

Chitosan-coated Liposomes of *Centella asiatica* Extract: An *In vitro* Formulation Design for Oral Delivery

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Abstract

Aim: A chitosan-coated liposomes of standardized *Centella asiatica* extract (CAE) was developed with the aim of improving the solubility of its phytoconstituents for the oral route with the intention to treat Alzheimer's disease. **Materials and Methods:** The CAE was obtained by the soxhletion process. The formulation was optimized using design-expert software, solvent evaporation, and ionotropic gelation method was adopted to prepare CAE liposome (CAEL) and chitosan-coated CAE liposomes (CCAEL), respectively. The prepared CCAEL was characterized for its vesicle size, entrapment efficiency, polydispersity index, drug content analysis, Fourier transform infrared (FTIR), differential scanning calorimetry (DSC), transmission electron microscopy (TEM), atomic force microscopy (AFM), *in vitro* drug release, *in vitro* antioxidant study, *ex vivo* permeation study, and stability study. **Results and Discussion:** The proper amalgamation of drug and chitosan-phospholipid mixture was confirmed by FTIR and DSC. The surface morphology of the prepared formulation was examined by TEM and AFM. The *in vitro* rate of drug release pattern was analyzed where CAE showed less rate of release of $35.34 \pm 0.30\%$ in about 10 h due to poor solubility, while approximately $58.6 \pm 0.42\%$ release was observed from optimized CCAEL. *In vitro* antioxidant study demonstrated that free radical scavenging activity of CAE was retained even after intricate it with the phospholipid, that is, % inhibition of 2,2-diphenyl-1-picrylhydrazyl at 50 $\mu\text{g/ml}$ was found 73.84% whereas 74.4% CAE. *In vitro* intestinal study proved that the permeation rate increases due to the encapsulation of chitosan-phospholipid complex. Stability study showed that chitosan coated liposomes were stable due to compact chitosan coating layer. **Conclusion:** The outcome of the current study is quite encouraging, shows better solubility and permeability. Further detailed preclinical studies are required to be conducted to ensure better product development.

Key words: *Centella asiatica*, chitosan, liposomes, phospholipid

INTRODUCTION

Alzheimer's disease (AD) is an irrevocable, progressive brain disease that deliberately destroys memory and thinking skills. In India, more than 4 million people are estimated to be suffering from AD and other forms of dementia, giving the country the third highest caseload in the world and estimated to reach almost 7.5 million by the end of 2030.^[1] When the age advances, function of mitochondria weakens and thereby causes lower ATP production and increased oxidative stress. The progressive degenerative mechanism involved disturbance in neurotransmission signals due to the deposition of amyloid plaques and neurofibrillary tangles (NFTs). Progressive accumulation of NFTs leads to the breakdown of neuronal functional signal and, finally, cell death.^[2]

At present, cholinesterase inhibitors such as rivastigmine, galantamine, and donepezil drugs are available for the treatment of mild to moderate AD. Its function is to increase the acetylcholine level by inhibiting the cholinesterase enzyme responsible for the breakdown of acetylcholine in

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the brain. The prolong therapy is required to treat the disease with the frequent dose, which may lead to side effects like gastralgia, nausea, cardiac arrhythmia, and loss of appetite. Concerning the factors such as blood-brain barrier (BBB), long-term treatment, side effects of synthetic drugs, and economic aspects, popped us to unleash the potential of herbal drugs to treat AD.^[3] *Centella asiatica* (CA) is creeping herbs belong to the family Apiaceae and since ancient time, CA categorized as “Medhya drugs” in Ayurveda. CA has been used for revitalizing the nerves and brain cells for memory enhancement, as it is reported that it has more than 70 phytoconstituents. Researchers have demonstrated that the activity of CA as it inhibits acetylcholinesterase, butyrylcholinesterase, and tyrosinase enzymes responsible for the development of AD.^[4] However, oral consumption of the *Centella asiatica* extract (CAE) has limited therapeutic actions due to its poor solubility and less bioavailability. Therefore, enhancing the solubility and stability of the extract is mandatory to get its maximum bioavailability.^[5]

Liposomes have been thus emerged as promising carriers due to its distinctive properties such as biodegradability and biocompatibility, holding hydrophilic and hydrophobic compounds. Bilayer phospholipid coat protects the drug from the external environment, the drug gets absorbed through inner intestinal absorptive cell; thus, it avoids the first metabolism and improves the solubility and thus enhances bioavailability. However, conventional liposomes may undergo enzymatical degradation during circulation leads to poor bioavailability. To overcome the problem, the strategy is adopted herein by coating the liposome with inert chitosan. Chitosan is a positively charged biodegradable polymer which gets easily interacted electrostatically with the negatively charged phospholipids and creates a stable biopolymer coat, thereby it prevents drug leakage and stabilizes the liposome.^[6] The positive charge of the chitosan supports in mucoadhesion with the negatively charged cell membrane causes prolong the drug retention time and enhances drug penetration and sustaining the release of the active component through mucus membrane.

Especially in the early stages of AD oral route should always be considered as a preferred route as it is convenient, economical, non-invasive, often considered as a safer route that requires no training. However, in this segment, various research studies suggest that oral drug delivery from the bloodstream to the brain across the BBB has long been a significant challenge to treat AD. Therefore, an attempt was made to develop chitosan-coated *Centella asiatica* extract liposomes (CCAEL) and its *in-vitro* evaluation for oral delivery.

MATERIALS AND METHODS

Materials

Dimethyl sulfoxide (DMSO), methanol, petroleum ether, ethyl acetate, n butanol, chloroform, and dichloromethane

were purchased from HiMedia Laboratory Pvt. Ltd. Mumbai, India. CAE was prepared and standardized in college. Asiatic acid was purchased from Sigma Aldrich USA. Chitosan (50 kDa, 75–85% deacetylated) were procured from Sigma-Aldrich, USA. Soya phosphatidylcholine (SPC) phospholipid “Phospholipon® 90 G” was a gift sample from Lipoid®, GmbH, Germany. Cholesterol was purchased from HiMedia Laboratory Pvt. Ltd, Mumbai, India. Acetonitrile, orthophosphoric acid, and all other chemicals/reagents were of analytical or HPLC grade with no additional purification process.

Plant material

The whole plant CA was identified, confirmed, and authenticated by Dr. Krishna Kumar G, [rofessor, Department of Applied Botany, Mangalore University, Karnataka, India. (Herbarium voucher specimen number: 16PH005R). The whole plant was used for the research activity.

Methods

Collection and preparation of CAE

The CA specimen was collected early morning from the fertilized land during the monsoon season. As per the Ayurveda medicinal system, collection of the CA in appropriate time, place, and season gives a maximum yield of phytoconstituents.^[7] Organoleptic characteristic was performed followed by determination of moisture content, total ash content, and acid insoluble ash content. The CA whole plant was dried for 2 weeks at a temperature of 45°C and powdered. About 50 g of sifted powder was placed in a Soxhlet extraction process using methanol as a solvent. The process runs 40 cycles at a temperature of 65°C.^[8] The dried extract was further fractionally extracted with five different solvents such as petroleum ether, chloroform, ethyl acetate, n-butanol, and methanol subsequently throughout 24 h for 5 days. The obtained CAE was filtered, dried, and exposed to phytochemical analysis. The major secondary metabolites such as carbohydrate, tannins, steroids, terpenoids, alkaloids, flavonoids, saponins, quinone, and phenolic compounds were assessed in five different extractions was confirmed by standard protocols.^[9]

Standardization of methanolic CAE

CA fresh methanolic extract was filtered and treated at 50°C with charcoal to remove the chlorophyll content. 0.1% CAE solution was prepared, filtered, and subjected into RP-HPLC at 205 nm for 30 min. The obtained data were used to identify the presence of active phytochemical asiatic acid (AA) by comparison with the standard drug. The analysis was carried out using Shimadzu UFLC pump LC 20AD HPLC system, using column Phenomenex Luna C18 250 × 4.6 mm (5 μ particle size), column temperature 30°C, injection volume was 10 μl, mobile phase was 0.1% orthophosphoric

acid:acetonitrile (50:50%), the flow rate was 1 ml/min, and wavelength 210 nm using PDA detector (SPD-M20A).^[10]

Formulation of CAE liposome (CAEL)

CAEL was formulated by the solvent evaporation method, with some modifications. Different ratios of CAE, SPC, and cholesterol were dissolved in dichloromethane (20 ml) in 100 ml round bottom flask. It was subjected to the rotary flash evaporator (Superfit Rotavap series, 6-BU, continental Pvt. Ltd., Mumbai, India) and the flask was rotated with 60 rpm speed at 55°C to obtain a thin film. The film was hydrated with phosphate buffer pH 6.5 at room temperature.^[11]

Design of experiments (DOE)

To analyze the various factors affect the formulation and to get an optimized formulation of CAE, Design-Expert® (Version 11.0.3.0 64-bit, Stat-Ease Inc. Minneapolis, USA) software was used. 3² Design consist of independent variable such as drug lipid molar ratio (X_1) and SPC: cholesterol ratio (X_2) at three different level -1 (low), 0 (middle), and +1 (high) to evaluate the effect of dependent variables such as vesicle size (Y_1) and entrapment efficiency (EE) (Y_2).^[12] Based on the software suggestion, total 9 formulations were formulated and evaluated the response. Resulting data were fitted into the Design-Expert® software and were validated using an analysis of variance. The implied model can be explained by following the quadratic equation exhibiting coefficient effects, interactions, and polynomial terms.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \quad (1)$$

Where “Y” is a measured response associated with each factor level combination, “ b_0 ” is an intercept, “ b_1 ” to “ b_{22} ” are regression coefficients computed from the observed experimental values of “Y” and “ X_1 ” and “ X_2 ” are the coded levels of independent variables. The experimental design and the actual values of the independent variables are given in Table 1.

Table 1: 3² Level factorial design variables for optimized CAEL: Coded levels and actual values for each other.

Factors	Levels, actual (coded)		
	-1 (Low)	0 (Medium)	+1 (High)
Independent variables			
A=Drug : SPC ratio (w: w)	1:5 70:30	1:10 60:40	1:15 50:50
B=SPC : Cholesterol ratio (w: w)			
Dependent variables	Goal		
Y1=Vesicle size (nm)	Minimize		
Y2=Entrapment efficiency (%)	Maximize		

Formulation of CCAEL

CCAEL was prepared as per the improved ionotropic gelation method.^[13] Chitosan was dissolved in acetic acid (0.5% w/v) and kept overnight at room temperature. An appropriate amount of chitosan solution was added drop-wise to the optimized CAE liposomal suspension under continuous magnetic stirring at room temperature for 1 h. It was undisturbed for 3–4 h to get a proper swelling of the liposomes. Finally, the liposomal suspension was sonicated using Ultra-Probe Sonicator (CV-18, Sonics and Materials Inc., USA) for 30 min to produce CCAEL. The final formulation was stored in a well-closed container at 4°C for further studies.

In vitro evaluation of optimized CCAEL

Vesicle size, polydispersity index (PDI), and zeta (ζ) potential

It is an indicator of the overall stability and physicochemical properties of the liposomes.^[14] The samples were diluted with deionized water in a 1:10 ratio before the measurement. Through using Malvern Zetasizer (Malvern Instruments, Malvern, UK), the mean vesicle size (in nm), size distribution as the PDI, and zeta potential of the optimized CCAEL were estimated. Measurements were performed at a fixed temperature of 25°C in triplicate.

% EE

The % EE of the optimized CCAEL was determined by a direct method.^[15] The sonicated optimized CCAEL (1 ml) was taken in microcentrifuge tubes, centrifuged at 20,000 rpm for 1 h at 4°C in a cold centrifuge (Remi Elektrotechnik Ltd., Vasai, India) to get a white pellet. The pellet was treated with 0.1 NaOH 500 μ l and thoroughly vortexed for 3 min to obtain a white suspension. To get a clear solution, 5 ml of Triton X-100 was added, vortexed again for 2 min to confirm the vesicles are lysed completely to discharge the drug. The % EE was evaluated using RP-HPLC method and calculated using the following formula:

$$(\%) EE = \frac{\text{Drug in pellet (entrapped drug)}}{\text{Total drug added}} \times 100$$

Drug content analysis

The AA content in the CCAEL was calculated using RP-HPLC method. The sample was diluted in 10 ml methanol and filtered through 0.2 μ m membrane filter. The resultant solution was diluted and subjected to RP-HPLC analysis.

Differential scanning calorimetry (DSC)

The thermal analysis of the CAE, SPC, physical mixture (PM), and CCAEL was carried out using a DSC (Q20, TA

Instruments Inc., New Castle, USA). The study was conducted with dry nitrogen gas being rid of. High pure indium was used to calibrate the instrument's heat flow and heat capacity. The samples (about 5 mg) were kept in aluminum pans of open quality. Each sample was subjected to a single heating cycle from 0°C to 400°C at a heating rate of 10°C/min. The peak transition onset temperatures of samples were analyzed using the universal analysis software (Version 4.5A, Build 4.5.0.5, TA Instruments Inc., New Castle, USA).^[16]

Fourier transform infrared (FTIR) spectroscopy

To detect the possible chemical interactions between formulation components, FTIR analysis was investigated. The IR spectra of CAE, SPC, cholesterol, chitosan, a PM, and formulation were done using FTIR spectroscopy (Alpha Bruker, Japan). According to the ATR method, below the FTIR spectrophotometer probe, a known amount of the sample was placed and analyzed at a wavelength region of 4000–500 cm⁻¹. The wavenumber of the characteristic peak of a PM was compared with the pure sample and interpreted.^[17]

Surface morphology

Transmission electron microscopy (TEM)

To analyze the morphological characteristics of optimized CCAEL TEM (JEM-100S, JOEL Ltd., Tokyo, Japan) was used.^[18] The sample was diluted with deionized water (1:20) and sonicated for 3 min before the analysis. A drop of the diluted solution of CCAEL was injected separately on a carbon-coated copper grid, forms a liquid film. By incorporating one drop of ammonium molybdate (2% w/w) in 2% w/v ammonium acetate buffer (pH 6.8), the film on the grid was made negatively stained. The excess stain was removed; dried; the stained film was examined.

Atomic force microscopy (AFM)

The 3D surface morphology of the optimized CCAEL was visualized using an atomic force microscope (Innova SPM, Bruker, Santa Barbara, USA). Using AFM tips at 267–328 kHz resonance frequency at a scan speed of 1.2 Hz, the sample was applied as a thin smear to the Mica Disk and visualized in contact mode. The AFM tip has direct contact with the sample when in contact mode. While the tip was scanned along the surface, the sample topography induces a vertical deflection of the cantilever. This deflection was measured by a fiber optical interferometer.^[19]

In vitro release studies

The *in vitro* drug release pattern of CAE and optimized CCAEL suspension was carried out using sigma dialysis membrane; in a vertical Franz diffusion cell. The sample (50 mg CAE and 1 ml CCAEL) was mounted on one side, another side of the membrane

had the dissolution medium in contact. The whole dissolution assembly was kept on a magnetic stirrer at a temperature of 37°C. To mimic the stomach and intestine condition, two different dissolution media were taken. For the initial 0–2 h, 200 ml of acidic buffer pH 1.2 was placed, and then it replaced by phosphate buffer pH 7.2 with 0.25% w/v sodium lauryl sulfate (2–24 h). At specific time intervals (5, 15, 30, 45, 60, 120, 240, and 480 min), aliquots (5 ml) of samples were withdrawn and replaced with an equal volume of fresh medium to maintain a sink condition. After the filtration and the suitable dilutions, drug concentration was analyzed by RP-HPLC at 210 nm and compared. The data obtained from *in vitro* release studies were subjected to *in vitro* kinetic release models (Zero-order, First-order, Higuchi, and Korsmeyer–Peppas model) to interpret the mechanism of drug release from the optimized CCAEL.^[20]

In vitro antioxidant activity study

As per the method described by Jamuna *et al.* 2012^[21] antioxidant property of CAE and CCAEL was evaluated, and the results were compared with ascorbic acid (standard) using stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The CAE and CCAEL (3.5 ml each) of different concentrations ranging from 10 to 50 µg/ml were treated with 1.5 ml 0.1 mM solution of DPPH in methanol. The absorbance of the standard and samples were measured at 517 nm using ELISA plate reader (AM-2100, Alere Inc., USA). The % DPPH scavenged was calculated using the following formula:

$$\% \text{ Inhibition of DPPH} = \frac{\text{Absorption (control)} - \text{Absorption (sample)}}{\text{Absorption (control)}} \times 100$$

In vitro intestinal permeation study

The CAE and optimized CCAEL were evaluated to assess *in vitro* intraduodenal permeability. The fresh duodenal part from goat intestine was obtained from the slaughterhouse. The intestinal tissue was cut into 6 × 6 cm then slightly washed with phosphate buffered saline solution (pH 7.2) to remove blood debris. It was mounted tightly between donor and receiver compartment. Both the samples approximately 1 ml was inserted separately into the lumen of duodenum with the usage of the syringe. The phosphate buffer pH 7.2 was filled in the receiver compartment, and the temperature was maintained at 37°C. The laboratory aerator system was exposed to aeration with 5% CO₂ and 95% O₂ (10–15 bubbles/min). The 1 ml sample was withdrawn from the medium (every 1 h, up to 8 h) and replaced with an equal volume of fresh medium. The obtained samples were filtered and analyzed by RP-HPLC method at a wavelength of 210 nm to assess asiatic acid concentration.^[22]

Stability study

Stability analysis was performed at two separate temperatures, that is, refrigeration temperature (4 ± 2°C) and

room temperature ($32 \pm 2^\circ\text{C}$) for 4 weeks, for the optimized CCAEL. Appropriate dilutions with phosphate buffer solution pH 7.4 were performed every week and assessed for drug entrapment, vesicle size, and ζ potential analysis.^[23]

RESULTS AND DISCUSSION

Preliminary qualitative test of CAE

Once the herb was identified, the whole plants were collected, dried, powdered, and exposed to preliminary quality standard tests. The mean value of CA total ash was found to be 16%, insoluble acid ash was 3%, and water-soluble ash was 3%. This percentage clearly shows that the plant is safest for drug action and its effect.^[24] Total moisture content was found to be 7.2% low moisture content, always desirable for better stability of the drug. The extraction process was carried out using a Soxhlet apparatus using methanol as a solvent. The dark green solvent extract was condensed, and methanol was allowed to evaporate results in dried powder (% yield 6% w/w) that was then contained in a desiccator. The CAE

exhibited significant phyto profiles with respect to the five different solvents. Methanol extract has shown the highest occurrence of phytoconstituents out of the five solvents used [Table 2]. Methanol dissolves a larger portion of polar compounds and a particular group of non-polar compounds because of its amphiphilic nature; therefore, methanol was selected for further analytical analysis.

Standardization of CAE

The RP-HPLC method is suitable for quality control of the raw material extracts and assay of the markers in CA. It provides a reliable, accurate, linear, precise, simple, quick, and within-range quantitative estimation of total triterpenes (madecassoside, asiaticoside, and asiatic acid) in CAE. AA was considered as a standard drug for further drug estimation in drug content, entrapment studies, and drug release studies. The identification of compound asiatic acid was done with the retention time 15.79 min with the approximate 10% availability in the total extract [Figure 1].

Table 2: Phytochemical screening of CA test

Test	Solvents				
	Petroleum ether	Chloroform	Ethyl acetate	n-butanol	Methanol
Alkaloids	-	+	+	-	+
Carbohydrate	-	-	-	+	+
Flavonoids	-	+	-	+	+
Glycosides	-	+	+	+	+
Phenolic compounds	-	-	+	-	+
Protein	-	+	-	-	-
Saponins	-	-	+	+	+
Steroids	-	+	+	+	+
Tannins	-	-	-	-	+
Terpenoids	-	-	-	-	+

+: Indicates presence, -: Indicates absence

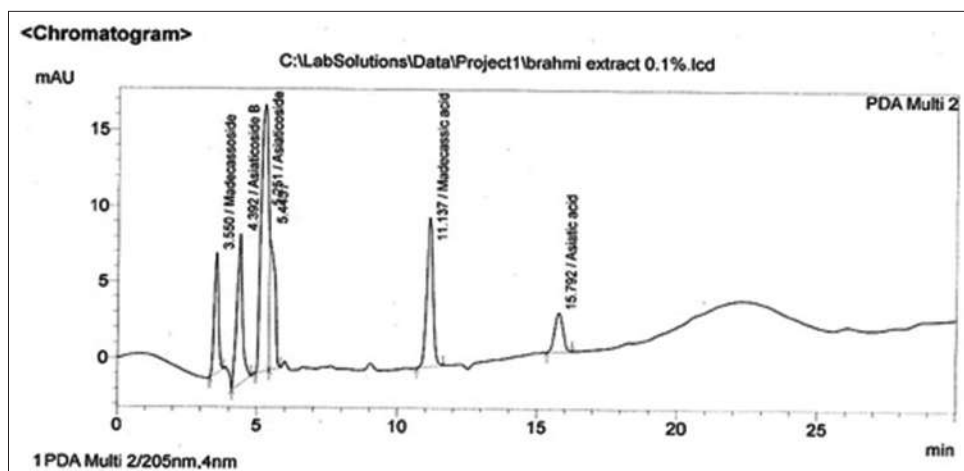


Figure 1: HPLC chromatogram of CAE

Formulation of conventional liposomes of CAE (CAEL)

The CAEL was formulated using the solvent evaporation method. Initial screening was carried out in many organic solvents such as ethanol, methanol, chloroform, DMSO, DMF, and dichloromethane. In dichloromethane, a clear solution was obtained, with a lower boiling point (39.6°C) with lower toxicity (LD₅₀ value 1.5 g/kg in rat). Therefore, dichloromethane was selected as a solvent to prepare the CAEL. Based on the literature survey, drug:SPC ratio was taken in the range of 1:5–1:15 and SPC:cholesterol ratio in the range of 70:30–50:50 that was further subjected to DOE. Mixtures were dissolved in dichloromethane solvent and evaporated using rotary flash evaporated. Before hydration, the film was dried at 45°C for nearly 45 min to remove the organic solvent. Cholesterol was added to improve liposomal stiffness and stability.^[25] The obtained multilamellar vesicles were pulverized using an Ultra-Probe Sonicator to get the desired size range of <250 nm.

DOE

Full factorial design (3²)

Based on experimental design,^[26] the results obtained from the experimental trials carried out using 3 level factorial randomized quadratic polynomial model for the two dependent variables for the extent of liposome formulations are shown in Table 3. The measured values from the experimental trails showed a significant variation in % EE (56.08–82.38%) and vesicle size (571–1000 nm). The quadratic equations (Eq. 1 and Eq. 2) were used to draw conclusions based on the magnitude of the coefficient, and positive sign before the variables show the linear association between response and factor, while the negative sign indicates the inverse relationship between the same.

$$\text{Vesicle size} = +226.56 + 42.50 * A - 20.17 * B \quad (2)$$

$$\% \text{ Entrapment efficiency} = +72.54 + 7.61 * A + 4.64 * B \quad (3)$$

Where A and B symbolize the coded values for CAE:SPC ratio and SPC:cholesterol ratio, respectively.

Optimization

The model generated for the vesicle size had a $P < 0.05$ and F-value of 7.87, indicating the model to be significant. The difference between the adjusted (R^2 0.6318) and the predicted (R^2 0.5257) model was found to be <0.2 , indicating a reasonable agreement between the two. The model generated for the EE had a $P < 0.05$ and F-value of 35.72, indicating the model to be significant. The difference between the adjusted (R^2 0.8967) and the predicted (R^2 0.8080) model found to be <0.2 . From the 3D surface plot of the effect of CAE:SPC ratio and SPC:cholesterol ratio on the response vesicle size and % EE is depicted in Figure 2a and b. The vesicle size of the prepared liposome was found to be in the range of 500–600 nm and % EE of 50–80%. The percentage error was calculated, and the optimized liposomes had a vesicle size of 209.8 nm and % EE to the extent of 80% [Table 4].

Formulation of chitosan-coated liposomes of CAE (CCAEL)

The positively charged chitosan was used to form a coat on liposomes to enhance its mucoadhesivity to negatively charged cell membrane and thereby prolong the release of the drug. Due to electrostatic interactions between the negatively charged SPC and the positive charges of primary amino groups of chitosan, it is useful in coating the surface of liposomes. Chitosan coating enhances the stability of liposomes and prevents drug leakage from the vesicular structure.^[27]

Table 3: Level factorial randomized quadratic design experimental trial batches, with obtained vesicle size (nm) and entrapment efficiency (%)

Std	Run	Factor 1	Factor 2	Response 1	Response 2
		A:Drug:SPC	B:SPC:Cholesterol	Vesicle size	Entrapment efficiency
		w:w	w:w	nm	%
1	1	-1	-1	482	56.08
9	2	1	1	376	82.38
4	3	-1	0	354	67.4
8	4	0	1	242	77.13
6	5	1	0	504	80.24
5	6	0	0	495	75.51
3	7	1	-1	505	75.76
2	8	0	-1	502	69.08
7	9	-1	1	571	69.24

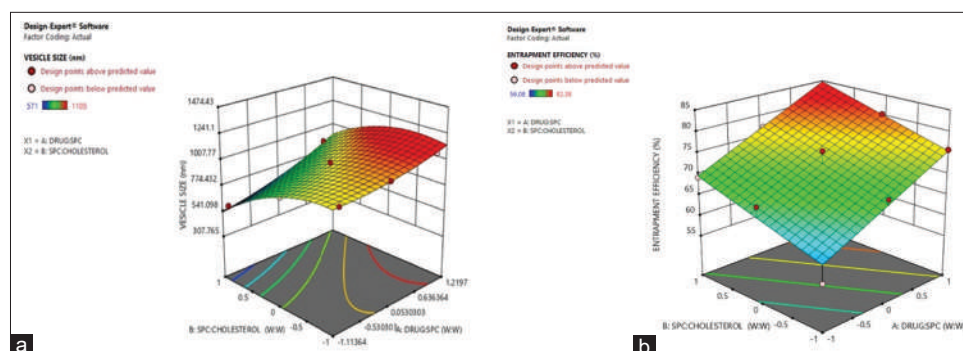


Figure 2: (a and b) 3D surface plot of vesicle size and entrapment efficiency

Table 4: Selected solution and the % error between the predicted and the observed values

Factors		Responses	
A: Drug:SPC (w/w)	B: SPC:Cholesterol (%w/w)	Vesicle size (nm)	Drug entrapment efficiency (%)
		Predicted	
1:6.88	50:50	212.86	82.40
		Actual	
		209.8	81.27
% Error:		1.40	1.45

Vesicle size, PDI, and zeta (ζ) potential

Vesicle size, PDI, and ζ potential are significant interpreters of the physical stability of liposomes in a medium.^[28] Chitosan coating enhances the thickness of the vesicular system as compared to conventional liposomes. The average vesicle size of sonicated optimized CAEL was found to be 201.4 nm. The average vesicle size of optimized CCAEL was found to be 678 nm that signifies the optimum complex formation. PDI value of optimized CAEL and CCAEL was found to be 0.493 and 0.457, respectively, that indicates a narrow range of vesicle size distribution. The surface charge of -20 to +20 mV was reported as a characteristic property of ζ potential for attraction, followed by flocculation exceeding repulsive forces.^[29] The chitosan coat attributes more cationic polymers adsorbed to the liposomal surface to show a slightly positive charge. The optimized formulation CAEL and CCAEL showed ζ potential of -20.8 mV and 22.3 mV, respectively.

% EE

The % EE was determined by lysing the optimized liposomes in Triton X-100. The total entrapment of AA in optimized CCAEL was found to be $60.3 \pm 0.03\%$ ($n = 3$). Optimized CCAEL showed better % EE because of the stable vesicular structure. AA is lipophilic and has poor water solubility, thus showing a higher affinity to be spotted in liposomes lipid bilayers. In this study, an untrapped drug was retained in the formulation to avoid drug loss.

Drug content analysis

Drug content was estimated using the RP-HPLC method. Since AA was slightly soluble in water, methanol was selected as a solvent. The standard calibration curve was plotted taking peak area on Y-axis and drug concentration on X-axis. The regression equation was found to be $Y = 3790.1x$ under a linearity range of 10–50 $\mu\text{g/ml}$. The retention time was 9.6 ± 0.22 min and the correlation coefficient (r^2) was 0.999. The AA available in optimized CCAEL formulation was found to be $63 \pm 0.02\%$ w/w.^[30]

DSC

DSC is an established tool for analyzing the approach to thermal behavior to define solid-state matter in a complex form.^[31] The obtained thermograms of CAE, SPC, PM of CAE and SPC, and optimized CCAEL are shown in Figure 3. CAE showed a broad endothermic peak at 94.04°C [Figure 3a] corresponding to its melting point. SPC showed that an endothermic peak at 172.02°C [Figure 3b] indicates the transformation of the physical state from a gel to a liquid-crystalline state. In the thermogram of the PM of CAE and SPC, two peaks were observed at 94.04°C and 171.96°C [Figure 3c], corresponding to the peaks of CAE and SPC, respectively. Further, thermogram of optimized CCAEL showed a partially fused two broad endothermic peaks at 86.9°C and 110.3°C [Figure 3d]. This shift from peak to lower temperature may be due to the drug's increased solubility and reduced formulating crystallinity. It may also be due to the

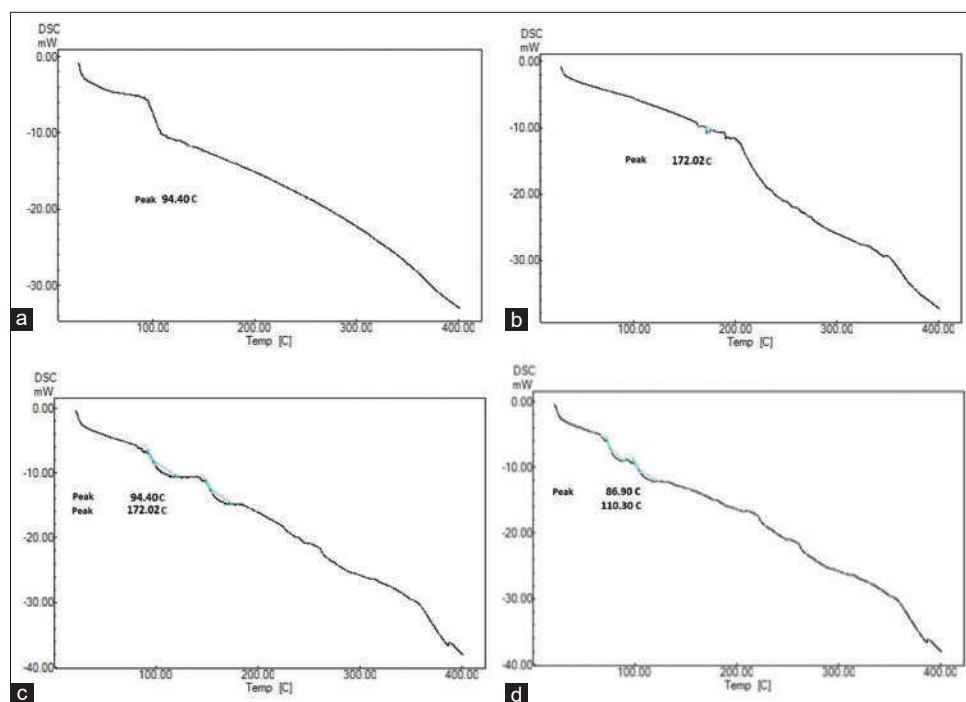


Figure 3: DSC thermogram of (a) CAE, (b) SPC, (c) physical mixture, (d) CCAEL

interactions among both hydrogen bonds or van der Waals forces and also due to the amalgamation of CAE with the long-chain hydrocarbon tail of phospholipid molecules and, therefore, the formation of the drug-phospholipid complex.

FTIR spectroscopy

FTIR spectroscopy was carried out to investigate the possible interactions between CAE and other components of the liposomes. The IR spectra of CAE, SPC, cholesterol, chitosan, a PM, and optimized CCAEL are shown in Figure 4a-c. A comparison of the FTIR spectra shows the changes to specific regions of the CAE molecules due to interaction with phospholipids. Changes were observed in the stretching frequency of the phenolic O-H of CAE from 3431.81 cm^{-1} to 3416.96 cm^{-1} in the CCAEL. The changes may be due to the formation of weak intermolecular interactions during the formation of CCAEL that leads to alteration in the absorption peaks of phospholipid at 1227.65 cm^{-1} and 1061.62 cm^{-1} . Weak intermolecular interactions between CAE and SPC molecules are attributable to the formation of CCAEL. These peaks were retained in the IR spectra of the PM of all the excipients and optimized formulation. This specifies that drug and other excipients were compatible with each other.^[32]

Surface morphology

TEM

TEM image of CAEL and optimized CCAEL [Figure 5 a and b] clearly shows the difference between coated and uncoated liposomes. Figure 5b was found to be well-formed,

separate vesicles without coagulation, whereas Figure 5a shows minute coagulation. TEM was used to investigate the spherical structure and thin films as it confirms from the image.^[33]

AFM

The AFM image of optimized CCAEL is depicted in Figure 6 showed well-formed, discrete vesicles. Clear vesicular spherical structures were observed when mounted on a glass slide, without any sign of aggregation or decomposition.^[34]

In vitro release studies

The study was carried out to analyze the drug release pattern from CAE and optimized CCAEL [Figure 7]. CAE showed a slow rate of release of $35.34 \pm 0.30\%$ in about 10 h due to poor solubility, while approximately $58.6 \pm 0.42\%$ release was observed from optimized CCAEL, probably due to better solubility. A further sudden decline of drug release was observed for CAE that lasts up to 16 h. accordingly; a sustained release pattern was observed in optimized CCAEL showed nearly $88.3 \pm 0.3\%$ release in 24 h. The sustained release of drugs from optimized CCAEL is probably due to physicochemical alteration, and complex occurred with SPC and chitosan between CAE. Moreover, this phenomenon increased the complex's solubility and wettability compared to pure CAE.^[35] The drug release kinetics from the optimized CCAEL was evaluated considering four kinetics models, including zero order, first order, Higuchi model, and Korsmeyer–Peppas model. The Higuchi's plot of the optimized CCAEL was found to be linear with the R^2 value 0.972 and confirmed diffusion

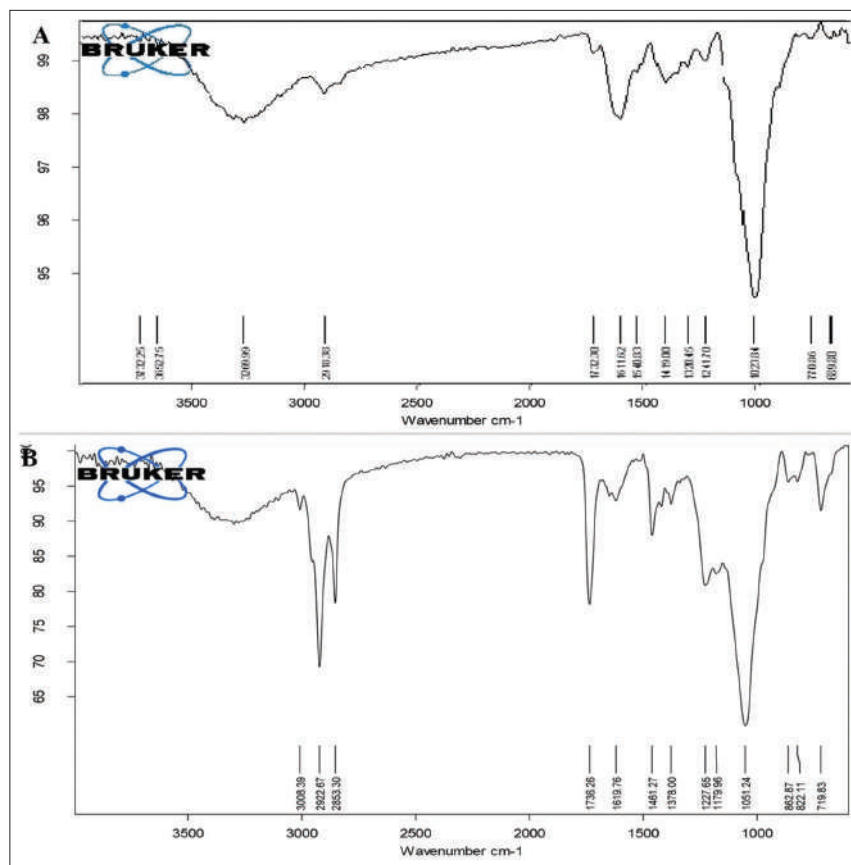


Figure 4a: FTIR of CAE and SPC

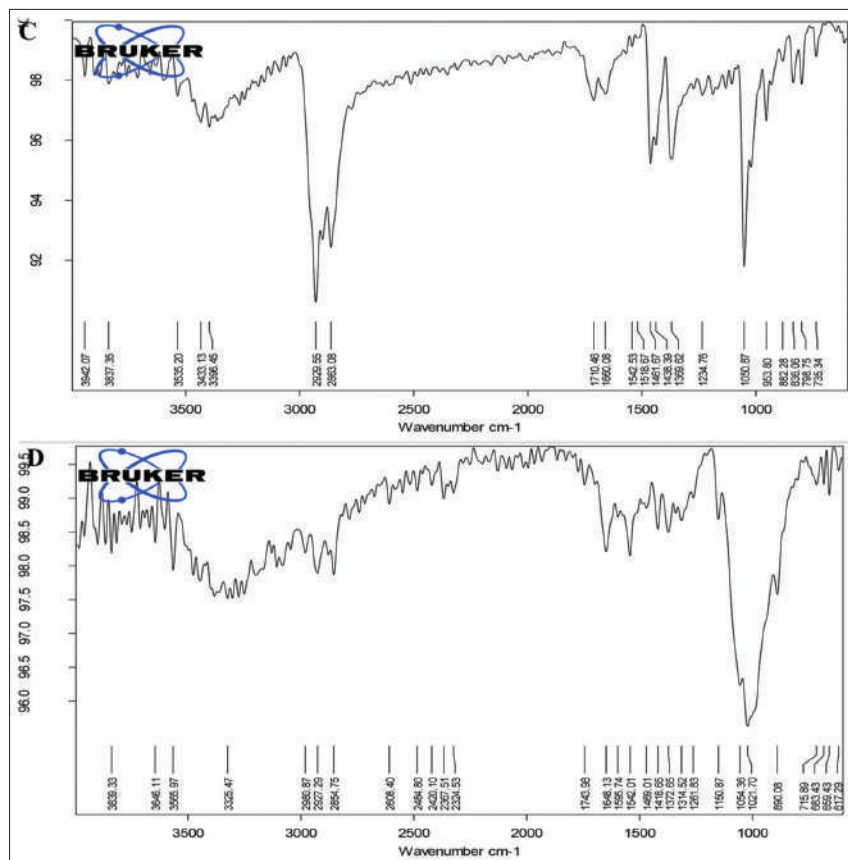


Figure 4b: FTIR of cholesterol and chitosan

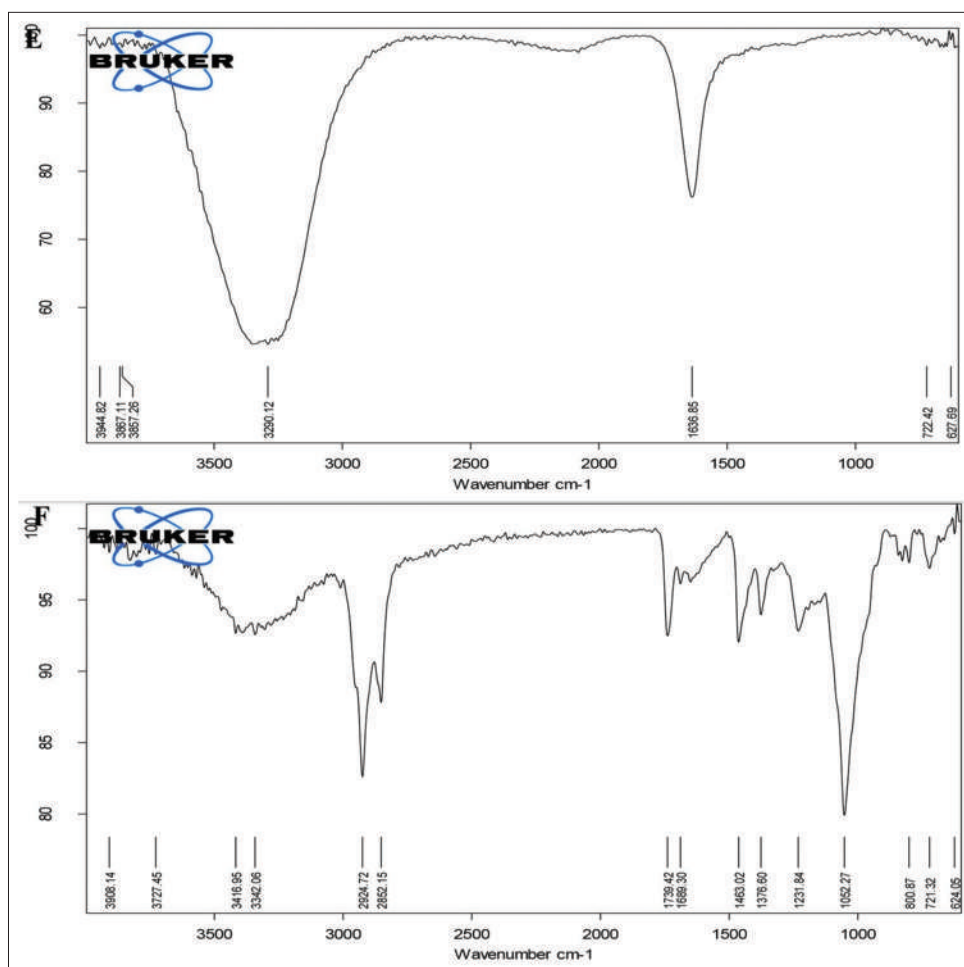


Figure 4c: FTIR of physical mixture and CCAEL

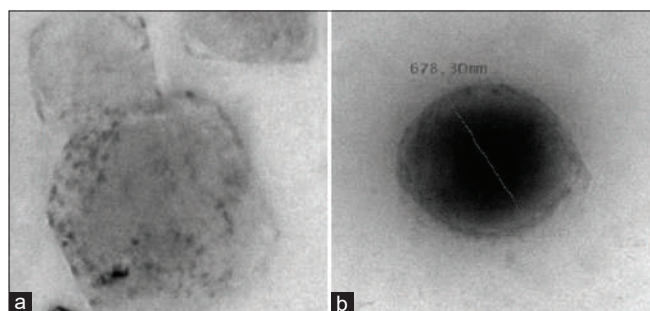


Figure 5: TEM images of (a) CAEL and (b) CCAEL

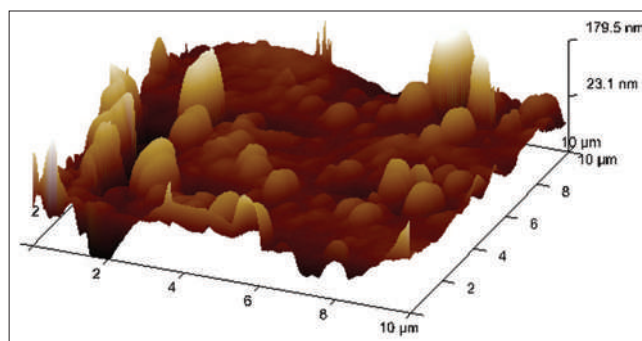


Figure 6: AFM image of CCAEL

controlled drug release based on Fick's law which is a square root time depended. In the Korsmeyer-Peppas model, the n value (0.3917) indicated the quasi Fickian model ($n < 0.4$); it represents the partial diffusion pattern of drug layer.^[36] Diffusion was the dominant mechanism followed and hence Higuchi was found to be the best fit model.

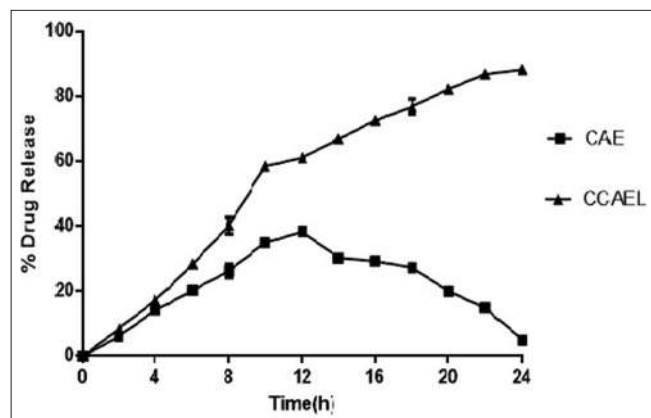
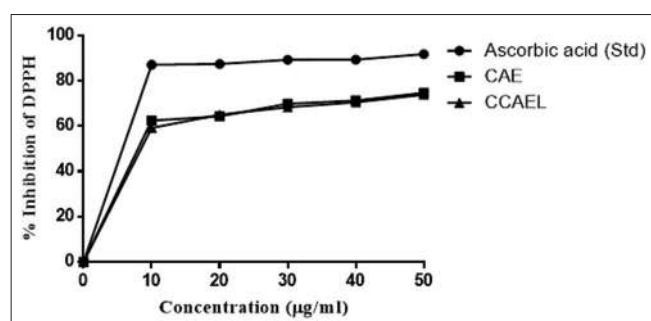
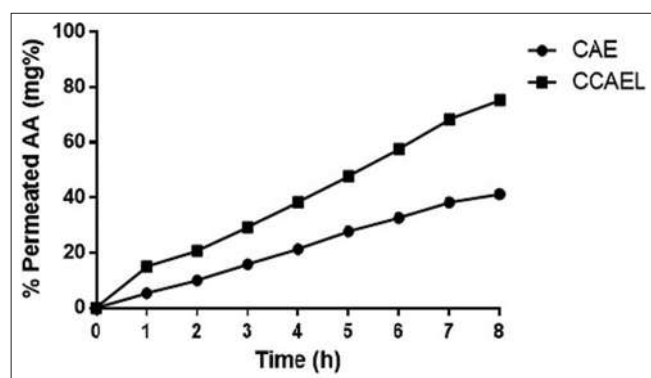
In vitro antioxidant activity study

The *in vitro* antioxidant activity of CAE and optimized CCAEL was assessed as a free radical scavenging ability based on % of DPPH free radical scavenged. The %

inhibition of DPPH by samples was compared with the standard ascorbic acid at the same concentration. The % inhibition of DPPH at 50 $\mu\text{g/ml}$ was found to be 91.75%, 74.4%, and 73.84%, respectively, for ascorbic acid, CAE, and optimized CCAEL [Figure 8]. The results indicated that standard drug and CAE exhibits substantial radical scavenging activity that has also been retained even after intricate it with the phospholipid without any adverse effect. It may be due to the increased capacity of CAE to donate hydrogen ions and reduce DPPH radicals to hydrazine equivalent.^[37]

Table 5: Stability study of the optimized CCAEL by considering entrapment efficiency, vesicle size, and PDI

	Entrapment study (%)									
	Initial		After 1 week		After 2 weeks		After 3 weeks		After 4 weeks	
Optimized CCAEL	4±2°C	32±2°C	4±2°C	32±2°C	4±2°C	32±2°C	4±2°C	32±2°C	4±2°C	32±2°C
	71.4±0.03	71.1	71.4±0.03	71.0	71.4±0.03	71.4	71.4±0.03	71.1	71.4±0.03	71.3
	Vesicle size (d.nm)									
	209.8	209.8	209.8	209.8	209.8	209.8	209.2	209.2	209.1	209.1
	Zeta potential (mV)									
	22.3	22.3	22.2	22.3	22.2	22.3	22.3	22.3	22.3	22.3


Figure 7: *In vitro* drug release study of CAE and CCAEL

Figure 8: *In vitro* antioxidant study of CAE and CCAEL

Figure 9: *Ex vivo* permeation study of CAE and CCAEL

In vitro intestinal permeation study

The amount of drug permeation through the intestinal region of both CAE and CCAEL was measured effectively using

a simple *in vitro* permeation study. The drug concentration permeated from optimized CCAEL over the duration of 8 h was found to be 75%; however, CAE exhibited less permeation of 41% [Figure 9]. A noticeable rise in the intestinal permeation rate possibly due to the encapsulation of chitosan-phospholipid complex which opens the compact junctions of cytoplasmic transmembrane proteins occludin, claudin-1, and ZO-1 of epithelium cells and supports CAE to transfer paracellular.^[38] The Bioadhesive nature of chitosan contributes to an improvement in residence time and a prolonged rate of adsorption at a mucosal site due to ionic interaction among positively charged alkaline polysaccharides of chitosan and negative charge of sialic acid groups in the intestine.^[39]

Stability study

The major problem in liposomes is that vesicles tend to fuse, grow larger, resulting in storage breakdown of the liposomes that lead to vesicle drug leakage. Stability at extreme temperatures (>room temperature) could not be achieved because phospholipid will deteriorate at higher temperatures.^[40] The optimized CCAEL formulation was evaluated for EE, vesicle size, and ζ potential. According to the data [Table 5], after a period of 4 weeks, formulations were stored at refrigeration temperature showed higher EE when compared to the room temperature. On the 4th week of storage, optimized CCAEL showed a high degree of stability in the EE throughout the stability study, that is, 71.4–71.3%. There was no such variation in vesicle size after the 4th week of storage (678.8 nm–679.2 nm). ζ potential of the optimized formulations showed marginal changes in the values after the 4th week of storage (+20.8–+23.1 mV) that characterize the strong stability of liposomes from flocculation when viewed in the context of its lower vesicle size. The stability of CCAEL is attributed to the chitosan coating.

CONCLUSION

Oral route is considered to be the most convenient route of drug administration, especially when long-term treatment is required. Keeping such a concept in mind, a CCAEL was formulated and evaluated *in vitro* to treat AD. The study

was found to be effective as it utilizes the chitosan-coated phospholipid base for the improvement of solubility, stability, and controlled site-specific absorption as compared with CAE. The CCAEL was evaluated for its successful formation of chitosan phospholipid coat through FTIR, DSC, TEM, and AFM. Drug release rate and membrane permeability of CCAEL were found to be better than CAE confirms the better solubility and permeability. The present study opened new vistas in the oral drug delivery segments. However, pre-clinical and clinical studies are required to confirm better product development. This study was an attempt to showcase the potential of CA as a mainline therapy for the treatment of AD.

REFERENCES

- Chen CS, Ouyang P, Yeh YC, Lai CL, Liu CK, Yen CF, *et al.* Apolipoprotein e polymorphism and behavioral and psychological symptoms of dementia in patients with Alzheimer disease. *Alzheimer Dis Assoc Disord* 2012;26:135-9.
- Yiannopoulou KG, Papageorgiou SG. Current and future treatments for Alzheimer's disease. *Ther Adv Neurol Disord* 2012;6:19-33.
- Aprahamian I, Stella F, Forlenza OV. New treatment strategies for Alzheimer's disease: Is there a hope? *Indian J Med Res* 2013;138:449-60.
- Mukherjee PK, Kumar V, Houghton PJ. Screening of Indian medicinal plants for acetylcholinesterase inhibitory activity. *Phytother Res* 2007;21:1142-5.
- Chandrika UG, Kumarab PA. Gotu Kola (*Centella asiatica*): Nutritional properties and plausible health benefits. *Adv Food Nutr Res* 2015;76:125-57.
- Mady MM, Darwish MM. Effect of chitosan coating on the characteristics of DPPC liposomes. *J Adv Res* 2010;1:187-91.
- Rao VR, Descamps O, John V, Bredesen ED. Ayurvedic medicinal plants for Alzheimer's disease: A review. *Alzheimers Res Ther* 2012;4:22.
- Chew KK, Ng SY, Thoo YY, Khoo MZ, Wan AW, Ho CW. Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of *Centella asiatica* extract. *Int Food Res J* 2011;18:571-8.
- Madhusudhan NC, Neeraja P, Devi P. Comparative analysis of active constituents in *Centella asiatica* varieties (Majjaposhak and Subhodak). *Int J Pharm Phytopharmacol Res* 2014;4:105-8.
- Hebbar S, Dubey A, Ravi GS, Kumar H, Saha S. RP-HPLC method development and validation of Asiatic acid isolated from the plant *Centella asiatica*. *Int J Appl Pharm* 2019;11:72-8.
- Prabhu P, Shetty R, Koland M, Vijayanarayana K, Vijayalakshmi KK, Nairy MH, *et al.* Investigation of nano lipid vesicles of methotrexate for anti-rheumatoid activity. *Int J Nanomedicine* 2012;7:177-86.
- Sailor G, Seth AK, Parmar G, Chauhan S, Javia A. Formulation and *in vitro* evaluation of berberine containing liposome optimized by 3 2 full factorial designs. *J Appl Pharm Sci* 2015;5:23-8.
- Giri TK. Alginate containing nanoarchitectonics for improved cancer therapy. In: *Nanoarchitectonics for Smart Delivery and Drug Targeting*. Amsterdam: Elsevier Inc.; 2016. p. 565-88.
- Peira E, Carlotti ME, Trotta C, Cavalli R, Trotta M. Positively charged microemulsions for topical application. *Int J Pharm* 2008;346:119-23.
- Tan Q, Liu S, Chen X, Wu M, Wang H, Yin H, *et al.* Design and evaluation of a novel evodiamine-phospholipid complex for improved oral bioavailability. *AAPS Pharm Sci Tech* 2012;13:534-47.
- Sri KV, Kondaiah A, Ratna JV, Annapurna A. Preparation and characterization of quercetin and rutin cyclodextrin inclusion complexes. *Drug Dev Ind Pharm* 2007;33:245-53.
- Ravi G, Krishna C, Charyulu R, Dubey A, Hebbar S. Matrix tablet-containing solid dispersion is not suitable for sustained release delivery of beclomethasone dipropionate. *J Pharm Negat Results* 2018;9:8-13.
- Fadel O, El Kirat K, Morandat S. The natural antioxidant rosmarinic acid spontaneously penetrates membranes to inhibit lipid peroxidation *in situ*. *Biochim Biophys Acta* 2011;1808:2973-80.
- Sikarwar MS, Sharma S, Jain AK, Parial SD. Preparation, characterization and evaluation of marsupsin-phospholipid complex. *AAPS Pharm Sci Tech* 2008;9:129-37.
- Maiti K, Mukherjee K, Gantait A, Saha BP, Mukherjee PK. Curcumin-phospholipid complex: Preparation, therapeutic evaluation and pharmacokinetic study in rats. *Int J Pharm* 2007;330:155-63.
- Jamuna S, Paulsamy S, Karthika K. Screening of *in vitro* antioxidant activity of methanolic leaf and root extracts of *Hypochoeris radicata* L. (Asteraceae). *J Appl Pharm Sci* 2012;2:149-54.
- Ruan LP, Chen S, Yu BY, Zhu DN, Cordell GA, Qiu SX. Prediction of human absorption of natural compounds by the non-everted rat intestinal sac model. *Eur J Med Chem* 2006;41:605-10.
- Zweers ML, Engbers GH, Grijpma DW, Feijen J. *In vitro* degradation of nanoparticles prepared from polymers based on DL-lactide, glycolide and poly (ethylene oxide). *J Control Release* 2004;100:347-56.
- Kaufmann B, Christen P. Recent extraction techniques for natural products: Microwave-assisted extraction and pressurised solvent extraction. *Phytochem Anal* 2002;13:105-13.
- Grit M, Crommelin DJ. Chemical stability of liposomes: Implications for their physical stability. *Chem Phys Lipids* 1993;64:3-18.
- Salazar J, Heinzerling O, Müller RH, Möschwitzer JP. Process optimization of a novel production method for nanosuspensions using design of experiments (DoE). *Int*

- J Pharm 2011;420:395-403.
27. Mengoni T, Adrian M, Pereira S, Santos-Carballal B, Kaiser M, Goycoolea F. A chitosan-based liposome formulation enhances the *in vitro* wound healing efficacy of substance P neuropeptide. *Pharmaceutics* 2017;9:56.
 28. Dubey A, Prabhu P, Patel J, Hebbar S, Shastry C, Charyulu R. Investigation of nano lipid vesicles of lornoxicam for targeted drug delivery. *Br J Pharm Res* 2016;11:1-15.
 29. Paolino D, Sinha P, Fresta M, Ferrari M. Drug delivery systems. In: Webster JG, editor. *Encyclopedia of Medical Devices and Instrumentation*. Hoboken, New Jersey, USA: John Wiley & Sons, Inc.; 2006.
 30. Volodkin D, Mohwald H, Voegel JC, Ball V. Coating of negatively charged liposomes by polylysine: Drug release study. *J Control Release* 2007;117:111-20.
 31. Giron D. Applications of thermal analysis in the pharmaceutical industry. *J Pharm Biomed Anal* 1986;4:755-70.
 32. Hamishehkar H, Hooresfand Z, Ghanbarzadeh S. Preparation and characterization of rutin-loaded nanophytosomes. *Pharm Sci* 2015;21:145-51.
 33. Ravi GS, Charyulu RN, Dubey A, Prabhu P, Hebbar S, Mathias AC. Nano-lipid complex of rutin: Development, characterisation and *in vivo* investigation of hepatoprotective, antioxidant activity and bioavailability study in rats. *AAPS Pharm Sci Tech* 2018;19:3631-49.
 34. Hossain S, Hashimoto M, Katakura M, Al Mamun A, Shido O. Medicinal value of asiaticoside for Alzheimer's disease as assessed using single-molecule-detection fluorescence correlation spectroscopy, laser-scanning microscopy, transmission electron microscopy, and *in silico* docking. *BMC Complement Altern Med* 2015;15:118.
 35. Jain S, Kumar D, Swarnakar NK, Thanki K. Polyelectrolyte stabilized multilayered liposomes for oral delivery of paclitaxel. *Biomaterials* 2012;33:6758-68.
 36. Dash S, Murthy PN, Nath L, Chowdhury P. Kinetic modeling on drug release from controlled drug delivery systems. *Acta Pol Pharm* 2010;67:217-23.
 37. Koşar M, Göger F, Baser KH. *In vitro* antioxidant properties and phenolic composition of *Salvia halophila* Hedge from Turkey. *Food Chem* 2011;129:374-9.
 38. Guan G, Wang H, Peng H, Guanya Li. Low dosage of chitosan supplementation improves intestinal permeability and impairs barrier function in mice. *Biomed Res Int* 2016;2016:4847296.
 39. Zare M, Samani SM, Sobhani Z. Enhanced intestinal permeation of doxorubicin using chitosan nanoparticles. *Adv Pharm Bull* 2018;8:411-7.
 40. Zhao X, Shi C, Zhou X, Lin T, Gong Y, Yin M, *et al.* Preparation of a nanoscale dihydromyricetin-phospholipid complex to improve the bioavailability: *In vitro* and *in vivo* evaluations. *Eur J Pharm Sci* 2019;138:104994.

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