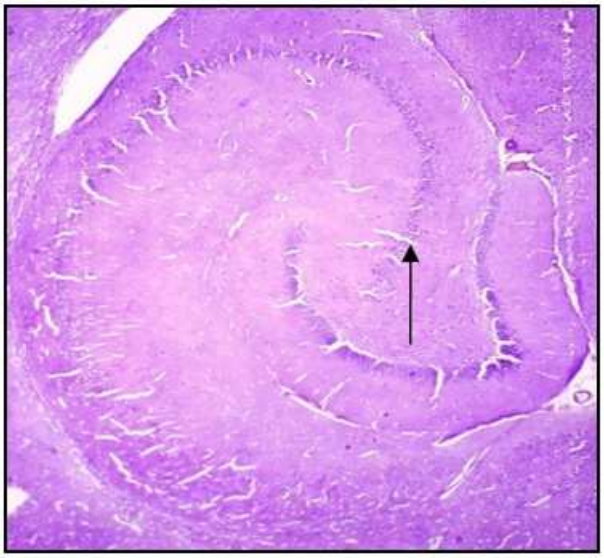


Fig. 15: Section studied from the hippocampus shows densely packed pyramidal cells in both CA1 and CA3 layers (Arrow) (H&E; mag. x50)

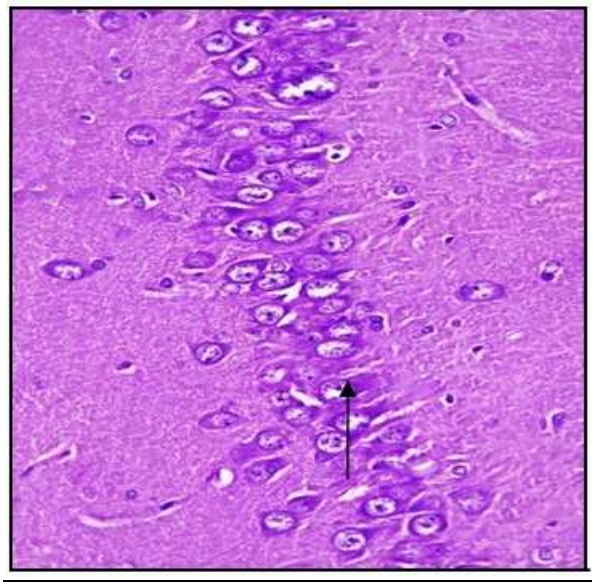


Fig. 16: The CA3 region shows intact pyramidal cells in tight clusters (Arrow). The interconnected neurophil fibers in CA3 region appear intact. (H&E; mag. x 400)

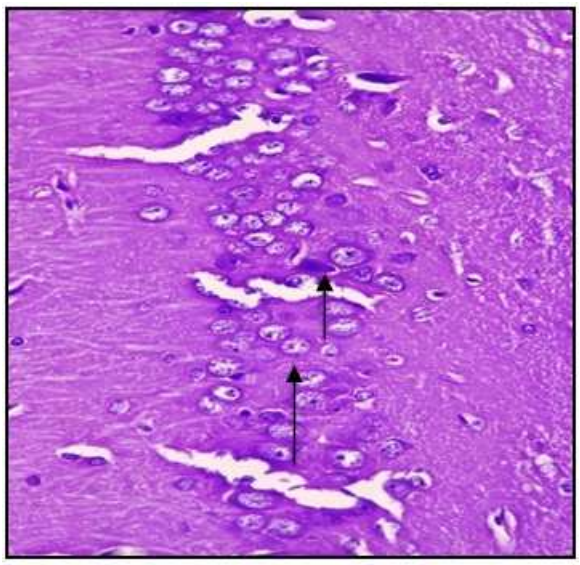


Fig. 17: The CA1 region shows intact pyramidal cells (Long Arrow) along with few neuritic plaques (Short Arrow). (H&E; mag. x 400)

Group 4: PLGA-Soya lecithin-Tween 80 NPs treated

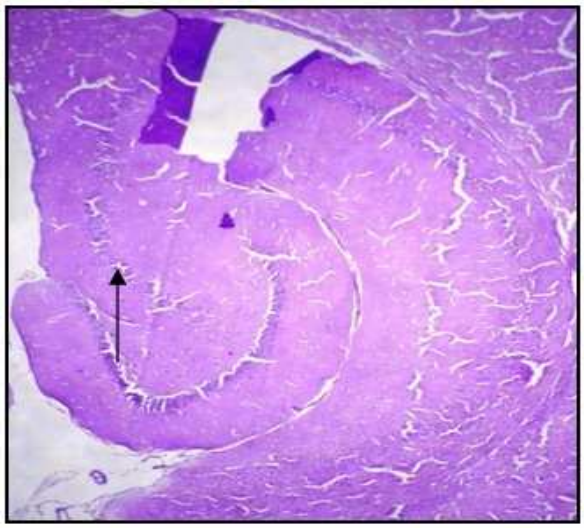


Fig. 18: Section studied from the hippocampus shows densely packed pyramidal cells in both CA1 and CA3 layers (Arrow). (H&E; mag. x50)

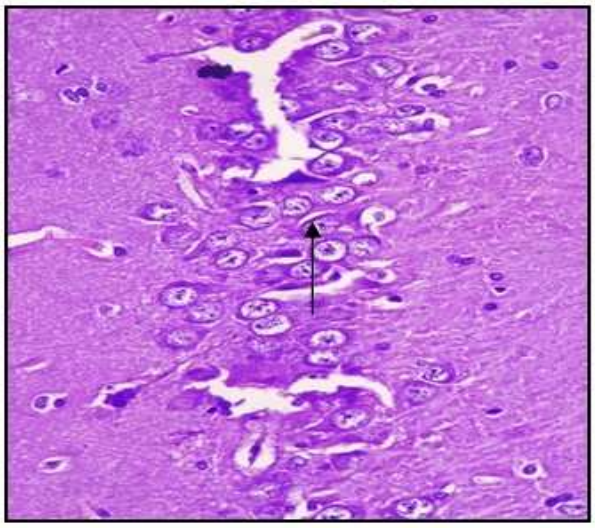


Fig. 19: The CA3 region shows intact pyramidal cells in tight clusters (Arrow). The interconnected neurophil fibers in CA3 region appear intact. (H&E; mag. x 400)

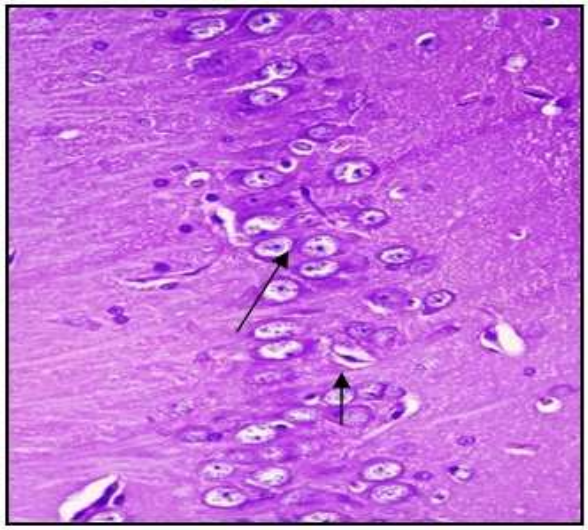


Fig. 20: The CA1 region shows intact pyramidal cells (Long Arrow) along with focal loss of neurophil fibers (Short Arrow). (H&E; mag. x 400)

Crapper *et al*., aluminium concentration was elevated in neurons containing neurofibrillary tangles and perhaps within senile plaques, however, aluminium might accumulate in neurons secondarily to intracellular degenerating changes and the neuropathological and behavioral changes following the aluminium exposure were similar to those observed in AD and the neurofibrillary changes observed in AD were found mostly within the cortical and hippocampal neurons.

Group 2 positive control (Figs. 13, 14(A), 14(B)) demonstrated cell deformation with high level of degeneration in neuronal cells and loosely packed pyramidal cells. Moreover, this group animal brain showed neuritic plaques and neurofibrillary tangles in CA1 and CA3 areas of hippocampus, which indicated the possible effect of acute oral administration of Aluminium chloride on the brain of the animal (Wistar rats). Based on hypothesis the AD model was developed into group 2. Groups 3 (Figs. 15, 16, 17) and 4 (Figs. 18, 19, 20) also demonstrated neuritic plaques and neurofibrillary tangles in CA1 and CA3 regions, which support the possible effect of acute administration of Aluminium chloride on the brain of animals.

While groups 3 and 4 have demonstrated significantly less amount of neuritic plaques and neurofibrillary tangles, which was due to standard drug RT and PLGA-Soya lecithin-Tween 80 NPs in groups 3 and 4, relatively. Figs. 16 and 19 of groups 3 and 4 showed the CA3 region with intact pyramidal cells in tight clusters. Figs. 17 and 20 showed the interconnected neurophil fibers in CA3 region appear intact. The CA1 region shows intact pyramidal cells along with few neuritic plaques and few neurofibrillary tangles. Which indicated that standard drug RT and

PLGA-Soya lecithin-Tween 80 NPs of RT showed anticholinergic activity of RT and suppress the progression of AD into aluminium chloride treated animal model (Wistar rats).

Activity of AchE

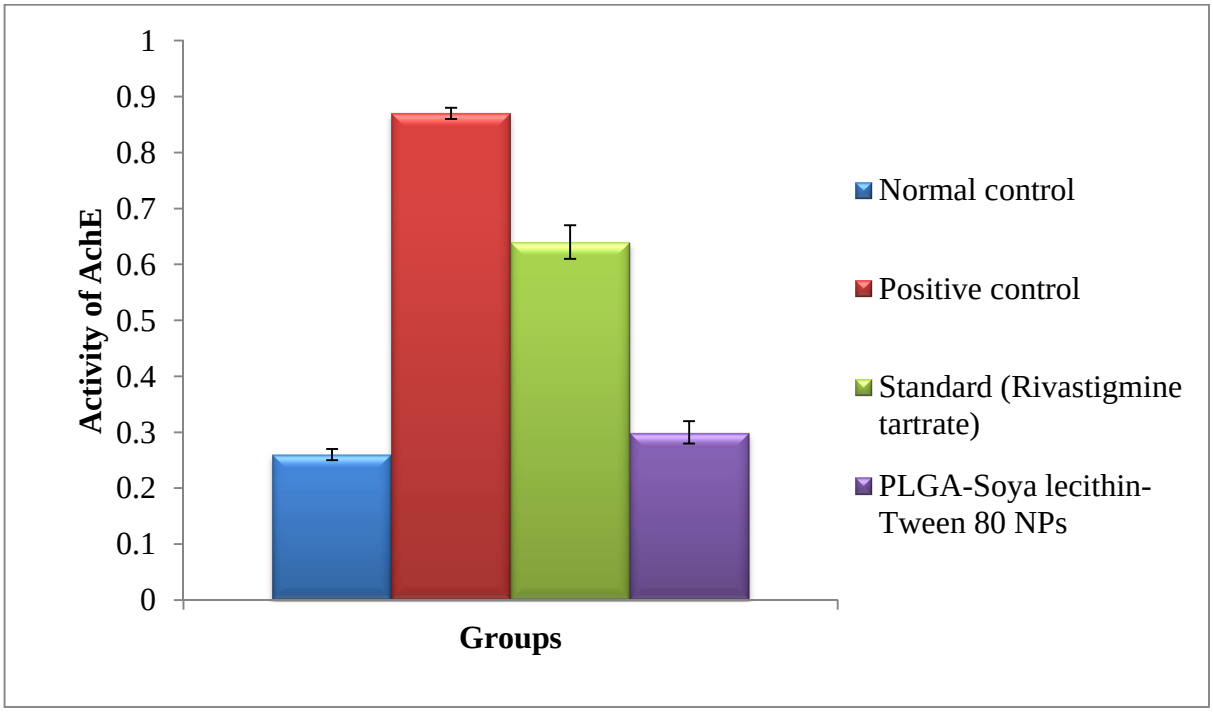


Fig. 21: Activity of AchE in brain region. Data represent means ± SEM (n = 6 animals each group) expressed as OD values/mg protein for activity of AchE

Cholinergic loss is well characterized and major component of neuropathology of AD, which already have been rationalized by the therapeutic effect of cholinesterase inhibitors[34].

Physiologically relevant concentration of Aβ related peptides have acute, negative effects on multiple aspects of acetylcholine (Ach) synthesis and release[35]. Neuromodulatory influence of the micromolar Aβ induces the toxicity of cholinergic neurons, possibly due to hyperphosphorylation of tau protein[36]. However, it has been well reported that the cholinergic therapy reduces amyloidal accumulation and have shown positive effects on Alzheimer's disease[37].

Based on this hypothesis, many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity through the cholinomimetic use of AchE inhibitors, Ach precursors and cholinergic antagonists. In this study, after Morris water maze test and Elevated plus maze test, animals sacrificed and their brains removed and evaluated for AchE activity was expressed as OD value/mg protein. After comparison with normal, standard and PLGA-Soya lecithin-Tween 80 NPs treated groups, AchE activity in the positive control group was more, which indicates that an animal model was built successfully. When compared with group 3 (Standard drug treated), PLGA-Soya lecithin-Tween 80 treated group demonstrated less AchE activity. It has been reported that the concentration of Ach rose with reduction of AchE activity under normal conditions, but both Ach and AchE concentration reduced under AD condition. The results showed that, compare to free RT, PLGA-Soya lecithin-Tween 80 NPs treated group, inhibits AchE effectively, and the reduction of AchE concentration results in slower degradation of Ach. Therefore, the concentration of Ach rose in rat's brain, and cholinergic system could reach a new equilibrium between Ach and AchE, which improved memory and cognitive deficits of rats under AD.

CONCLUSION

RT was successfully loaded into PLGA-Soya lecithin–Tween 80 NPs, was prepared using modified nanoprecipitation technique combined with self assembly. The formulations were prepared using 3^2 factorial design, to achieve with narrow size distribution (<200 nm), higher entrapment efficiency and percentage drug release. The FTIR and DSC study demonstrated there was no interaction between drug and polymers and are compatible with each other. Prepared nanoparticle of optimized formulation (D10) showed particle size 171.74 nm, polydispersity

0.154, entrapment efficiency 66.171 %, *in vitro* drug release 67.336 ± 0.254% (60h) and zeta potential -35.79 mV. The SEM study showed that particles were spherical in shape with smooth surface. The stability study for six months demonstrated that the formulations were stable at refrigerator (3-5°C) condition is the most suitable for storage of optimized PLGA-Soya lecithin-Tweeen 80 NPs. Administration of PLGA-Soya lecithin-Tween 80 NPs optimized formulation in Aluminium chloride treated animals results in enhancement in learning and memory capacity by reduction of AchE concentration and slowing down degradation of Ach. It antagonized the toxic effect of Aluminium chloride by reduction in escape latency, compared to standard drug solution treated animals. PLGA-Soya lecithin–Tween 80 NPs could be effective in brain targeting and sustained release of RT for prolong period and could be a significant improvement for treating Alzheimer's disease.

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