Chapter 3

# Therapeutic Evaluation of Solid Lipid Nanoparticle of Cycloastragenol in Streptozotocin Induced Vascular Dementia in *Danio rerio*

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## Abstract

Cycloastragenol is a molecule isolated from disparate species in the genus Astragalus purported to have telomerase activation activity. It has numbers of pharmacological actions like anti-oxidant, cell cycle regulation; ions channel modulators and vascular integrit. The present study is designed for the evaluation of Cycloastragenol (CAG) in streptozotocin induced vascular dementia (VaD) in *Danio rerio*. The vascular dementia was induced by intraperitoneal injection of streptozotocin (STZ; 350 mg/kg). The pre-treatment of cycloastragenol CAG (20 and 40 mg/kg; *i.p.*) was administered for 28 consecutive days. The reference control *i.e.,* insulin (10 mM/kg) and donepezil (10 mg/kg) was also administered for 28 consecutive days. The sign of VaD *i.e.,* learning and memory levels were evaluated with different behavioural tests like light & dark chamber test; partition preference test; three (horizontal) compartment tests; and T-maze test. In addition, the STZ induced biochemical changes such as glucose &homocysteine levels in plasma; and acetylcholinesterase activity, lipid peroxidation & reduced glutathione levels in brain samples of zebrafish were assessed. The CAG found to possess the attenuating effect in STZ induced VaD along with alterations of biochemical changes. This effect is similar to that of reference control *i.e.,*

donepezil pre-treated group. Therefore, this CAG can be used as future natural medicine for various neurovascular disorders like VaD due to its potential anti-hyperglycemic, reduction of homocysteine, anti-lipid peroxidative, anti-oxidative and acetylcholinesterase inhibitory actions.



**Keywords:** Cycloastragenol; Danio rerio; Homocysteine; Streptozotocin; Vascular Dementia

**Abbreviations:** AChE: Acetylcholinesterase; AD: Alzheimer's disease; BBB: Blood brain barrier; CAG: Cycloastragenol; SLN-CAG: Solid lipid nano particle Cycloastragenol; DTNB: 5,5'-Dithiobis-(2-Nitrobenzoic Acid); ETC: Entry to the target chamber; GSH: Reduced glutathione; HcY: Homocysteine; HPLC: High-performance liquid chromatography; i.p.: Intraperitoneal; NEDC: Number of entry to the dark chamber; ODS: Octadecylsilane; SDS: Sodium dodecyl sulphate; STZ: Streptozotocin; TBARS: Thiobarbituric acid reactive substances; TL: Transfer latency; TPSA: Target preference of short arm; TSLC: Time spent in the light chamber; TSLS: Time spent in lower segment; TSTC: Time spent in the target chamber; TSUS: Time spent in upper segment; VaD: Vascular dementia.

## 1. Introduction

The impairment of cerebral blood flow is one of the primary pathophysiological events for the development of vascular dementia (VaD) [1]. Which leads to the decline the thinking ability and skills due to lack of cerebral blood flow in various parts of the brain viz deprivation of brain cellular oxygen and nutrients supply. The primary symptoms are lack of reasoning, planning, judgment, memory and other thoughtful processes. The global incidence of VaD is higher than prevalence rate. Worldwide it is estimated around 6 to 12 cases per every 1000 population and it occurs above the 70 years of age [2]. The average duration of the development of VaD pathogenesis is around 5 years and the survival rate is lower than Alzheimer's disease (AD). In India, VaD is preventable with some alternative therapy like herbal approach, aroma therapy, Ayurvedic treatments and other life style management [3]. However, the availability of literature is limited.

The VaD associated memory impairment comprises the lack of registration, consolidation and retrieval process of information by brain tissue. In addition, it is called as cognitive dysfunction [4]. The various non-rodent animal models such as *Drosophila melanogaster* (fruit fly) and *Caenorhabditis elegans* are employed for the screening of various nootropic agents [5]. Currently, zebrafish (*Danio rerio*) is also employed in the assessment of neurovascular dysfunction associated dementia [6]. Further, this model is well accepted for the drug discovery process due to closely mimicking function human genome as well as neurophysiology actions [7]. Neurological system is one of the high energy utilizing tissue; and lack of nutrients and blood flow leads to produce the VaD [8]. Streptozotocin (STZ) is potent toxicant for beta cells of pancreatic gland viz raise the diabetic complication leads to VaD [9]. In addition, it also modulates the metabolic alteration and reduction of neurotransmitters levels leads to accumulation of homocysteine and reduction acetylcholine level respectively [10].



Currently, the AD medications are recommended for the treatment of VaD like donepezil

[Aricept®: acetylcholinesterase (AChE) inhibitor]; galantamine (Reminyl®: partial AChE inhibitor, positive allosteric modulator of nicotinic acetylcholine receptors and inhibitor of beta-amyloid aggregation); and rivastigmine (Exelon®: dual inhibitor of acetylcholinesterase and butyrylcholinesterase). Paradoxically, the long term usage of these conventional nootropic agents is also documented to produce the various unwanted serious side effects [11-14]. Hence, the newer drug discovery is essential for the neurovascular disorders like vascular dementia. Therefore, the present study designed to evaluate the role of cycloastragenol (CAG) in STZ induced vascular dementia in *Danio rerio*.

## 2. Materials and Methods

### 2.1. Animals

Wild-type adult (< 8-month-old male) Zebrafish was used in this study. The animal kept in 10 liters (potable water) housing tank. Further, the tank was maintained with aerator for aeration (for making oxygen-enriched environment) process; the temperature i.e., 25 ± 2 ºC was maintained with aquarium temperature; and 11 to 12 light and dark cycle of photoperiod for maintaining the normal circadian rhythmicity. The home cage tank was closed with rubber board to avoid diving outside of the tank and damage to the tissue. All the animals were acclimatized for 2 weeks before the performance of cognitive study test. All behavioural observation was performed between the 09.00 AM to 01.00 PM to avoid the hormone associated neurobiological interaction and neurobehavioral abnormalities.

### 2.2. Drugs and Chemicals

Trichloro acetic acid, 5,5-dithibis(2-nitrobenzoic acid), reduced glutathione, thiobarbituric acid, 1,1,3,3-Tetra methoxy propane, acetylthiocholine iodide and streptozotocin (STZ) were purchased from Sigma chemical, India. Cycloastragenol (CAG) was procured from Jarrow formulas, Los Angeles, California, USA. CAG dissolved in distilled water. The reference control drug *i.e.,* donepezil was procured from Intas pharmaceutical Ltd, India.

### 2.3. Preparation of solid lipid nanoparticle of cycloastragenol (SLN-CAG)

The SLN-CAG was prepared by solvent evaporation method as described by Agra *et al.* [15]. Briefly, about 4 mg of poloxamer-188 was dissolved in 10 ml of water. The poloxamer-188 was acted as a surfactant. And, 3 ml of co-solvent *i.e.,* propylene glycol was added with surfactant solution. This solution was used for the lipid formulation with 50 mg of stearic acid. Then, 10 mg of CAG was dissolved in the 10 ml of ethanol and mixed with above lipid mixture. This formulation was kept for 45 minutes in magnetic stirrer. Thereafter, the formulation was sonicated for further 15 minutes and centrifuged at 11200 G-force at room temperature (37 ˚C) for 15 minutes. The solid pellet was kept for freeze drying. The prepared SLN-CAG was

stored at 4 °C for further physiochemical testing (*i.e.,* particle size, zeta potential, entrapment efficiency and drug loading capacity) and therapeutic evaluation.

### 2.4. Particle size and zeta potential of SLN-CAG

The particle size and zeta potential of SLN-CAG were assessed by dynamic light scattering method by using Microtrac-Nanotrac Wave Nanotechnology Particle Size Analyzer (Betatek Inc., Toronto, Canada). The mean diameter of ultrafine particles of SLN-CAG was analyzed by Nanotrac FLEX software with Nanotrac Wave Particle Size Analyzer. The laser light frequency was shifted according to the Doppler effect corresponding to the relative velocity of the particle. Laser diode light was scattered in all directions including 180 degrees backward. The power spectrum interference signal was calculated with high-speed FFT (Fast Fourier Transform) with digital signal processors. Particle size was evaluated corresponding to the volume of distribution. Further, the zeta potential of SLN-CAG were also analyzed by using same Microtrac-Nanotrac Wave Particle Size Analyzer (Betatek Inc., Toronto, Canada) with optical electric probes device. The pair of optical electric probes was arranged opposite manner to the sample cell. This probe was creating the electric field and induce the particle movements towards the opposite electrode charge. The motion of particle was analyzed by dynamic light scattering method. Even, the particle size distribution was determined by the velocity of particle distribution in dispersing medium. For zeta potential assessment, the particle electrophoretic mobility was calculated by this additional velocity component. Zeta potential was calculated by measurement of particle mobility and zeta potential relationships. This relationship was calculated by Smoluchowski equation: $\zeta = \mu\eta/\varepsilon$. Hence, $\zeta$ is zeta potential; $\mu$ is mobility of particles; $\eta$ is the viscosity of particle; and $\varepsilon$ is dielectric constant [$\varepsilon$ = Vacuum permittivity: $8.854 \times 10^{-12}$ F/M (farads per meter)] at 25 ˚C in water.

### 2.5. Entrapment efficiency and drug loading capacity of SLN-CAG

The SLN-CAG was separated from the free drug by using the Sephadex G-50 minicolumn centrifugation technique [16]. Briefly, 0.3 ml of SLN-CAG suspension was placed in Sephadex G-50 minicolumn. This column was presaturated with empty nanoparticles with the same composition of suspension. The minicolumn was centrifuged at 112 G-Force for 1 minute, and the column washed seven times with 0.1 ml of distilled water. Elute contains the drug-loaded nanoparticles. The elute volume was adjusted to 25 ml with ethanol solution. These solutions were vortexed for 3 minutes for the breakdown of SLN-CAG and complete dissolving of CAG in the medium. Then, the mixture was centrifuged at 16128 G - a force for 10 minutes to precipitate the crystallized salt and lipid particles. The amount of entrapped CAG was assessed in the supernatant liquid by using an ultraviolet-visible spectrophotometer (DU 640B, UV-Spectrophotometer, Beckman Coulter Inc., CA, USA) at a wavelength of 373 nm. The entrapment efficiency and drug-loading of CAG in the nanoparticles were calculated

according to the following equations [17].

$$EE = \frac{\mathbf{W}(\text{Entrapped drug})}{\mathbf{W}(\text{Intial drug})} X\ 100\ \%$$

$$DL = \frac{\mathbf{W}(\text{Entrapped drug})}{\mathbf{W}(\text{Nanoparticles})} X\ 100\ \%$$

Where W (Initial drug) represented the initial amount of CAG added. W (Entrapped drug) represented the amount of CAG entrapped in the nanoparticles. W (Nanoparticles) represented the total weight of nanoparticles with all components.

### 2.6. *In-vitro* drug release effect of CAG from SLN-CAG

The release of CAG from SLN-CAG was assessed by an *in-vitro* method using diffusion cell apparatus (EMFD-08, Orchid scientific & Innovative India Pvt. Ltd. Nasik, Maharashtra, India) with a dialysis membrane (molecular weight cut off 10,000 Da). Briefly, the membrane-membrane was kept in double distilled water for 24 hours. Then, the membrane was fixed in diffusion cell apparatus. The 2 ml suspension of SLN-CAG was loaded in donor compartment and receptor compartment was filled with the dissolution medium i.e., phosphate buffer at pH 7.4. The temperature was maintained at 35 ± 0.5 °C with the continuous stirring process at 100 rpm. The 2 ml of aliquots were withdrawn at different time points 0, 1, 2, 3, 6 and 12 hour. Thereafter, aliquots were filtered and replenished with 2 ml of the fresh buffer medium. The aliquots solution was mixed with 250 ml of ethanol and shaking for 15 minutes in ultrasonic bath; then, filtered through Whatman (No 1) filter paper. The obtained solution is diluted with 1:1 ratio of 50 % v/v ethanol and water mixture. The cycloastragenol concentration was estimated by spectrophotometrically (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA) at 370 nm wavelength.

### 2.3. Induction of vascular dementia (VaD)

The diabetes was induced by intraperitoneal injection of STZ to zebrafish as described method of Intine *et al.* [18]; and Heckler and Kroll, [19]. Briefly, 350 mg/kg of STZ was injected in zebrafish under anaesthetic condition (ice cold solution). Stock solution was prepared by mixing of 7 mg of STZ in 1ml of normal saline (0.9 % w/v sodium chloride). One gram of zebrafish was injected (*i.p.*) with 50 microliter of stock solution by using insulin syringe (27 ½ gauge needle). After 24 hours, blood glucose was estimated by using glucometer (drop of blood was obtained by applications of lancet needle side of the back fins).The diabetic conditions of zebrafish were considered when stable blood glucose level raised above 15mM of glucose.

### 2.4. Experimental protocol

Five groups of adult male zebrafish (n = 20), were employed in the present study. Group I: Normal control group; Group II:  STZ (350 mg/kg; *i.p.*) treated group; Group III and IV: STZ

+ cycloastragenol (CAG; 20 and 40 mg/kg; *i.p.* for 28 consecutive days) Group V and VI: STZ + SLN-CAG ( 20 and 40 mg/kg; *i.p.* for 28 consecutive days) treated group; Group VII: STZ + Insulin (100 mM, *i.p.* for 28 consecutive days) treated group; Group VIII: STZ + Donepezil (10 mg/kg; *i.p.* for 28 consecutive days) treated group. Thereafter, the behavioural parameters were assessed on 28$^{th}$ day; whereas, T maze training made on 28$^{th}$ day and assessment of vascular dementia made on day 29. On 29$^{th}$ day, all the animals were sacrificed and blood samples were collected for the glucose and homocysteine estimation.

### 2.5. Assessment of neurocognitive functions

The cognitive functions are evaluated with different tests like light & dark chamber test; partition preference test; three (horizontal) compartment tests; and T-maze Test. Zebrafish was engaged for the evaluation of neurocognitive functions with different test apparatus after exposing of STZ. All animals were acclimatized for 5 minutes in all behavioural test apparatus to minimize the error report.

#### 2.5.1. Light and dark chamber test

Light and dark chamber test is one of the methods of spatial memory function as described by Dubey *et al.* [20]. Briefly, the dimension of this apparatus is 20 cm length, 10 cm width,and 23 cm height. The water level was maintained up to the level of 12 cm and it is divided into the two equal (10 cm) vertical segments. One half is covered with back paper and it is considered as dark chamber. Another half is allow passing the natural light (50 Lux). The cognitive function was evaluated by assessment *"Time spent in the light chamber"* termed as TSLC and *"Number of entry to the dark chamber"* termed as NEDC. Generally, when animal placed in the new chamber it prefers the darker side for few seconds (it may be due to fearful about newer environment); within few seconds it will move towards the light chamber. If animal not move to the light chamber it has poor memory. If animal preferred light chamber indicates that memory function improved.

#### 2.5.2 Partition preference test

Partition preference test is another method for the assessment of working and spatial memory function as described by Dubey *et al.* [20]. Briefly, the dimension of this apparatus is 20 cm length; 10 cm width; and 23 cm height. The water level was maintained up to the 3 cm. And, it is divided in to two equal (10 cm) vertical segments with glass (slide movement) and gap was maintained for 1 cm from ground level of the tank. The cognitive function was evaluated by assessment of number of entry to the target chamber and calculated as *"Time spent in the target chamber"* (TSTC), and another one is named as *"Percentage entry to the target chamber"* (% ETC). Generally, when animal placed in the home chamber; it prefers to go target chamber. If animal not enter to home chamber within 60 seconds; animal was keeping

in target chamber by blocking of way with glass slid. Thereafter, the way was open for further 60 seconds for home chamber entry. The preference of both chambers indicates the enhanced memory function. The preference of home chamber is the lack of memory function.

### 2.5.3 Three horizontal compartment test

Three horizontal compartment test is another method for the assessment of neurocognitive function as described by Dubey *et al.* [20]. Briefly, training section one day before fish were placed in the test chamber and guided to swim in all segments. This is achieved by side way movement of red colour (like / dislike) object. This training period was allowed for 60 seconds. Generally animal prefer in the bottom segment for few seconds (due to fearful newer environment). If animal not move in the upper two segments can be train by application of food pellet on the top of the surface or show the external object in different segment. It is required only few seconds. Thereafter, animals are ready to swim in all segments with normal swimming speed. This training will be successful if using single animal species. Paradoxically, if more than one animal species placed; the segment preference varies based on other animal movement. In addition, the other factors like age, gender,and races of the animal are also make the variable of segment swimming. The cognitive function was evaluated by assessment of "*time spent in upper segment"* (TSUS); and "*time spent in lower segment"* (TSLS). Generally, when animal placed in the test chamber; it prefers to swim in upper segment of chamber within 15 seconds. It indicates that the animal has normal or improved memory function. If animal not prefer the upper segment (even guided with likable stick or food); and animal swim in middle or lower segment indicates that loss of memory. The present study employed the individual assessment in trial as well as in test to achieve accurate results.

### 2.5.4. T-maze test

T-maze test is another method for the assessment of neurocognitive function. The T-maze test is one of the conventional methods of memory assessment in rodent species. T-maze test was performed as described method of Buccafusco *et al.* [21] and Colwill *et al.* [22]. Briefly, T maze apparatus consist of two short arms (10 cm length; 6 cm width; and 10 cm height) with different colour (one arm with red colour glass; and another end arm coloured with green colour). And one long arm (20 cm length; 10 cm width; and 10 cm height). One short arm (green colour arm) employed as favourable environment with reward of food pellet (if preferred) and another short arm (red colour) employed as punishment by string with glass rod. One day before test assessment; all animal were allowed learn the T maze environment and trained for the learning process. Next day, animals were placed in the corner of long arm (starting point) and target point was identified for either any one of the short arm. The starting point and target point were separated by vertical slide control pattern. Each fish was explored for 2 minutes for the learning and memory assessment. The transfer latency (TL) and percentage target (green)

preference of short arm (% TPSA) were noted for the assessment of cognitive function. The animal movements were tracked by USB camera with computer software (12 Megapixel USB Camera, Intex, India).

### 2.6. Estimations of biomarker changes in blood

#### 2.6.1 Blood sample collection

The blood samples were collected at the end of the study protocol from zebrafish as described method of Pedroso *et al.* [23]; and Babaei *et al.* [24]. Briefly, fish were placed on the petri dish and with steel blade, the diagonal incision made between the anal fin and the caudal fin and placed in centrifugal tube. The centrifuge tube was prefilled with 100 µl of anticoagulant (11 % w/v of sodium citrate). This setup was allowed to centrifuge at 1000 rpm for 10 minutes. Then, zebrafish removed from the centrifuge tube and blood samples were centrifuged with 2500 rpm for 15 minutes to collect the plasma for the estimations of glucose and homocysteine levels. The dilution was made with 1: 4 ratio with anti-coagulant solution.

#### 2.6.2. Estimation of plasma glucose

The plasma samples were used for the assessment of glucose estimation as described method *i.e.,* glucose oxidase and glucose peroxidase (GOD–POD) reactions [25]. Streptozotocin treated zebrafish raise the blood glucose levels [18]. The GOD–POD reaction associated colour development with glucose levels were estimated by spectrophotometric method (DU 640B Spectrophotometer, Beckman Coulter Inc., 263 CA, USA) at 505 nm. The glucose estimation kit was commercially available in market (Reckon Diagnostics Pvt. Ltd. Vadodara, India).

#### 2.6.3. Estimation of homocysteine level

The plasma samples were used for the assessment of homocysteine estimation by HPLC method as described by Devi *et al.* [26]. Briefly, reverse-phase HPLC with fluorescence detection was used for plasma homocysteine estimation. The 50 µL plasma was mixed with 50 µl of 0.8 M perchloric acid and vortexed vigorously. Then centrifugation was made at 1700 × g for 10 min. The 50 µL of supernatant was mixed with 20 ml of SBDF (ammonium 7-flurobenzo-2-oxa-1, 3-diazole-4sulphonate; 1 mg/ml in 125 mM boric acid) was added and incubated at 60 °C for one hour. Then samples were injected in HPLC column with mobile phase at a flow rate of 1.5 ml per min. The column equilibration time was maintained for 15 minutes with ambient temperature. The retention time of each sample was calculated by using homocysteine standard plot with the help of calibration curve (made with different concentrations). Stationary phase *i.e.,* octadecylsilane (ODS) 250 X 4.6 mm column; and mobile phase *i.e.,* 4 % acetonitrile in potassium dihydrogen orthophosphate (0.2 M, pH: 2.1) was used in this study. The excitatory and emission light beam source were detected with fluorescent detector at 385nm and 515 nm

wavelength respectively.

## 2.7. Estimations of biomarker changes in brain

### 2.7.1. Brain sample collection

After assessment of behavioural assessment and blood sample collection; the zebrafish brain samples were isolated immediately by microsurgical method and freeze dried at -4 ˚C. Next day, all samples were homogenate with phosphate buffer solution. The supernatant were collected by centrifugation at 1372 g force for 15 minutes. The supernatant of zebrafish brain samples were used for estimation of tissue biomarker changes *i.e.,* thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and acetylcholine esterase (AChE) activity levels.

### 2.7.2. Estimation of TBARS level in zebrafish brain

The TBARS level of zebrafish brain samples were estimated by spectroscopic method as described by Ohkawa *et al.* [27]. Briefly, 0.2 ml of tissue supernatant was mixed with 0.2 ml of 8.1 % w/v of sodium dodecyl sulphate (SDS), 1.5 ml of 30 % v/v of acetic acid (pH 3.5), 1.5 ml of 0.8 % w/v of thiobarbituric acid and volume made up to 4 ml with distilled water. All the test tubes were incubated at 95 ˚C for 1 hour. Then cooled with tab water and further added 1 ml of distilled water and 5 ml of n-butanol-pyridine (15:1 v/v) mixture. After 10 minutes, tubes were centrifuged at 1372 g force for 15 min. The clear pink colour chromogen of supernatant was collected. The formation of pink colour and their intensity is based on the presence of TBARS content in tissue sample. The change of absorbance was noted by spectrophotometrically (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA) at 535 nm wavelength. These readings were used for the further calculation of TBARS levels in tissue samples. The results were expressed as nM per mg of protein.

### 2.7.3. Estimation of GSH level in zebrafish brain

The GSH level of zebrafish brain samples were estimated by spectroscopic method as described by Ellman [28]. Briefly, 0.5 ml supernatant was mixed with 2 ml of 0.3 M of disodium hydrogen phosphate solution and 0.25 ml of 0.001 M of freshly prepared DTNB solution to develop the yellow colour chromogen. The formation of yellow colour and their intensity is based on the presence of GSH content in tissue sample. The changes of absorbance were noted by spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA) at 412 nm wavelength. These readings are used for the further calculation of GSH levels in tissue samples. The results were expressed as μM of GSH / mg of protein.

### 2.7.4 Estimation of AChE level in zebrafish brain

The AChE activity level of zebrafish brain samples were estimated by spectroscopic method as described by Ellman *et al.* [29]. Briefly, 500 µl of brain supernatant was mixed with 0.25 ml of 0.001 M of DTNB and allow developing the yellow colour chromogen products. The formation of yellow colour and their intensity is based on the ability of AChE to conversion of acetylthiocholine content. It indicates the presence of active AChE level. The change of absorbance was noted by spectrophotometrically (DU 640B Spectrophotometer, Beckman Coulter Inc., CA, USA) at 420 nm wavelength. These readings were used for the further calculation of AChE activity levels in tissue sample. The AChE activity was calculated by using the specific formula *i.e.,* R = δ O.D X Volume of assay (3 ml) / ε X mg of protein. Hence, R is representing the rate of enzyme activity in 'n' mole of acetylthiocholine iodide hydrolyzed per minute per mg protein. O.D. is representing a change of absorbance per minute. ε is representing the extinction coefficient value *i.e.,* 13600 per mole per centimetre. The results were expressed as µM of acetylthiocholine hydrolysis per milligram of protein per minute.

### 2.7.5. Estimation of total protein level in zebrafish brain

The total protein level of zebrafish brain samples were estimated by spectroscopic method as described by Lowry *et al.* [30]. Briefly, 300 µl of zebrafish brain supernatants were diluted with distilled water upto 1 ml. Further, 5 ml of Lowry's reagent was mixed with solution and allow standing for further 15 minutes at room temperature (37 ˚C). Then 0.5 ml of Folin-Ciocalteu reagent was added slowly and vortexed vigorously at room temperature (37 ˚C) for 30 min. The clear purple chromogen was formed. The formation of purple colour and their intensity is based on the presence of protein content in tissue sample. The change of absorbance was noted by spectrophotometrically (DU 640B, UV-Spectrophotometer, Beckman Coulter Inc., CA, USA) at 750 nm wavelength. These readings were used for the further calculation of total protein levels in tissue samples. The results were expressed as mg of protein per ml of supernatant.

## 2.8. Statistical analysis

All the results were expressed as mean ± standard deviation (SD). Data obtained from behavioural tests were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test were applied by using Graph pad prism Version-5.0 software. The data of tissue biomarker *i.e.,* TBARS, GSH and AChE levels were analyzed using one way ANOVA followed by Tukey's multiple range tests were applied for Post-hoc analysis by using Graph pad prism Version-5.0 software. A probability value of $p < 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Assessment of physiochemical parameters of SLN-CAG preparation

The optimized solid lipid nanoparticle of CAG (SLN-CAG) was prepared by solvent evaporation method as predicted experimental designing patterns. The physiochemical parameters of SLN-CAG were tested for assessment of particle size, zeta potential, entrapment efficiency and drug loading capacity. The values are depicted in **Figure 1** and **Table 1**. The mean particle sizes, zeta potentials of the SLN-CAG formulations were obtained; and mean particle sizes range is 347.98 nm and mean zeta potentials is – 14.12 (3.29 mV). Similarly, the mean percentage entrapment efficiency and drug loading capacity of SLN-CAG are 82.9 and 93.78 respectively.

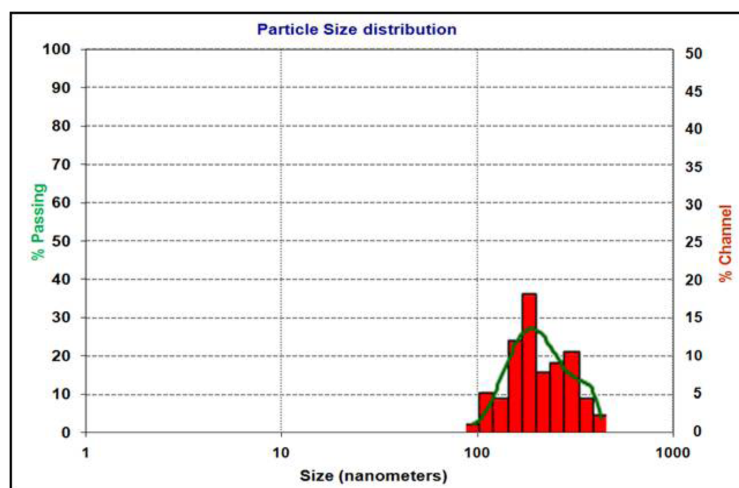


**Figure 1:** Assessment of particle size. This illustration revealed that, SLN-CAG possess the nanoparticle range *i.e.,* mean value was 347.98 nm.

**Table 1:** Physiochemical parameters of SLN-CAG preparation.

| Formulation of SLN-CAG | Mean Particle Size (nm) | Zeta Potential (mV) | Entrapment Efficiency (%) | Drug loading Capacity (%) |
|---|---|---|---|---|
| SLN-CAG$_1$ | 256.4 ± 1.4 | - 11.2 ± (- 2.04) | 82.8 ± 2.2 | 96.4 ± 1.1 |
| SLN-CAG$_2$ | 167.1 ± 3.7 | - 9.4 ± (- 1.37) | 86.5 ± 1.4 | 84.6 ± 1.2 |
| SLN-CAG$_3$ | 248.8 ± 1.6 | - 16.4 ± (- 3.72) | 80.7 ± 06 | 94.8 ± 1.1 |
| SLN-CAG$_4$ | 382.9 ± 2.8 | - 15.8 ± (- 4.23) | 88.4 ± 1.8 | 92.3 ± 3.4 |
| SLN-CAG$_5$ | 538.2 ± 1.9 | - 14.3 ± (- 3.96) | 84.9 ± 0.4 | 99.2 ± 1.6 |
| SLN-CAG$_6$ | 494.5 ± 3.3 | - 17.6 ± (- 4.47) | 74.1 ± 2.5 | 95.4 ± 1.4 |

In this table, value express the physiochemical parameters of SLN-CAG of various preparations. Abbreviation: SLN-CAG, Solid lipid nanoparticles of cycloastragenol.

### 3.2. Assessment of drug release potential of SLN-CAG preparation by *in-vitro* method

The percentage drug release potential of SLN-CAG was assessed by using *in-vitro* diffusion cell-based study with membrane dialysis process. The values are depicted in table 2. The first three hours, *in vitro* release of CAG from SLN-CAG, is faster about ≈ 40 to 50 % followed by release the CAG is sustained manner. Within 12 hours, the cumulative percentage

drug release potential of SLN-CAG range is 81.9 to 93.7 in a phosphate buffer of pH 7.4 at 35 ± 0.5 ˚C.

**Table 2:** Drug release potentials of SLN-CAG preparation.

| Formulation of SLN-CAG | Drug Release (%) | | | | |
|---|---|---|---|---|---|
| | 1 h | 2 h | 3 h | 6 h | 12 h |
| SLN-$CAG_1$ | 20.2 ± 2.1 | 38.2 ± 1.2 | 50.3 ± 1.7 | 70.1 ± 1.8 | 96.7 ± 2.1 |
| SLN-$CAG_2$ | 22.6 ± 2.5 | 42.3 ± 1.3 | 56.1 ± 1.6 | 72.6 ± 2.0 | 82.3 ± 1.5 |
| SLN-$CAG_3$ | 16.2 ± 1.8 | 40.1 ± 1.6 | 52.5 ± 1.3 | 76.5 ± 1.6 | 94.2 ± 2.8 |
| SLN-$CAG_4$ | 24.3 ± 2.7 | 46.3 ± 1.5 | 60.6 ± 1.4 | 74.3 ± 2.1 | 86.4 ± 1.7 |
| SLN-$CAG_5$ | 28.7 ± 1.5 | 34.7 ± 1.4 | 64.3 ± 1.5 | 78.7 ± 1.5 | 82.9 ± 2.4 |
| SLN-$CAG_6$ | 26.1 ± 2.4 | 43.2 ± 1.2 | 54.7 ± 1.3 | 84.4 ± 1.7 | 84.3 ± 1.3 |

In this table, value express the drug release potentials of SLN-CAG with variable preparation. Abbreviation: SLN-CAG, Solid lipid nanoparticles of cycloastragenol.

**Table 3:** Effect of SLN-CAG in STZ induced diabetic vascular dysfunction

| Groups | Glucose (µM / L) | HcY (µM / L) |
|---|---|---|
| Normal | 5.80 ± 0.6 | 12.8 ± 0.07 |
| STZ (350) | 30.2 ± 0.12 [a] | 42.7 ± 0.004 [a] |
| STZ+ CAG (20) | 22.4 ± 0.2 [a] | 24.9 ± 0.4 [a] |
| STZ+ CAG (40) | 18.9 ± 0.3 [a] | 17.2 ± 0.4 [a] |
| STZ + SLN-CAG (20) | 11.2 ± 0.4 [b] | 6.4 ± 0.003 [b] |
| STZ + SLN-CAG (40) | 7.96 ± 0.9 [b] | 8.6 ± 0.007 [b] |
| STZ + Insulin (100 mM) | 9.22 ± 0.4 [b] | 12.40 ± 0.007 [b] |
| STZ + Donepezil (10) | 7.24 ± 0.7 [b] | 9.5 ± 0.009 [b] |

In this table, value express the effect of SLN-CAG on tissue biomarker changes. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 20 zebrafish per group. [a]$p < 0.05$Vs normal group. [b]$p < 0.05$Vs STZ control group.Abbreviation: STZ, streptozotocin; CAG, Cycloastragenol; SLN-CAG, Solid lipid nanoparticles of cycloastragenol; HcY, homocysteine; and AChE, acetylcholinesterase.

### 3.4. Assessment of STZ induced vascular dysfunction associated dementia

The administration of STZ (350 mg/kg; *i.p.*) produce the significant ($p < 0.05$) rising of blood glucose and homocysteine levels which leads to raise the diabetes associated VaD. The VaD type of abnormal memory function was assessed by different test such as light & dark chamber test; partition preference test; three (horizontal) compartment tests; and T-maze test. The role of SLN-CAG in STZ induced diabetic VaD type of cognitive impairment expressed in following section.

#### 3.4.1. Effect of SLN-CAG in light and dark chamber test

The intraperitoneal injection of STZ (350 mg/kg) produces the significant ($p < 0.05$) impairment of vascular dementia type of memory. And, it is tested in light and dark chamber

test apparatus; the results are indicates the decreasing TSLC and raising NEDC values as compared to normal control group. The pretreatment of SLN-CAG (20 and 40 mg/kg; *i.p.*) shown to produce the ameliorative effect in STZ induced cognitive impairment in dose dependent manner. In addition, the pretreatment of reference control *i.e.,* insulin (100 mM) and donepezil (10 mg/kg; *i.p.*) also produce the significant attenuation of STZ induced cognitive impairment changes. It indicates that the administration of SLN-CAG possess the significant role in the improvement of STZ associated VaD type of memory. The results are illustrated in **Figure 2a** and **2b**.

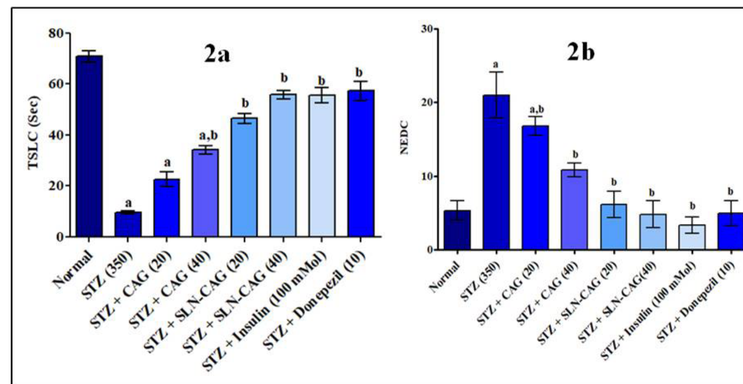


**Figure 2:** Effect of SLN-CAG on light and dark chamber test. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. [a]*p*< 0.05 Vs normal group. [b]*p*< 0.05 Vs STZ control group. Abbreviation: STZ, streptozotocin; TSLC, time spent in light chamber; and NEDC, number of entry to the dark chamber.

### 3.4.2. Effect of SLN-CAG in partition preference test

The intraperitoneal injection of STZ (350 mg/kg) produces the significant ($p< 0.05$) impairment of vascular dementia type of memory. And, it is tested in partition preference test apparatus; the results are indicates the decreasing TSTC and % ETC values as compared to normal control group. The pretreatment of SLN-CAG (20 and 40 mg/kg; *i.p.*) shown to produce the ameliorative effect in STZ induced cognitive impairment in dose dependent manner. In addition, the pretreatment of reference control *i.e.,* insulin (100 mM) and donepezil (10 mg/kg; *i.p.*) also produce the significant attenuation of STZ induced cognitive impairment changes. It indicates that the administration of CAG possess the significant role in the improvement of STZ associated VaD type of memory. The results are illustrated in **Figure 3a** and **3b**.

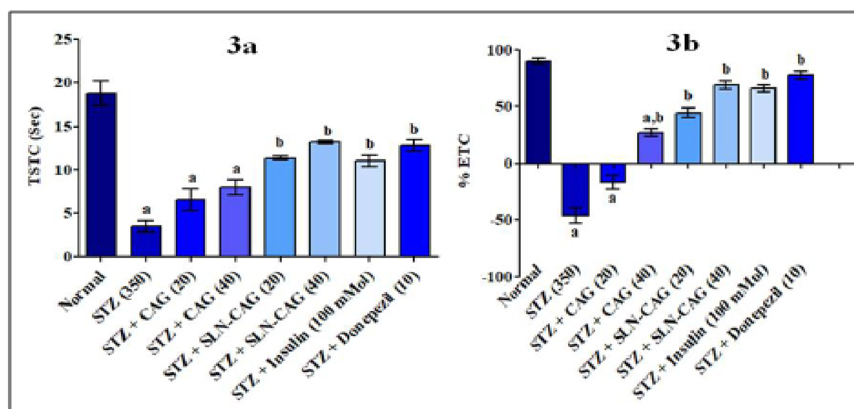


**Figure 3:** Effect of SLN-CAG on partition preference test. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. [a]*p*< 0.05 Vs normal group. [b]*p*< 0.05 Vs STZ control group. Abbreviation: STZ, streptozotocin; TSTC, time spent in target chamber; and % ETC, entry to the target chamber.

### 3.4.3. Effect of SLN-CAG in three horizontal compartment test

The intraperitoneal injection of STZ (350 mg/kg) produces the significant ($p < 0.05$) impairment of vascular dementia type of memory. And, it is tested in three horizontal compartment test apparatus; the results are indicates the decreasing TSUS and increasing the TSLS values as compared to normal control group. The pretreatment of SLN-CAG (20 and 40 mg/kg; *i.p.*) shown to produce the ameliorative effect in STZ induced cognitive impairment in dose dependent manner. In addition, the pretreatment of reference control *i.e.,* insulin (100 mM) and donepezil (10 mg/kg; *i.p.*) also produce the significant attenuation of STZ induced cognitive impairment changes. It indicates that the administration of SLN-CAG possess the significant role in the improvement of STZ associated VaD type of memory. The results are illustrated in **Figure 4a** and **4b**.

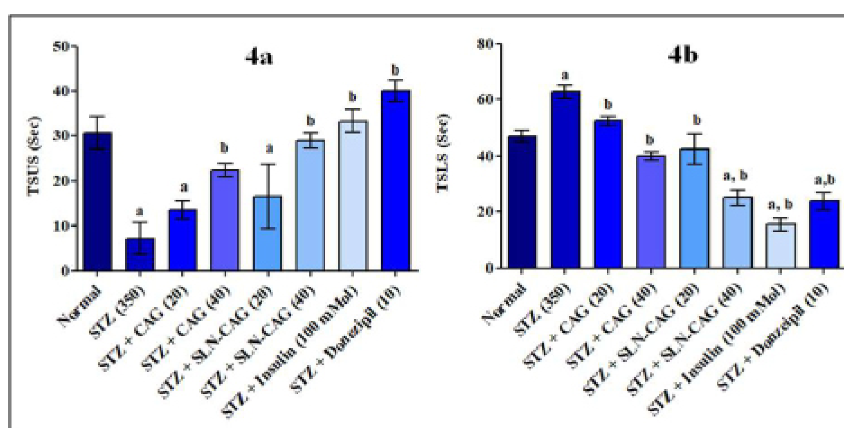


**Figure 4:** Effect of SLN-CAG on horizontal compartment test. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. $^{a}p< 0.05$ Vs normal group. $^{b}p< 0.05$ Vs STZ control group. Abbreviation: STZ, streptozotocin; TSUS, time spent in upper segment; and TSLS, time spent in lower segment.

### 3.4.4 Effect of SLN-CAG in T-maze test

The intraperitoneal injection of STZ (350 mg/kg) produces the significant ($p< 0.05$) impairment of vascular dementia type of memory. And, it is tested in T-maze test apparatus; the results are indicates the increasing TL and decreasing % TPSA values as compared to normal control group. The pretreatment of SLN-CAG (20 and 40 mg/kg; *i.p.*) shown to produce the ameliorative effect in STZ induced cognitive impairment in dose dependent manner. In addition, the pretreatment of reference control *i.e.,* insulin (100 mM) and donepezil (10 mg/kg; *i.p.*) also produce the significant attenuation of STZ induced cognitive impairment changes. It indicates that the administration of SLN-CAG possess the significant role in the improvement of STZ associated VaD type of memory. The results are illustrated in **Figure 5a** and **5b**.

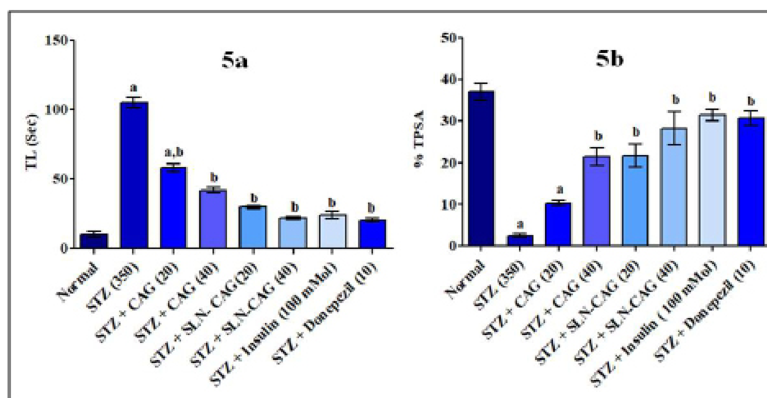


**Figure 5:** Effect of SLN-CAG on T-maze test. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 6 zebrafish per group. $^{a}p < 0.05$ Vs normal group. $^{b}p < 0.05$ Vs STZ control group. Abbreviation: STZ, streptozotocin; TL, transfer latency; % TPSA, percentage target preference of short arm

## 3.5 Effect of SLN-CAG in biomarker changes in zebrafish brain

The intraperitoneal injection of STZ (350 mg/kg) produces the significant ($p < 0.05$) raising of brain TBARS and AChE activity levels, and decrease the GSH level when compared to normal control group. The pre-treatment of SLN-CAG (20 and 40 mg/kg; *i.p.*) shown to produce the ameliorative effect in STZ induced biochemical changes in dose dependent manner. In addition, the pretreatment of reference control *i.e.,* insulin (100 mM) and donepezil (10 mg/kg; *i.p.*) also produce the significant attenuation of STZ induced biomarker changes in zebrafish brain samples. It indicates that the administration of SLN-CAG possess the significant role in the improvement of STZ associated VaD type of memory via anti-oxidant, anti-inflammatory and enzymatic regulation cholinergic neurotransmission. The results are tabulated in **Table 4**.

**Table 4:** Effect of SLN-CAG in STZ induced vascular dysfunction associated tissue biomarker changes.

| Groups | GSH (µM / mg of protein) | TBARS (nM / mg of protein) | AChE (µM / mg of protein / min) |
|---|---|---|---|
| Normal | 5.46 ± 0.03 | 0.17 ± 0.007 | 18.3 ± 1.21 |
| STZ (350) | 1.22 ± 0.08 $^{a}$ | 1.92 ± 0.002 $^{a}$ | 63.2 ± 0.51 $^{a}$ |
| STZ + CAG (20) | 1.82 ± 0.02 $^{a}$ | 1.68 ± 0.012 $^{a}$ | 46.1 ± 0.21 $^{a}$ |
| STZ + CAG (40) | 2.41 ± 0.03 $^{a}$ | 1.09 ± 0.008 $^{a}$ | 32.8 ± 0.11 $^{a}$ |
| STZ + SLN-CAG (20) | 3.63 ± 0.06 $^{b}$ | 0.30 ± 0.009 $^{b}$ | 27.2 ± 1.09 $^{b}$ |
| STZ + SLN-CAG (40) | 4.47 ± 0.01 $^{b}$ | 0.21 ± 0.006 $^{b}$ | 24.7 ± 0.96 $^{b}$ |
| STZ + Insulin (100 mM) | 6.95 ± 0.02 $^{b}$ | 0.16 ± 0.008 $^{b}$ | 28.7 ± 1.08 $^{b}$ |
| STZ + Donepezil (10) | 5.72 ± 0.02 $^{b}$ | 0.16 ± 0.008 $^{b}$ | 19.6 ± 1.15 $^{b}$ |

In this table, value express the effect of SLN-CAG on tissue biomarker changes. Digits in parenthesis indicate dose mg/kg. Data were expressed as mean ± SD, n = 20 zebrafish per group. $^{a}P < 0.05$ Vs normal group. $^{b}P < 0.05$ Vs STZ control group. Abbreviation: STZ, streptozotocin; CAG, Cycloastragenol; SLN-CAG, Solid lipid nanoparticles of cycloastragenol; GSH, reduced glutathione; TBARS, thiobarbituric acid reactive substances; and AChE, acetylcholinesterase.

## 4. Discussion

The present study revealed that SLN-CAG (20 and 40 mg/kg) significantly ameliorate the STZ (350 mg/kg; *i.p.*) induced diabetes and vascular dysfunction by reducing the levels

of plasma glucose and homocysteine levels. The VaD associated cognitive impairment also assessed by using various test apparatus *i.e.,* decreasing TSLC and raising NEDC values in light and dark chamber test; decreasing TSTC and % ETC values in partition chamber test; decreasing TSUS and increasing the TSLS values in three horizontal compartment test; and increasing TL and decreasing % TPSA values in T-maze test. Moreover, the STZ induced alteration of TBARS, GSH and AChE activity levels also ameliorated by SLN-CAG treatment in zebrafish. The present results are supported by the readings of reference control *i.e.,* insulin and donepezil treated group.

STZ is one of potent toxic agents for β cells of Langerhans of islets leads to enhance the type-I diabetic mellitus. In addition, the chronic abnormalities of metabolic changes i.e., accumulation of glucose, proteins and other metabolic waste products in the circulation induce the endothelial cell damage [32]. The hallmark of vascular damage is rising of plasma homocysteine. In addition, STZ potentially alter the cognitive centre of the brain i.e., hippocampus via over activation of post-synaptic glutamate and N-methyl-D-aspartate (NMDA) receptors [33]. Paradoxically, STZ directly act on neuronal tissue leads to alter the cellular molecules as well as cytoskeletal protein which in turn cause the neurovascular damage and VaD [34]. Furthermore, the multiple neurovascular complications occur due to STZ induced metabolic abnormalities followed by enhance the accumulation of free radical, inflammatory mediators and neurodegenerative prion proteins [35-36]. In diabetes condition, the various cellular and molecular changes of tissue leads to cause the multiple tissue damage like heart, kidney and brain including vascular system [37]. The administration of insulin is known to enhance the tissue function via multiple pharmacological mechanisms. STZ associated metabolic dysfunction reported to produce the vascular dysfunction as well as AD progression [38]. In addition, insulin resistance also raises the risk of AD progress via down regulation insulin receptors in brain [38]. Currently, novel insulin delivery mechanism and usage of insulin sensitizers are well documented to attenuate the AD progress [39-40]. The primary hallmark of VaD is alteration brain acetylcholinesterase activity. The inhibitor of acetylcholinesterase activity such as donepezil is widely accepted for the cognitive impairment disorders in preclinical and clinical conditions [41-42]. In the present study, donepezil is used as reference control. In addition, insulin and donepezil has own anti-oxidant, anti-inflammatory and neuroprotective actions [40, 43]. The similar pharmacological actions are observed in the present study. Cycloastragenol has potential neuroprotective action via anti-oxidant, anti-inflammatory and anti-apoptotic actions [44-45]. However, the primary drawback of this medicine in clinical setup is blood brain barrier (BBB) crossing ability [46]. In this study, SLN-CAG was employed to prove the beneficial effect in STZ induced VaD. The pretreatment of SLN-CAG revealed the ameliorative effect in STZ induced VaD with regulation biochemical marker changes.

The cycloastragenol is documented to ameliorate neuronal damage and vascular complication [46-47]. In addition, it also documented to enhance the memory function against STZ associated cognitive impairment in rodent [48-49]. The established mechanism of cycloastragenolis is free radical scavenger and anti-inflammatory actions. However, beyond the anti-oxidant and anti-inflammatory actions of cycloastragenol is also playing a key role in multiple cellular and molecular actions such as regulation of neurotransmitter, hormonal modulation, free calcium mobility, apoptosis and necrotic actions [50-51]. The cognitive improving action of SLN-CAG in STZ model reveals that it possess the endogenous anti-oxidant *i.e.,* raise the reduced glutathione action, reduction of inflammatory reactions *i.e.,* decrease the lipid peroxidation and neurotransmitter regulatory actions *i.e.,* reduction of AChE activity. Crucially, the SLN-CAG is key molecule to enhancement of cognitive function against STZ toxicity. The primary outcome of this research work proves that SLN-CAG attenuate the vascular damage associated cognitive dysfunction. So it can be evaluated in clinical condition of VaD due to its solving nature of clinical problem *i.e.,* lack of bioavailability and poor BBB crossing ability. Hence, SLN-CAG may be a future medicine for the treatment of neurodegenerative disorders like memory disorders due to its potential anti-diabetic, reduction of homocysteine accumulation, anti-oxidative, anti-lipidperoxidative and acetylcholine esterase inhibitory actions.

## 5. Conflict of interest

The authors declare that there is no conflict of interest in the present study.

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