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REVIEW ARTICLE

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Development of a Eudragit-Chitosan Nanosystem for the pH-Dependent Transport of Duloxetine to the Brain: Synthesis, Characterization and *In Silico* Modeling Analysis

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ABSTRACT

The purpose of this study was to synthesize duloxetine (DLX)- loaded Eudragit-Chitosan (Eud-CHT) nanoparticles enclosed in an oral gelatin capsule and to evaluate the potential to transport DLX to the blood-brain barrier (BBB)for improved neuro-availability. The utilization of Eudragit® with chitosan offers a pH-dependent controlled drug release. The physicochemical properties of the formulated DLX-loaded Eud-CHT nanosystem were confirmed using various characterization techniques. SEM confirmed the nanoparticle morphology and pore size distribution. The particle size was 100 ± 73,41 nm, with a polydispersity index (PDI) of 0,283 and zeta potential of 16±2,79 mV. Drug entrapment efficacy (DEE) of 72% was attained, and molecular modelling predicted an efficient and controllable drug delivery system. The release of DLX from the nanosystem was evaluated at pH1.2, pH 6.8 and pH 7.4. At a pH of 6.8, 40 % of DLX was released, with only 20 % at pH 1.2 and 35% at pH 7.4. This demonstrated DLX's pH-dependent release and the Eud-CHT nanosystem's shielding effect at gastric pH. In addition, HEK 293 neural cells confirmed the non-toxicity of the DLX-Eud-CHT nanosystem. *In silico* modelling revealed a DLX-Eud-CHT composite with an outer cationic surface attributable to the EUD moieties on nanoparticles for preferential cell recognition and uptake at the anionic cell interface. The combined trials and results from the synthesis of DLX-Eud-CHT nanoparticles showed that these nanoparticles could be utilized as a potentially invaluable formulation for oral drug delivery of duloxetine with improved neuro-availability.

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oral delivery;
pH-dependent

Introduction

Depression is a mental disorder in which patients experience chronic symptoms of low mood, reduced interest in previously enjoyed activities, loss of appetite, insomnia, exhaustion, and more severely suicidal thoughts (Akechi et al., 2019; Dening et al., 2016). In depression disorder, patients often require pharmacological treatment for at least 6-9 months (Akechi et al., 2019;

Sakurai et al., 2020). Duloxetine (DLX) is a novel antidepressant from the class of selective serotonin, and noradrenaline reuptake inhibitors (SNRIs) used as a first-line treatment of a depressive disorder. It functions in the brain to inhibit the reuptake of noradrenaline and serotonin to alleviate symptoms of depression (Paulzen et al., 2016). DLX has the edge over other existing antidepressants, including amitriptyline, citalopram, clomipramine, dosulepin and doxepin due to its favorable pharmacodynamic features such as dual inhibition, improved efficacy, tolerability, safety, faster recovery, fewer side effects, and low affinity for other neuronal receptors (Posadas et al., 2016). In this study, DLX was used as the model antidepressant drug for treating a depressive disorder, analyzing it is *in vitro*, *in silico* and cytotoxicity profiles.

However, because of the systemic use of DLX, side effects such as liver damage, weight gain, drowsiness and fatigue, dry mouth, and altered sexual function are experienced (Chahal et al., 2020). This often results in patient non-compliance and the possibility of relapse (Akechi et al., 2019; Boisseau & Loubaton, 2011). DLX is susceptible to hydrolytic degradation, which is more pronounced at low pH states.

Current orally administered DLX formulations are largely metabolized in the liver leading to poor bioavailability. A steady-state plasma concentration of DLX is desired to effectively treat depression. Three per daily dosing of DLX may cause fluctuation in the plasma concentration with reduced patient compliance due to multiple doses needed over an extended period (Akechi et al., 2019). Approximately 90% of drugs are administered via the oral route because of better patient compliance, cost-effectiveness, no sterility restrictions, and the most convenience for treating chronic disorders (Indurkhya et al., 2018). However, the oral route of drug delivery is not always favorable due to challenges of drug dissolution, permeability and solubility in the gastrointestinal tract (GIT) (Viswanathan et al., 2017). Many drugs are not stable in low pH conditions and experience chemical degradation. Even following absorption, some drugs face substantial first-pass metabolism in the liver. These factors adversely affect the bioavailability and efficacy of the drug.

Advances in nanomedicine provide an opportunity to improve the efficiency of orally administered drugs such as DLX. Numerous nano-enabled delivery systems for brain targeting have been developed, including branched nanoemulsions, dendrimers, nanoliposomes, nanomicelles, nanogels, nanocapsules, nanovesicles and carbon nanotubes. These nanosystems have proven to be very successful in transporting drugs across the blood-brain barrier (BBB) due to their small size (< 200 nm) with improved drug solubility, absorption and decreasing drug-related side-effects (Khosa et al., 2018). Hence, nanomedicine can significantly improve drug delivery to facilitate targeted CNS delivery with controlled release characteristics and improved drug stability.

The purpose of this study was to synthesize a DLX-loaded nanoparticle system using a pH-responsive polymer blend of an ammonium methacrylate copolymer (Eudragit RL100) and chitosan (EUD-CHT) loaded into a gelatin capsule for the oral delivery of DLX, intended to transport DLX to the brain with improved efficacy and reduced side-effects, as an *in vitro* prototype nano-formulation.

CHT is a natural carbohydrate-based polymer that is non-toxic, biodegradable and can control drug release when incorporated as a nanosystem (Patel et al., 2015; Xu et al., 2018). Eudragit RL100 (EUD) (poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonio ethyl methacrylate chloride) is an FDA-approved copolymer used for biomedical applications (Dong et al., 2019). It is bio-adhesive and can provide pH-responsive (site-specific) drug-release properties. EUD is cationic and therefore interacts favorably with anionic cell surfaces of targeted tissues to increase cellular uptake of drug-loaded nanoparticles (Adibkia et al., 2011). Incorporating DLX within the copolymeric nanoparticles provides great potential to improve the DLX solubility and absorption through the GIT wall for preferential transport to the brain. The polymeric structure of the EUD-CHT nanoparticles can also protect DLX from the harsh acidic conditions in the gastric region due to the pH-responsive behavior of EUD. The DLX-loaded EUD-CHT nanoparticles were prepared by solvent emulsion evaporation, and synthesis was confirmed by analyzing various physicochemical properties, including surface morphology by Scanning Electron Microscopy (SEM), chemical structure stability and integrity

by FT-IR, particle size and zeta potential, thermal stability by Thermogravimetric Analysis (TGA) and Differential Scanning Calorimeter (DSC). The synthesized DLX-loaded EUD-CHT nanoparticles were then studied against three pH environments, simulating the gastric (pH 1.2), intestinal (pH 6.8) and systemic physiological (pH 7.4) environments. DLX release was determined by dissolution and analyzed by UV spectroscopy. The degree of particle dispersion/ agglomeration was evaluated, in line with optical interactions, looking at the stability of the DLX-loaded EUD-CHT nanoparticles, utilizing a Turbiscan™ LAB. In addition, HEK 293 neural cells were treated with the DLX-loaded EUD-CHT nanoparticle formulation and evaluated for cytotoxicity utilizing a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT) assay and absorbance measured at 570 nm.

Materials and Methods

Materials

Eudragit RL-100 (Eud), polyvinyl alcohol (PVA) (Av. Mw 30 – 70 kDa), chitosan (CHT) (medium Mw; 450 kDa), HPLC grade acetone, acetonitrile, acetic acid, chloroform, hydrochloric acid, sodium phosphate, Dimethyl sulfoxide, Fetal bovine serum (FBS), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT), 0.25 % w/v trypsin and mannitol were purchased from Sigma (Sigma-Aldrich, Missouri, USA). Human Embryonic Kidney (HEK) 293 cells (CRL# 1573, passage 21, Batch # 203970) were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and Duloxetine HCL was purchased from Sigma (Sigma-Aldrich, Missouri, USA). All reagents and chemicals used were of high analytical grade.

Synthesis of the DLX-Loaded EUD-CHT Nanoparticles

The DLX-loaded EUD-CHT nanoparticles were prepared using the single emulsion solvent evaporation (Fig. 1), reported by Wang *et al*. (2013). EUD (100 mg) was weighed and mixed with 2.5 mL of acetone until dissolved, then mixed with 10 mg of duloxetine HCL and added to an aqueous phase of 0.5 % v/v acetic acid solution, which contained PVA (0.5 % w/v) and CHT (0.5 % w/v). The resultant emulsion was then vortexed for 10 min and then sonicated. The rotary evaporation and vacuum method were used to remove the acetone. Ultracentrifugation was conducted on the mixture to obtain the nanoparticles using the Beckmann coulter, USA, at 4 °C for 18 min per 23,000 g. The downstream processing procedure of centrifugation, washing and lyophilization was followed as per the emulsification solvent evaporation protocol (Wang et al., 2016). The concentrated nanoparticles were freeze-dried using mannitol to protect the formulation from damage caused by freezing (2.5 % w/v) and to ensure stabilization within the nanoparticle and loaded drug (Chaves et al., 2018; Szymusiak et al., 2016; Tong et al., 2017). The freeze-dried nanoparticles were subsequently lyophilized (FreeZone® 2.5, Labconco®, Kansas City, MS, USA) at -80 °C for 48 h to yield a powder.

Physicochemical analysis employing FTIR on pristine polymers and the DLX-loaded EUD-CHT nanoparticles

Pristine polymers and different nanoparticle formulations were analyzed using Attenuated

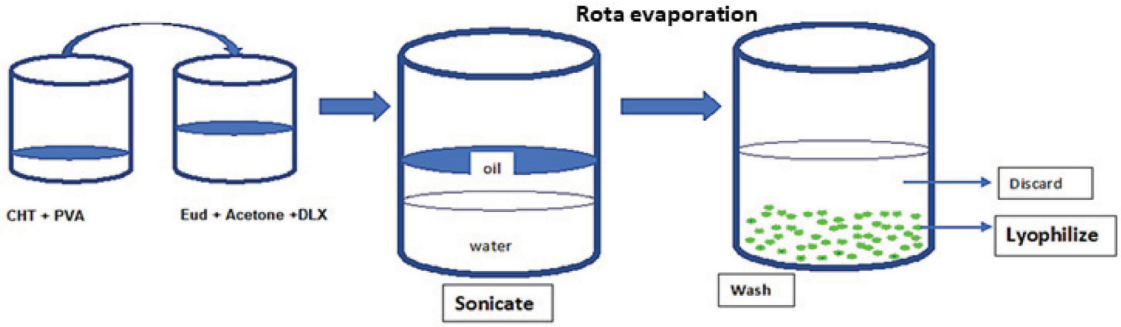

Figure 1. Schematic illustration of solvent emulsion evaporation synthesis and physicochemical characterization of the DLX-loaded EUD-CHT nanoparticles.

Total Reflectance Fourier Transformation Infrared (ATR-FTIR) analysis to evaluate the chemical and stability properties of the DLX-loaded EUD-CHT nanoparticles. Characteristic peaks were compared to confirm the molecular vibrations and transitions of the polymers in the formulations. The Perkin Elmer Spectrum 2000 FTIR Spectrometer was employed, applying a single-reflection diamond MIRTGS detector (Perkin Elmer Spectrum 100, Llantrisant, Wales, UK). Samples were analyzed at a resolution of 4 cm^{-1} using a universal ATR polarization accessory. Samples of each polymer and the DLX-loaded EUD-CHT nanoparticles were placed on a diamond crystal, and each ran at a scan rate of 100 to minimize signal-to-noise ratio in the wavelength range of 4000 - 600 cm^{-1} at a constant pressure of 120 psi (Kondiah et al., 2013). It was determining the Particle size and zeta potential of the DLX-loaded EUD-CHT nanoparticles. The zeta potential of nanoparticles and particle size distributions were evaluated using a Zetasizer NanoZS analyser (Malvern Instruments Ltd. UK) that functions by dynamic light scattering. A light scattering angle of 90° and a temperature of 25 °C were settings used to confirm the size of the particles. The nanoparticles were dissolved (1 mg of each formulation in 1 mL of Millipore water). Samples were diluted and sonicated before measurement, with the sample temperature maintained at 25 °C throughout the analyses. A 2 mL sample of the nanoparticle suspension was then filtered through a 0.22 µm filter and placed in disposable cuvettes. Particle size, polydispersity index and zeta potential values were analyzed to screen formulations and select the most favorable preparation parameters, such as the organic solvent, the concentration of stabilizer and sonication for the emulsification solvent evaporation approach.

Morphological analysis of the DLX-loaded EUD-CHT nanoparticles

The DLX-loaded EUD-CHT nanoparticles were evaluated using a JEOL 840 SEM equipment (JEOL, Japan), under various magnifications at 20 keV. The dry formulation, in lyophilized form, was loaded on stubs and sputter-coated with gold-palladium shadowing. Micrographs of the formulation were then critically evaluated, determining the surface morphology of the synthesized nanoparticles.

Thermal analysis of the DLX-loaded EUD-CHT nanoparticles

The DLX-loaded EUD-CHT nanoparticles were analyzed by Differential Scanning Calorimetry (DSC) to evaluate its glass transition temperature and the melting point of the copolymers. Data was collected using the Mettler Toledo DSC-1 STARe system, heated from 25-300 °C at a heating rate of 10 °C per minute, resulting in thermograms of heat flow versus temperature. Thermogravimetric analysis (TGA) was conducted on the DLX-loaded EUD-CHT nanoparticles. Samples of 10 mg were heated at 10 °C/min in an aluminum pan, using nitrogen as a purge gas (flow rate of 25 mL/min). The percentage of copolymer mass loss over temperature heating conditions was evaluated (Nasef et al., 2017).

Optical characterization and stability of the DLX-loaded EUD-CHT nanoparticles, utilizing a Turbiscan

The degree of particle dispersion/ agglomeration was evaluated, in line with optical interactions, looking at the stability of the DLX-loaded EUD-CHT nanoparticles, utilizing a Turbiscan™ LAB (Formulation, L'Union, France). A 20 mL aliquot of DLX-loaded EUD-CHT nanoparticles and duloxetine-free nanoparticle suspensions were introduced into sample holders. The Turbiscan measures stability based on turbidity with transmission light or backscattering light transmitted from a sample (Gagliardi et al., 2019; Sumaila et al., 2019). The turbiscan can scan the entire length of a sample at 40 µm intervals. 10 mg of the DLX-loaded EUD-CHT nanoparticles and duloxetine-free nanoparticles were dissolved in a buffer solution in their respective beakers. Samples were analyzed for 60 min utilizing the Turbiscan™ LAB. Backscattering results were used to measure its stability in terms of its nature to remain dispersed in solution.

Evaluation of the DLX-loading efficiency within the DLX-loaded EUD-CHT nanoparticles

A mass of 15 mg of the nanoparticulate formulation was weighed and mixed in phosphate buffer saline (10 mL) at a pH of 7,4 and 37 °C. The suspension was then centrifuged for 60 min at a speed of 10 000 rpm. The resultant solution

was filtered through a 0.22 mm Millipore filter to remove residue that may not have dissolved (Tong et al., 2017). The Calibration curve was used to determine drug entrapment efficiency (DEE) at 290 nm, utilizing the calculated linear Equations (1 & 2) where $R^2 = 0.9962$. The specific absorptivity can be used to determine the drug concentration released, using the constructed standard calibration curve relating absorbance to concentration by Beer Lamberts Law of linearity (Equation 1).

$$y = mx + C \quad (1)$$

DLX Concentration

$$\text{Concentration of Duloxetine} = \frac{y}{m} - b \quad (2)$$

Drug Entrapment Efficiency

$$\text{DEE}\,(\%) = \frac{\text{Mass of DLX in nanoparticles}}{\text{Mass of nanoparticles}} \times 100 \quad (3)$$

DLX Loading Capacity

$$\text{DLX Loading}\,(\%\,\frac{w}{v}) = \frac{\text{Mass of DLX in nanoparticles}}{\text{Mass of nanoparticles}} \times 100 \quad (4)$$

In silico analysis of DLX interactions with the polymeric nanoparticle structure

The molecular interaction of DLX with the DLX-loaded EUD-CHT nanoparticles, was evaluated by molecular modelling technology using Schrondinger's Material Sciences docking software (2018-2). The mol file of CHT (CT1078683894) was retrieved from Mol-Instincts to the docking workspace. The 2D sketcher was used to generated EUD structure, and finally, CHT and EUD were crosslinked in the 3D Builder platform. The generated co-polymer was further prepared with a protein preparation wizard (Rants'o et al., 2022; Marwaha et al., 2020). On the other hand, the SDF molecular structure of DTX was obtained from PubChem and prepared by the LiqPrep tool. Polymer Builder was used to generating a polymer from the prepared Eud-CHT subunits. The final polymer was allowed to form an amorphous cell under OPLS3e force field at a cut-off van der Waals clash scale factor of 0.5, the temperature of 300 K for Boltzmann constant, and density of 0.5 g/cm^3 (DuBay et al., 2012; Mavrantzas, 2021). This polymer was then processed for spherical nanoparticle formation under the Nanoparticle Builder tool at a cut-off radius of 5 Å. Lastly, the nanoparticle-DTX interaction was assessed through the rigorous Disordered System Builder, where DLXas immersed into the nanospheres and the complex processed under the OPLS3e forcefield, after which the crystal pose, polymer configuration and intermolecular interactions were analyzed (Lolicato et al. 2017; Gartner and Jayaraman, 2019; Choi et al., 2021).

In vitro release analysis of the DLX-loaded EUD-CHT nanoparticles

In vitro release studies of DLX from the DLX-loaded EUD-CHT nanoparticles were analyzed using a USP 2 apparatus (ERWEKA, USP Apparatus II, DT 126, UK), operated at a paddle revolution of 50 rpm over a 24h period. Thirty milligrams (30 mg) of the DLX-loaded EUD-CHT nanoparticles were weighed and encapsulated into hard gelatin capsules. With 30 mg of DLX, Capsules were placed into vessels containing 900 mL PBS buffer of three pH mediums, pH 1.2 simulating gastric gastric stomach fluid, pH 6.8 simulating the small intestinal environment and pH 7.4 for human physiological settings with the temperature at 37 °C. Subsequently, samples were exposed to the dissolution media separately for 2 hrs. Five (5 mL) of the sample in triplicate were collected at predetermined time intervals, 0 min, 5 min, 30 min, 60 min, 1 hr, 2 hrs, 4 hrs, 8 hrs and 24 hrs. At each time interval of sampling, the sample quantity (5 mL) in triplicate was pipetted out and replaced with the same amount of buffer medium to maintain sink conditions. A UV spectrophotometer was used to measure the absorbance at a wavelength of 290 nm (Samal & Prusty, 2019). The DLX stock solution was prepared by dissolving duloxetine HCL (10 mg) in 20 % v/v acetonitrile in a 100 mL volumetric flask. Serial dilutions of 1- 5 mL were prepared from the stock solution and made up to 50 mL with stock solution (Nasef et al., 2017; Samal & Prusty, 2019). Absorbance values were measured at the obtained maximum wavelength of 290 nm for duloxetine, and the release curves demonstrate the concentration of duloxetine at different time intervals. Each point is depicted as a mean ± Standard Deviation SD (n=3).

Kinetic modelling of *in vitro* DLX release from the DLX-loaded EUD-CHT nanoparticles

Drug release kinetics were determined using a zero-order release model, first-order release kinetics, Fick's law of diffusion, Korsmeyer-Peppa's equation, and Hixson-Crowell equation, [Equations (5) – (9)]. Statistics and arithmetic's were determined using Systat SigmaPlot software 12 (Systat Software Inc., CA, USA).

The zero-order model (Equation (5)) represents a stable release process, where:

$$Y = a_0 + K_0 t \quad (5)$$

Equation 6 (first-order release kinetics) defines the spread of the drug within the nanoparticulate structure with a drug release rate correlating with its concentration.

$$\text{Log } Y = \text{Log } a_0 + K_1 t \quad (6)$$

Fick's law of diffusion (Equation (7)) gave way to the establishment of Higuchi's square root of time model to determine drug release from a complex network.

$$Y = a_0 + Kt^{1/2} \quad (7)$$

For authentication of the therapeutic release steps, the initial 35 % of the released therapeutic was substituted in the Korsmeyer-Peppas model (Equation (8)).

$$F = \frac{Mt}{M\infty} = Kt^n \quad (8)$$

Where $Mt/M\infty$ represents the fraction of released drug at time t, Mt represents the amount of released drug at time t, $M\infty$ represents the maximum amount that can be released, t represents the time intervals of release in hours, k represents the kinetic constant, and n represents the release exponent. There was an expectation of nanoparticle diameter increase due to DLX incorporation. Therefore, the Hixson-Crowell law (Equation (9)) was used to describe the release of the drug by dissolution, impacted by surface area and diameter of nanoparticle changes.

$$\sqrt[3]{Qa} - \sqrt[3]{Qa} = K_1 t \quad (9)$$

The highest R^2 value determined from the kinetic model was chosen to represent the most appropriate model for authenticating the release of DLX from the nanoparticles. There are different release profiles, namely Fick's diffusion, anomalous diffusion or erosion. The release exponent value (n) for the Korsmeyer-Peppas law was used in determining the nature of release from the nanoparticles.

***In vitro* cytotoxicity studies the DLX-loaded EUD-CHT nanoparticles utilizing HEK 293 cells**

Cellular toxicity studies were conducted utilizing HEK 293 cells, and the MTT assay was carried out, as described by Jafari et al. (Jafari et al., 2016). HEK 293 cells were in a subculture, in a 96-plate well apparatus, at a seeding density of 695000 cells/mL in a trypsin medium. The cells (HEK 293) remained at 37 °C and CO_2 of 5 % in a controlled incubator. The cells were treated with three variant concentrations of duloxetine HCL, the DLX-loaded EUD-CHT nanoparticles, and blank nanoparticles. The treatment period was 48 hrs, after which the culture medium was removed, then substituted with fresh medium and MTT reagent, and further incubated for 4 hours. Before crystallization, all medium was aspirated, dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. Absorbance was then measured at 570 nm utilizing the Thermo Lab systems Multiskan MK3 microplate reader (Pantshwa et al., 2017). Percentage cell viability was calculated using the number of cells counted over the count of quadrants, shown in Equations 10 and 11. DF represents the dilution factor that was used as10^4 is the constant.

Calculation of viable cell number:

$$\text{Total Cell Number} - \text{Dead Cell Number} = \text{Viable Cell Number} \quad (10)$$

Calculation of percentage cell viability (11)

$$\%\ \text{Viable cells} = \frac{\text{Number of cells counted}}{\text{Number of Quandrants counted}} \times DF \times 10^4\ \text{cells/ml}$$

Statistical Analysis

Origin software (version 8.5.0 SR1, Origin Lab Corporation, Northampton, MA, USA) processed

and analysed data for the preparation and characterisation of nanoparticles and drug release studies. All results were represented by the mean ± standard error of three experiments.

Results and Discussions

Characterization of the synthesized DLX-loaded EUD-CHT nanoparticles, utilizing FTIR spectroscopy

The molecular composition and characteristics of the DLX-loaded EUD-CHT nanoparticles were evaluated for their chemical characteristics. FTIR spectra of starting material Eudragit, chitosan and combinational blank of the DLX-loaded EUD-CHT nanoparticles, as well as unloaded DLX nanoparticles, are shown in Fig. 2. A comparable peak in Eud and Eud-chitosan of methylene stretching due to ester bonds is also observed at 2952 cm^{-1}. FTIR spectral evaluation of duloxetine HCL and duloxetine-loaded nanoparticles shows comparable peaks at 1281 cm^{-1} and 1724 cm^{-1}. The first peak can be attributed to C-O vibration, while the second peak^{-1} is attributed to -C-O stretch. A peak at 3328 cm^{-1} is observed and attributed to the N-H stretching of duloxetine HCL. The CH_2 bending can be observed at peaks 1324 and 1321 cm^{-1}. The results observed were in correlation with those concluded in studies by Sindhu *et al*. (2018) and Mani *et al*. (2014). A comparison of the DLX-loaded EUD-CHT nanoparticles and chitosan (Fig. 2) depicts similarities in peaks at 1377 and 1374 cm^{-1} for C-H bending, 1147 and 1144 cm^{-1} for the C-O-C stretching band and 888 and 892 cm^{-1} for C-N vibration of chitosan (Menezes et al., 2020). Comparing Eudragit FTIR spectra and the FTIR spectra of the DLX-loaded EUD-CHT nanoparticles (Fig. 3), they thus displayed distinct peaks noted at 2936 and 2952 cm^{-1} attributed to the C-C chains stretching vibration, 1451 cm^{-1} and 1448 cm^{-1} for the OR-C=O group, and 1147 and 1150 cm^{-1} for stretching of the ester from the Eudragit (Guo et al., 2017; Mohammadi et al., 2017). The results of the FITR characterization, thus, indicate the synthesis of the DLX-loaded EUD-CHT nanoparticles.

Evaluation of particle size, zeta potential and SEM morphological analysis of the DLX-loaded EUD-CHT nanoparticles

The nanoparticles, both unloaded and loaded DLX EUD-CHT nanoparticles, were evaluated for size and zeta potential, employing the Zetasizer NanoZS analyzer (Malvern Instruments Ltd. UK). Fig. 4 (I & ii) illustrates the particle size and distribution of the placebo nanoparticles, with a particle size of 100 nm and with a polydispersity index (PDI) of 0.445. A PDI less than 0.5 indicates narrow size distribution, that is, uniform/ homogenous and dispersed with acceptable

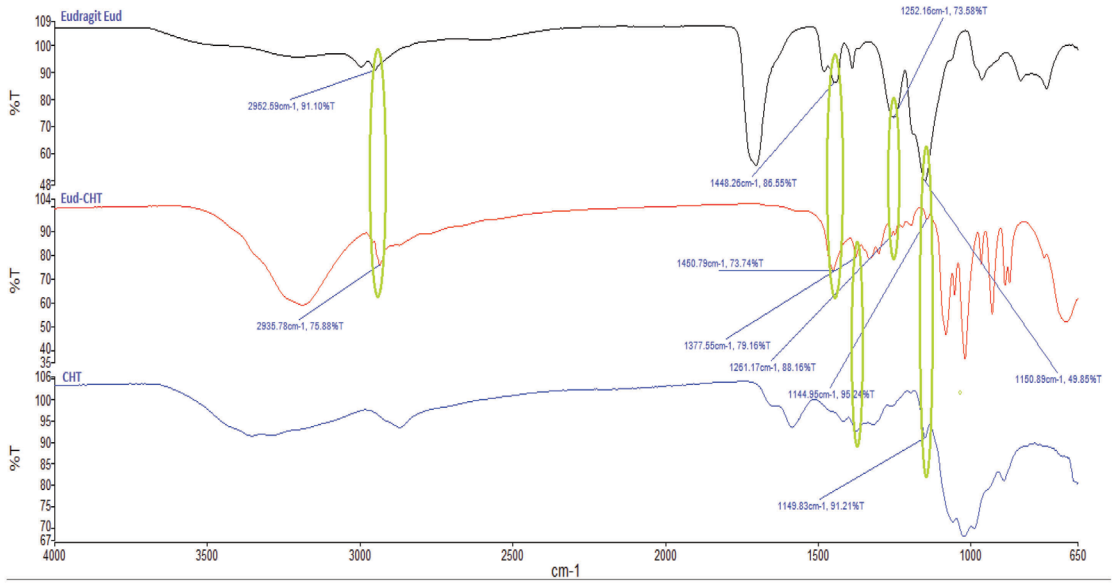

Figure 2. FTIR Spectra of Eudragit RL-100 (Eud), Chitosan (CHT) and blank EUD-CHT nanoparticles.

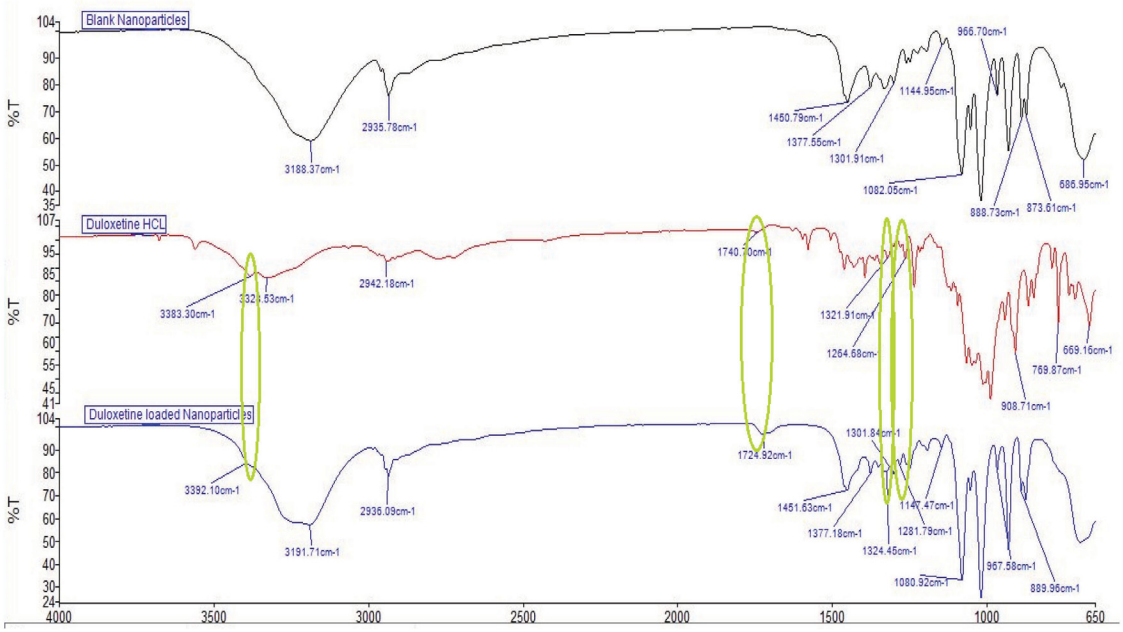

Figure 3. FTIR Spectra of synthesized DLX-loaded EUD-CHT nanoparticles and DLX-unloaded EUD-CHT nanoparticles.

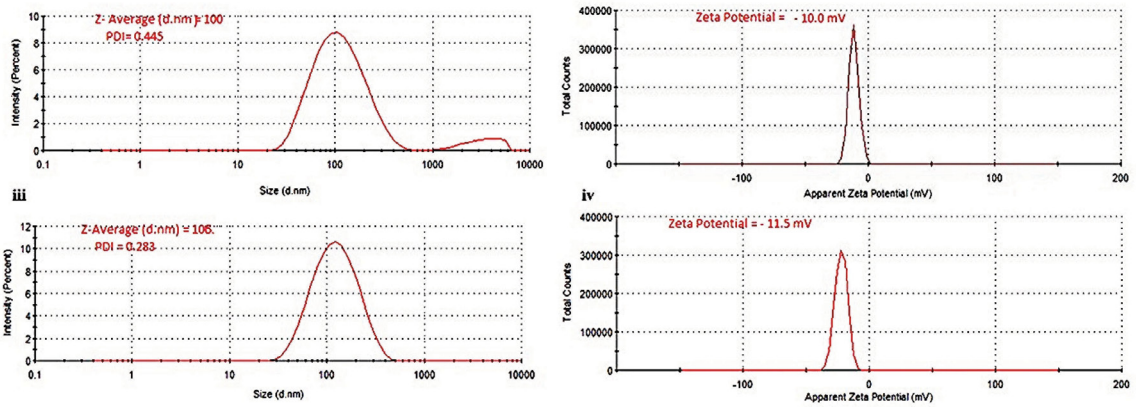

Figure 4. Graphs depicting **(i)** Particle size distribution of placebo nanoparticles, **(ii)** Zeta potential graph of placebo nanoparticles, **(iii)** Particle size distribution graph of the DLX-loaded EUD-CHT nanoparticles, **(iv)** Zeta potential graph of the DLX-loaded EUD-CHT nanoparticles.

stability of the particle size distribution from its synthesis. The observed particle size for the DLX-loaded EUD-CHT nanoparticles in Fig. 4 (iii) was evaluated to be 106 nm with a PDI of 0.283. Thus, the average particle size for drug-loaded nanoparticles was comparable to its placebo nanoparticles, regardless of duloxetine HCL loading. A particle size of < 200 nm is favorable for BBB penetration (Zaman et al., 2018). The particle surface morphology was investigated and characterized utilizing SEM. The particle size, compared with SEM images (Fig. 5), shows spherical-shaped particles with a narrow size distribution and a mean particle size of 100 nm. This observation shows a corresponding result to the Zetasizer study. The observed zeta potential for placebo and drug-loaded nanoparticles (Fig. 4 (ii) & b(iv)) was -10 ± 3.26 mV and -11.5 ± 2.79 mV, respectively. This indicates a good range of stability of the particles, with a charge to protect against agglomeration (Sindhu et al., 2018; Tong et al., 2017). It can be deduced that

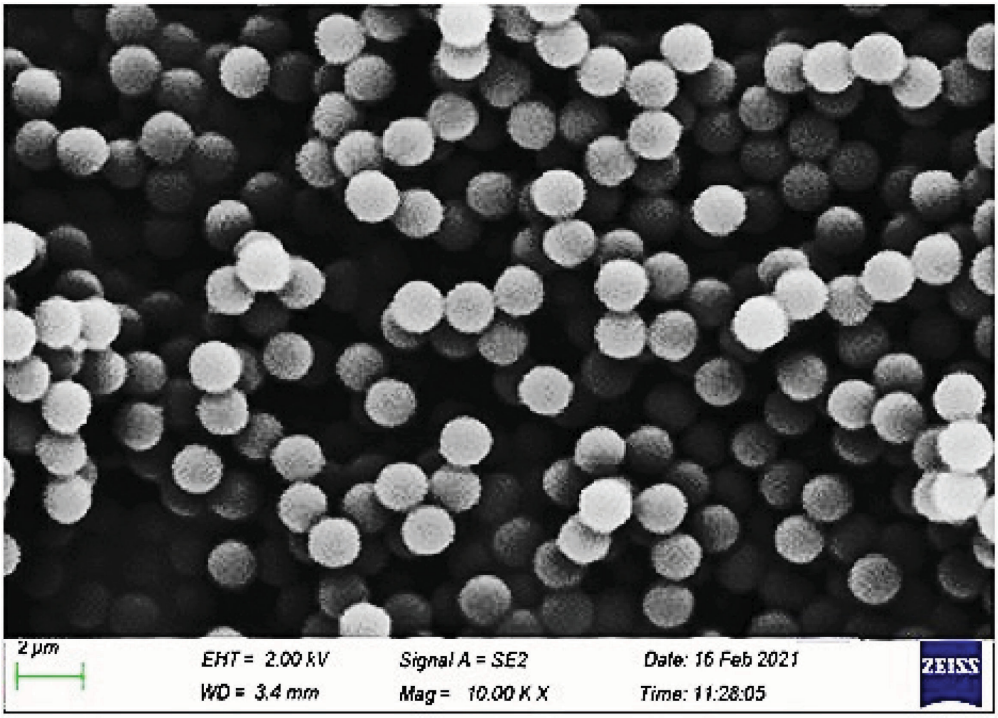

Figure 5. Microscopy images of the DLX-loaded EUD-CHT nanoparticles, post lyophilization.

the concentrations of the polymers used also affected the level of stability due to interference of cohesion (Keck & Müller, 2006). Thus, to prevent aggregation of the nanoparticles, it was observed that a vast degree of cryoprotectants was not necessary to maintain the uniformity of charge distribution of the DLX-loaded EUD-CHT nanoparticles (McNally et al., 2003)**.** The chitosan coating provided a positive charge which can further favor increased interactions with negative cell membranes. Studies also reflected that if too high concentrations of chitosan was used, adhesion of the particles was observed, thus, the final formulation proved to be optimum for effective particle size and zeta potential characteristics (Keck & Müller, 2006).

Thermogravimetric analysis of the DLX-loaded and unloaded EUD-CHT nanoparticles.

Thermal stability analysis was conducted to evaluate the integrity and changes in the mass of the polymeric nano-formulation during the application of thermal temperature ramping conditions (Jothimani, Sureshkumar, & Venkatachalapathy, 2017). Utilizing TGA, the thermal degradation of the DLX-loaded EUD-CHT nanoparticles generated in Fig. 6a (red solid) illustrated a one-step degradation with a weight loss of 62 % on heating from 36.2–907.5 °C. However, its related derivative TGA (DTGA, blue) demonstrates a degradation in two specific steps shown by DTGA peaks at 68.1 °C and 310 °C, respectively. These points correspond to the points of inflexion, for moister and degradation aspects. The thermograph for the model drug Duloxetine HCL shows a two-step weight loss, beginning at 40 and in the region of 310 °C, where the first and second step reflects moisture and degradation, respectively The extrapolated end of the degradation of Duloxetine was observed to be in the region of 400 °C, and the total Duloxetine weight loss on heating from 40-910 °C was calculated to be 77.935 %.

Blank nanoparticles show a two-step weight loss and degradation, the onset at 190–210 °C, with a total loss of 85 % of weight, which is attributed to the copolymeric structural degradation, and following between 210–250 °C. A comparison between DLX loaded, and blank nano-formulation shows a difference of a one-step degradation at 330–450 °C, which can be attributed to the inclusion of the drug in the formulation. A similar result of a two-step degradation of Duloxetine, between 200 and 400 °C, was found in a study

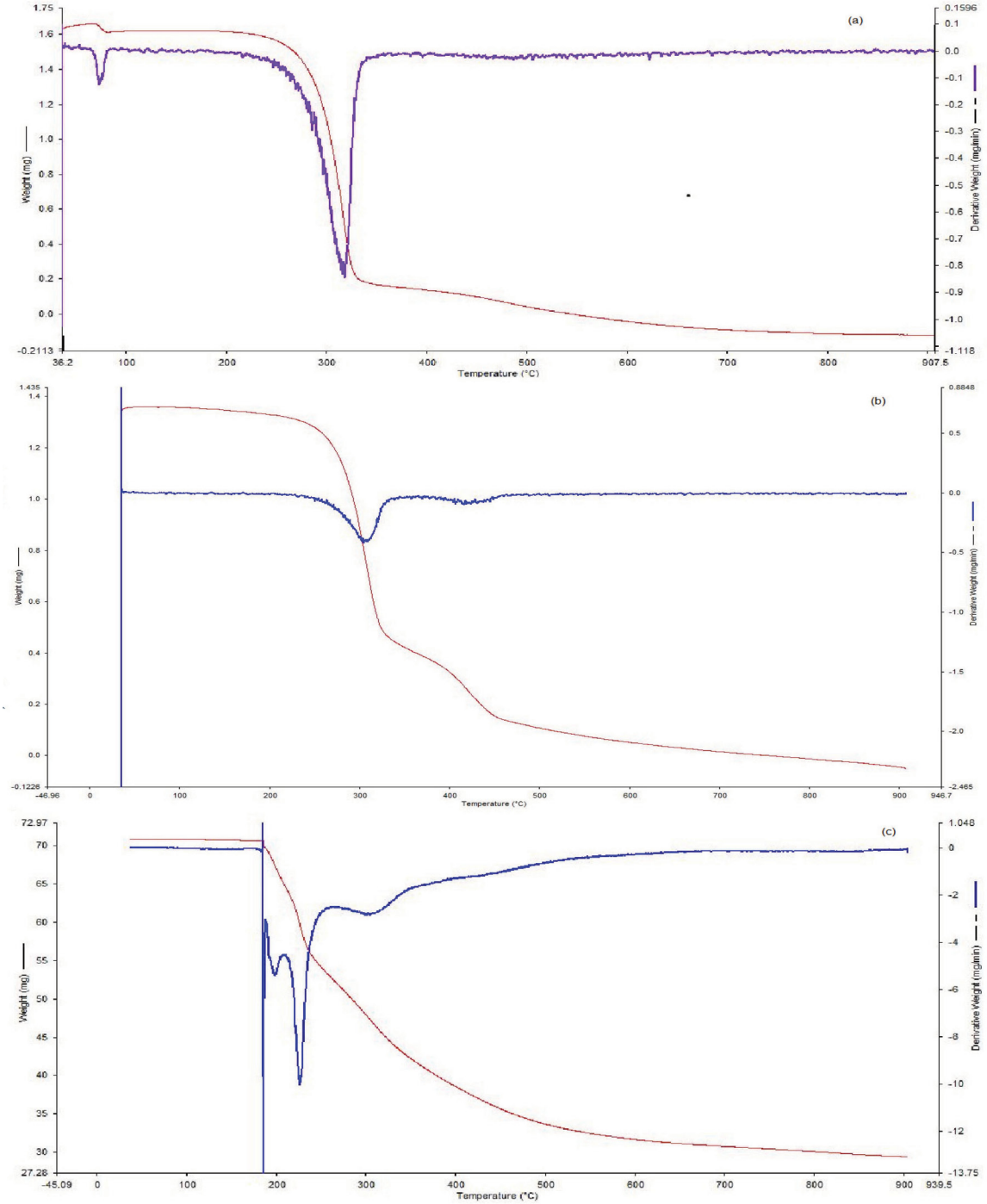

Figure 6. Thermographs (TGA: red) and derivatives (DTGA: blue), depicting thermal degradation of **(a)** the DLX-loaded EUD-CHT nanoparticles, **(b)** Plain Duloxetine drug **(c)** unloaded DLX- EUD-CHT nanoparticles.

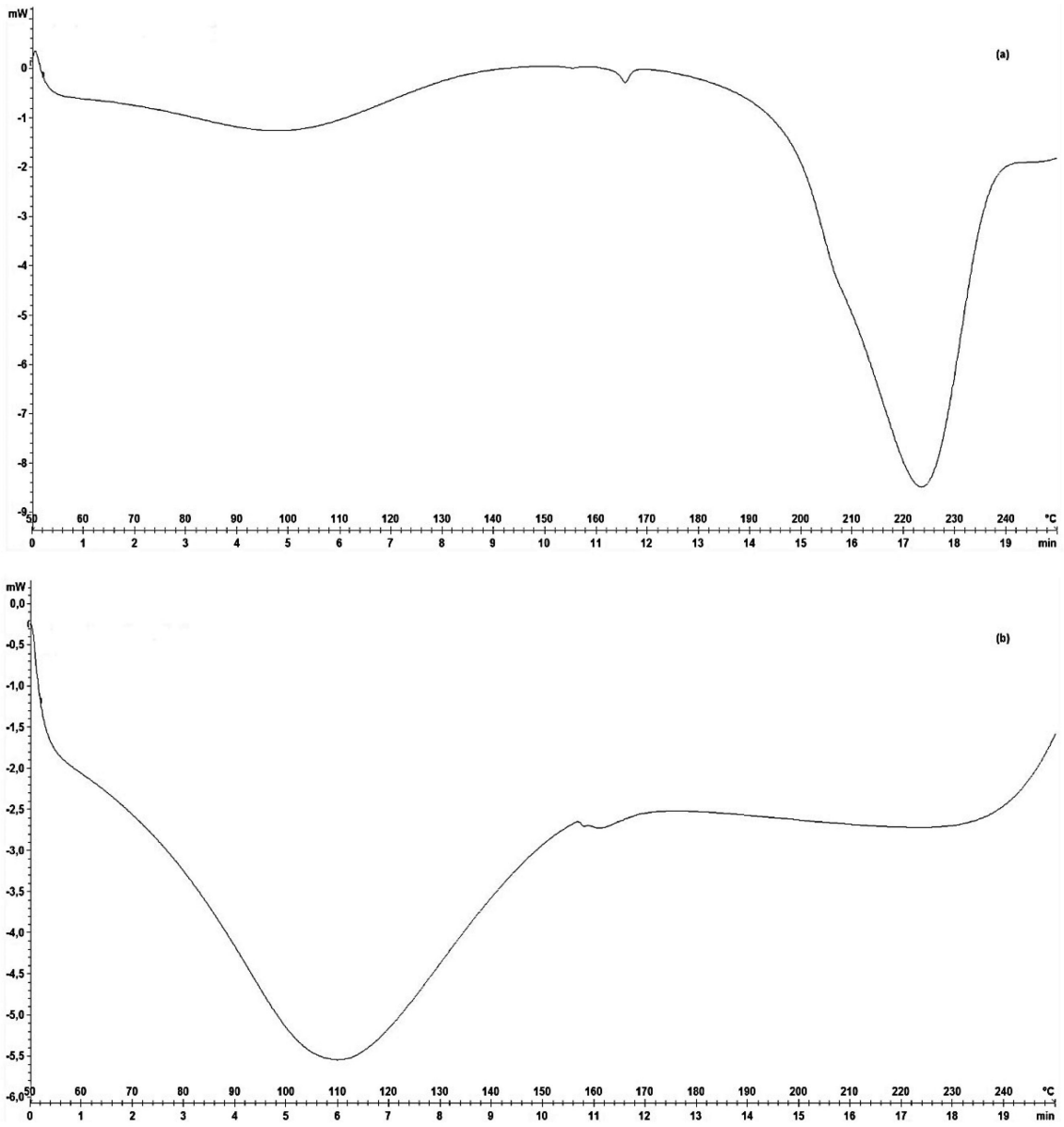

Figure 7. Differential Scanning Calorimetry graphs for **(a)** Eudragit RL 100 and **(b)** chitosan polymer.

conducted by Mani et al. (2014). The initial loss is associated with the melting point of Duloxetine HCL at 118–162 °C (Chahal et al., 2020). The inclusion of Duloxetine into the nanoparticulate structure thus showed enhancement in the thermal stability of Duloxetine, to a moderate extent (Akhtar et al., 2020).

Thermal analysis by Differential Scanning Calorimetry (DSC)

DSC analysis allowed the interpretation of glass transitions and melting points in response to the DLX-loaded EUD-CHT nanoparticles and DLX encapsulation. Condensed differential scanning calorimetry thermographs of blank nanoparticles, DLX and the DLX-loaded EUD-CHT nanoparticles are seen in Fig. 7. The glass transition of Eudragit RL was observed at 62 °C similarly demonstrated by Jana *et al*. (2014). An Endothermic peak was observed between 195 °C – 235 °C (Fig. 7a). Fig. 7b shows a thermograph for chitosan and an endothermic peak observed at 60 °C – 150 °C due to chitosan's dehydration phase (Chatzitaki et al., 2020). Fig. 8 shows the thermograph for Eudragit/chitosan

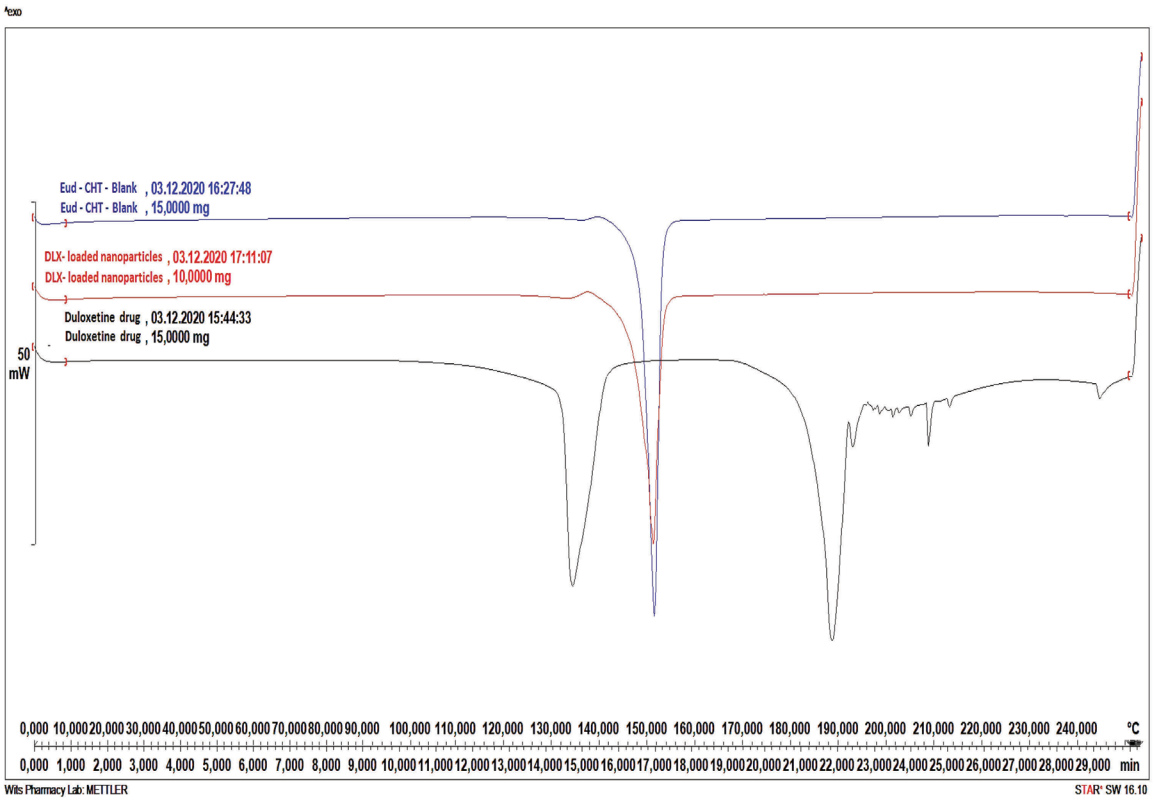

Figure 8. Condensed differential scanning calorimetry thermograph for blank nanoparticles, the DLX-loaded EUD-CHT nanoparticles and pure duloxetine drug.

blank nanoparticles, demonstrating a distinct endothermic peak between 145 °C–160 °C. The DLX-loaded EUD-CHT nanoparticles show a slight decrease in temperatur a slight temperature at the point of the complete phase change (Mani et al., 2014).

Investigation of stability of nanoparticles utilizing the Turbiscan™ LAB

The DLX-loaded EUD-CHT nanoparticle suspension was analyzed for its aggregation in response of its stability, using light scattering analysis. The light scattering analysis is based on the principle of particle dispersion within a solution. It analyses flocculation and coalescence at different heights of the sample. The relationship between Back Scattering (ΔBS) vs Transmission (T) determines the level of aggregation at the top and middle of the sample and sedimentation at the bottom of the sample (Sumaila et al., 2019). Figs. 9 and 10 represent the Light Transmission graph for the DLX-loaded EUD-CHT nanoparticles and Duloxetine HCL plain drug respectively. In both graphs, there is a form of destabilization. The two graphs show a similar trend, a destabilization at the bottom of the sample and a decrease in ΔBS and T in the middle of the sample. The plain drug and the DLX-loaded EUD-CHT nanoparticles exhibited changes in ΔBS from the bottom to the middle of the sample of 20 % and 30 %, respectively. This may be due to the sedimentation kinetics of nanoparticles and the heterogeneous particle size in the nanoparticulate formulation, as noted in the Zeta size analysis, of size range 100.6nm. The plain drug exhibits an increase in ΔBS in the middle of the sample, t demonstrating coalescence in the plain drug solution. Nanoparticles having Eudragit hydrophobic core and hydrophilic chitosan shell could explain the properties of the particles to exist in solution, with charge repulsion, to prevent aggregation.

Molecular modelling of duloxetine interactions with the polymeric nanoparticle carrier

The co-polymer generated from the crosslinking of Eudragit and CHT was processed under

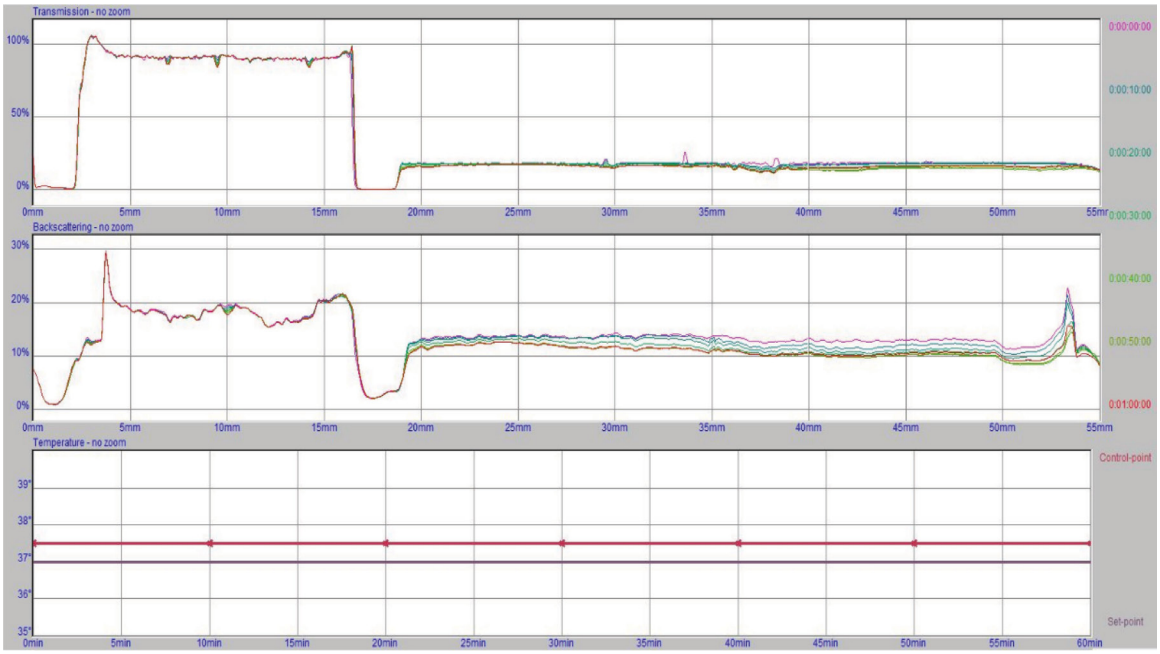

Figure 9. Representation of destabilization kinetics of the DLX-loaded EUD-CHT nanoparticles, indicating ΔBS (Back Scattering) and T (Transmission) measured at 37 °C for 1 hour.

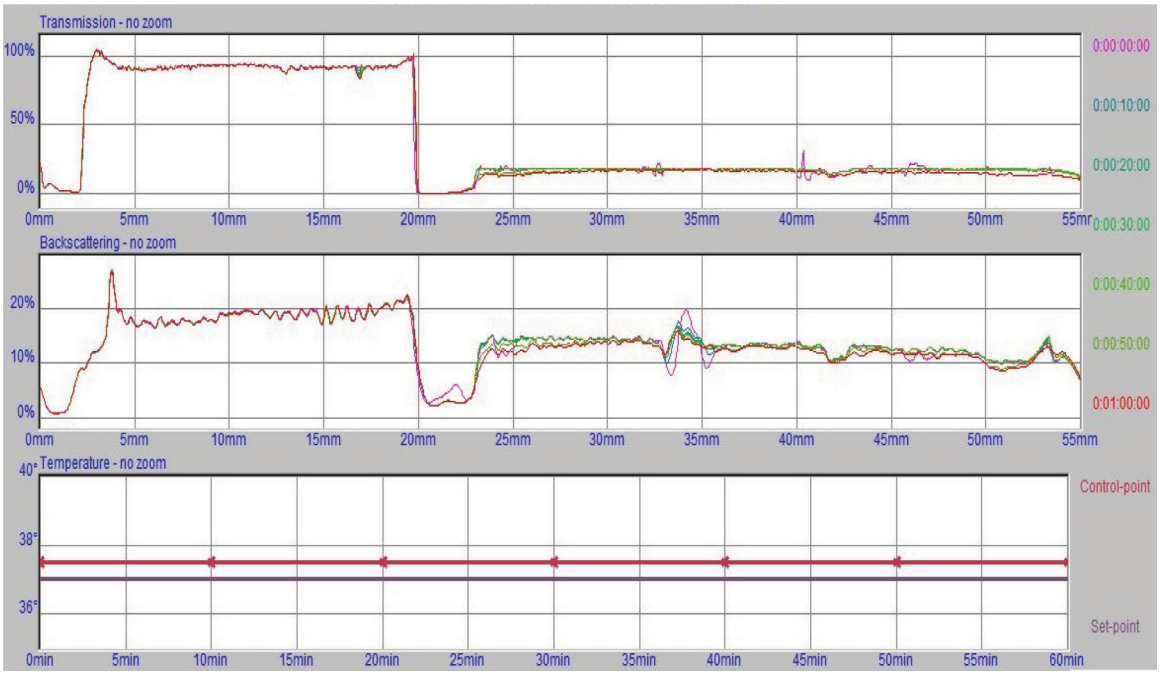

Figure 10. Representation of destabilization kinetics of duloxetine plain drug, indicating ΔBS (Back Scattering) and T (Transmission) measured at 37 °C for 1 hour.

Nanoparticle Builder. It successfully generated a nanosphere showing the positively charged Eudragit head and CHT tail, congruent with previous studies. (Fig. 11a) (Ghaffari et al., 2007; Nataraj et al., 2018). The Disordered System Buidler showed a central nanoparticle composite of DLX where the polymer monomers assummed a configuration that placed the positively charged Eudragit heads on the nanoparticle surface (Fig. 11b).

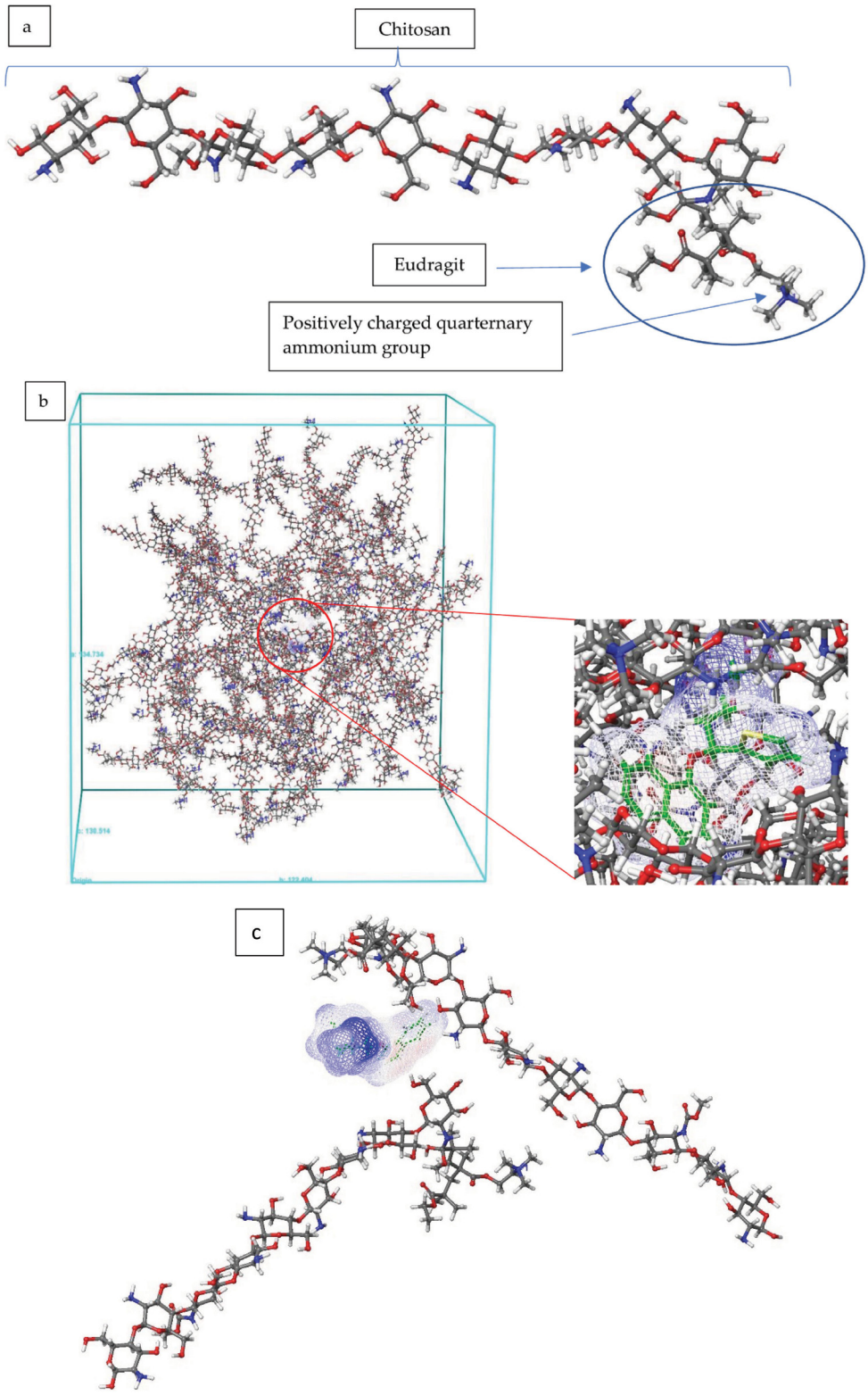

Figure 11. Molecular visualization of the Eud-CHT copolymer **(a)**, a central composite of Eud-CHT-DLX nanoparticles (DLX shown in green inside a mesh surface) **(b)**, polymer configurations in the nanoparticle **(c)**, and SwissADME bioavailability radar (left) and neuro-availability BOILED-Egg graphical illustration (right) **(d)**.

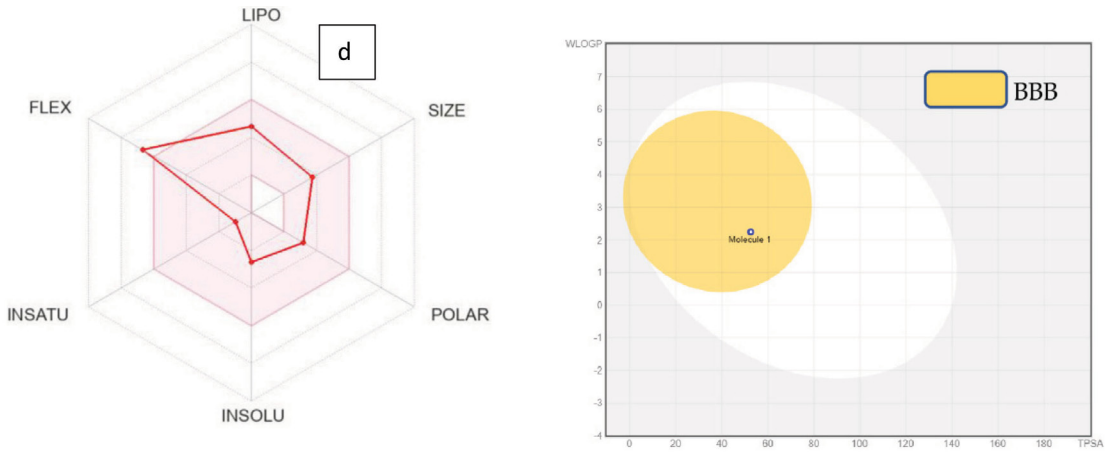

Figure 11 (continued). Molecular visualization of the Eud-CHT copolymer **(a)**, a central composite of Eud-CHT-DLX nanoparticles (DLX shown in green inside a mesh surface) **(b)**, polymer configurations in the nanoparticle **(c)**, and SwissADME bioavailability radar (left) and neuro-availability BOILED-Egg graphical illustration (right) **(d)**.

Two monomers in close were selected to display the general crystal pose to further assess the interaction of DLX and nanoparticles and the polymer configurations (Fig. 11c). Interestingly, the co-polymers interacted with DLX mainly with the terminal glucosamine group from the chitosan monomer before Eudragit cross-linkage. Furthermore, as these exposed glucosamine groups extended towards the central DLX ligand, the Eudragit portion bent towards the outer surface. This exposed their positively charged quarternary ammonium groups to the nanoparticle surface. This is of great importance as the increased ionic interactions of the positively charged nanoparticles with negatively charged cellular membranes will potentially enhance cellular recognition and uptake of DLX (Abdelfatah et al., 2017). This will be even more advantageous for CNS uptake of DLX. To further asses for the increased CNS uptake, the Eudragit monomer was submitted to the SwissADME web portal. This produced a favorable bioavailability radar within the required physicochemical properties for GIT absorption and egg-shaped plot (BOILED-Egg) model showing the polymer in the BBB region (Fig. 11d) (Daina et al., 2017). This indicates an excellent potential for both high GIT absorption and BBB permeation of the formed nanoparticle, hence high DLX concentrations in the CNS. Also, DLX mainly interacted with the nanoparticle through weak van der Waals forces, weakening the energy barrier required for its release at the site of action (Abdelfatah et al., 2017).

***In vitro* drug release studies undertaken on the DLX-loaded EUD-CHT nanoparticles.**

The *in vitro* release of duloxetine from the DLX-loaded EUD-CHT nanoparticles was undertaken and reflected at 3 different pH values of the formulation, as seen in Fig. 12. The DLX-loaded EUD-CHT nanoparticles was dissolved in mediums simulating gastric, intestinal and normal physiological pH at 1.2, 6.8, and 7.4, respectively. The study was conducted over 2 hrs., observing the release at hourly intervals. Each point represents mean ± SD (n=3).

The purpose of using EUD-CHT polymer in designing and synthesizing the DLX-loaded nanoparticles was to protect the antidepressant drug from degradation in the stomach acid, improve bioavailability and potentially create a controlled-release drug delivery system. The pH-responsive carriers for oral drug delivery have been found to enhance drug delivery stability in the stomach and achieve controlled release in the intestines. However, it is commonly impossible for a single polymer to meet all of the desirable qualities in a formulation, such as pH response, mucoadhesion and drug release profile. The combination of different polymers allows systems to be obtained with improved properties. The systems such as the Eudragit-Chitosan Nanosystem consisting of two layers, each with other physicochemical properties, which gives the formulation greater functional versatility by merging the advantages

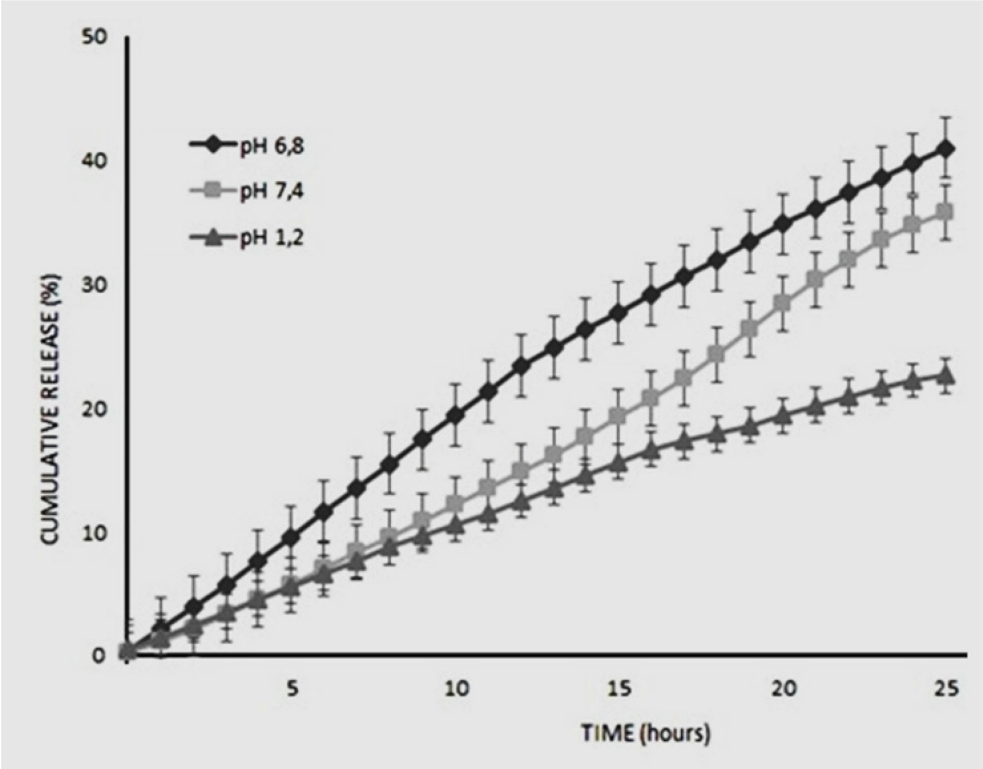

Figure 12. Duloxetine release from the DLX-loaded EUD-CHT nanoparticles at simulated conditions of the stomach pH, intestinal pH, and normal physiological pH (1.2, 6.8 & 7.4). Each point represents: mean ± SD (n=3).

and characteristics of both materials (https://doi.org/10.1016/j.ijpharm.2022.121554). The use of pH-dependent polymers like Eudragit® makes the system offer a controlled release depending on the pH. Because this polymer becomes soluble at a pH over 6, it represents an exciting excipient for achieving controlled release in an acidic environment (Martín-Illana et al., 2022). Percentage drug release was analyzed for up to 24 hrs at different pH mediums, representing the stomach, intestinal, and neutral environments. Fig. 12 shows the drug release up to 20 % after 24 hrs for nanoparticles placed at a medium of pH 1.2. It can be observed that the nanoparticulate system has protected duloxetine from the harsh pH environment. At pH 6.8, the percentage of drug release was 40 % higher than at pH 7.4, which only recorded 35% drug release after 24 hrs. The DLX-loaded EUD-CHT nanoparticles released the drug faster at elevated alkaline pH, and less drug was released at an acid pH. The release mechanism in different pH conditions can be attributed to the presence of eudragit L100, an anionic copolymer with low solubility in acid condition but readily dissolves at high alkaline pH conditions. Under acid conditions, eudragit L100 gets deprotonated, forming a dense film on the nanoparticle surface, which helps to nanoparticle surface and helps to nanoparticle surface and helps protect the integrity of nanoparticle structures, thus reducing the amount of the drug released. Similar release profiles of eudragit coated chitosan nanoparticles under similar pH conditions were observed and reported by Unsoy *et al*., (2014) and Chen *et al*., (2017). Compared to a study conducted by Rana *et al*. (2020), solid lipid nanoparticles (SNL) are loaded with duloxetine for targeted delivery to the brain (Rana et al., 2020). SLN formulation released up to 52.9 % of the drug and, after 24 hrs, at pH 7.4, while Eud-CHT-DLX nanoparticle released only 35 % over the same period and pH conditions. This was an indication that Eud-CHT-DLX nanoparticles does not only provide protection to the drug under harsh GIT condition but also have a show a more sustained and a controlled release profile. This can reduce frequency of administration and subsequent toxic side effect.

NM *in vitro* release conditions	Zero-order	First-order	Higuchi	Korsmeyer-Peppas	Hixson-Crowell n-value	n-value
37°C, pH 6,8	0,592	0.711	0.812	0.781	0.671	0.482
37°C, pH 7,4	0,652	0.750	0.845	0.830	0.712	0.461
37°C, pH 1,2	0,891	0.901	0.971	0.961	0.894	0.531

Table 1. Drug release kinetics results for several models of duloxetine-loaded nanoparticles for targeted drug delivery. **Abbreviation:** NM-nanoparticles.

Kinetic modelling of drug release of the DLX-loaded EUD-CHT nanoparticles

Drug release kinetics were determined using the Zero-order release model, First-order release kinetics, Fick's law of diffusion, Korsmeyer-Peppa's equation, and Hixson-Crowell equations. The obtained data from drug release studies at pH 6.8, 7.4 and 1.2 at 37 °C was used in the above equations to determine the values in Table 1. The release profile of the DLX-loaded EUD-CHT nanoparticles over 24 hrs was observed to display a controlled release profile attributed to the dissolution of the DLX-loaded EUD-CHT nanoparticles. Higuchi's model best describes the release of DLX from the nanostructure. DLX was released through the disintegrated outer hydrophobic Eudragit layer at 37 °C, dependent on the square root of time according to Fick's law of diffusion. At 6.8, 1.2, and 7.4, Korsmeyer-Peppa's model clearly defined the release kinetics.

According to the Korsmeyer-Peppas model, if the release power coefficient (n value) is more significant than 0.45 and smaller than 0.89, it implies that the therapeutic release is due to anomalous transport behavior (Non-Fickian diffusion). In contrast, $n < 0.45$ means that the drug release was due to Fickian diffusion. For release from the DLX-loaded EUD-CHT nanoparticles, the n value was close to 0.45 at both pH values, implying that the drug release was mostly due to Fickian diffusion. DLX was released from the inner core by the disintegrated Eudragit, whose disintegration is temperature controlled, consistent with Higuch's square root law. There was no distinguished effect of pH on the release behavior kinetics. This suggests that the DLX-loaded EUD-CHT nanoparticles provided favorable release of the hydrophobic drug, through a proposed GIT membrane, for a hypothesis of improved penetration across the BBB.

Evaluation of Cytotoxicity of the DLX-loaded EUD-CHT nanoparticles utilizing HEK 293 cells

A cytotoxicity assay was conducted to evaluate the impact of the DLX-loaded EUD-CHT nanoparticles on the viability of HEK-293 cells. After 48 hrs of treatment with duloxetine HCL, the DLX-loaded EUD-CHT nanoparticles and blank nanoparticles at concentrations of 1.5, 3, and 6 µg/mL; bright field microscopic images were captured (at 10X), and an MTT assay was conducted as described above. Fig. 13 depicts a graph for the MTT assays for plain duloxetine HCL, the DLX-loaded EUD-CHT nanoparticles, and blank nanoparticles. Fig. 14. Displays light microscopic images of the HEK 293 cells treated with a) Untreated cells, b) 1.5 µg/mL DLX-loaded EUD-CHT nanoparticles, c) 3 % DMSO, d) Plain duloxetine drug and e) 1.5 µg/mL blank nanoparticles. All treated and untreated cells show a comparable pattern of cell viability. As expected, the positive control cells treated with 3 % DMSO displayed high cytotoxicity. On the graph, the plain duloxetine HCL drug shows a dose-dependent effect, in that at 1.5 µg/mL and 3 µg/mL, cell viability was high, the assay was 131 % and 125 % compared with untreated cells (100 %), and at 6 µg/mL duloxetine concentration, the assay shows a decline at 98 %. The DLX-loaded EUD-CHT nanoparticles showed increased cell viability at 140, 119 and 155 for the 1.5, 3 and 6 µg/mL concentrations. The blank nanoparticle treatment slightly inhibited growth compared with untreated cells. he DLX-loaded EUD-CHT nanoparticles and blank nanoparticles at 3 % concentration showed less cell viability than at lower (1.5 µg/mL) and higher (3 µg/mL) doses. Cells treated with 3 % DMSO were used as a positive control and showed low viability of 2 %. The DLX-loaded EUD-CHT nanoparticles depicted an increase in cell viability compared to plain duloxetine. This suggested that the

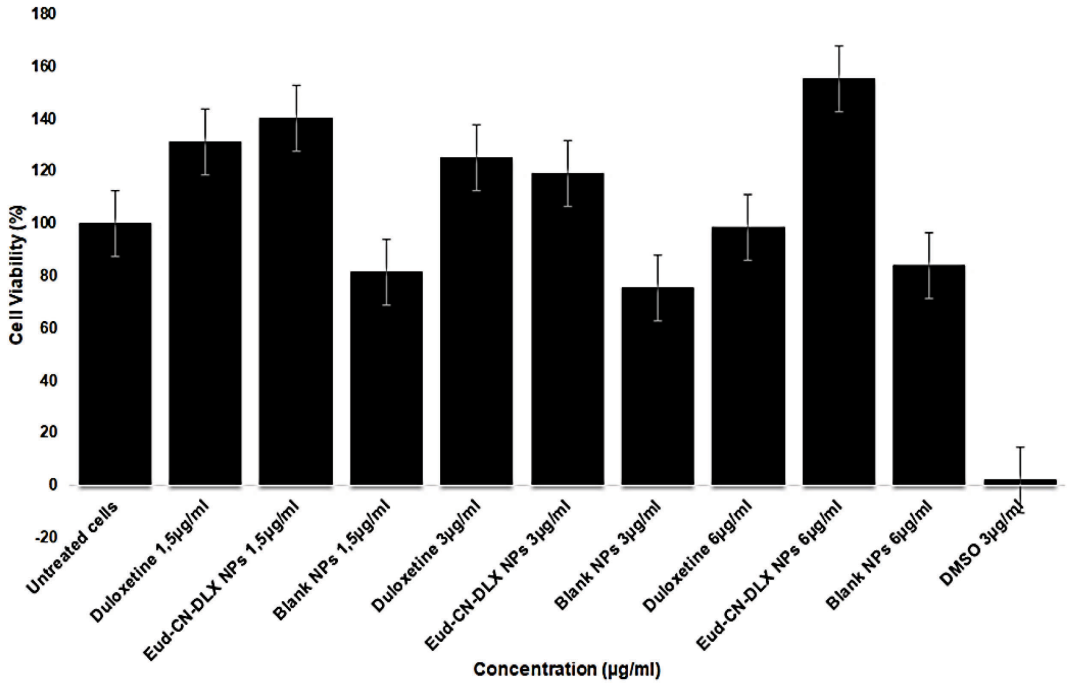

Figure 13. Tetrazolium salt MTT assay for evaluating the outcome of duloxetine-loaded nanoparticles (DLX-loaded EUD-CHT nanoparticles), blank nanoparticles and duloxetine plain drug on percentage viability of HEK 293 cells. The cells treated with various formulations were incubated for 48 hrs before evaluation of cell viability. Each percentage cell viability point represents average ±SD (n=3)

DLX-loaded EUD-CHT nanoparticles are less cytotoxic than the pure drug, as reported previously (Jafari et al., 2016). The data indicate that the DLX-loaded EUD-CHT nanoparticles can be an effective and safe carrier for the delivery of duloxetine to the brain. Moreover, a lower cytotoxic profile was observed with the nanoparticles compared to the plain duloxetine HCL (Jafari et al., 2016).

Conclusions

In this study, a DLX-loaded EUD-CHT nanosystem was formulated to demonstrate sustained release of DLX over 24 hours. In acidic pH, DLX release from the nanosystem reached 20 % after 24 hours, suggesting pH-dependent DLX release with gastric protection. TGA and DSC analysis revealed desirable thermal stability of the DLX-loaded EUD-CHT nanosystem compared to native DLX. MTT analysis demonstrated that the DLX-loaded EUD-CHT nanosystem was non-toxic to HEK cells. Molecular modeling confirmed that the nanosystem generates a cationic surface at the nanosystem generates a cationic surface imparted by EUD's quaternary ammonium moiety, which may enhance cell recognition and uptake at anionic cell membranes. Furthermore, the SwissADME assessment of EUD indicated the potential for enhanced oral absorption and transport of DLX to the BBB for superior neuro availability. This study's results strongly suggest that the DLX-loaded EUD-CHT nanosystem can provide better DLX bio- and neuro-availability after oral administration. The next phase of this study will focus on preclinical *in-vivo* pharmacokinetics/pharmacodynamics evaluation of DLX after EUD-CHT nanosystem oral dose.

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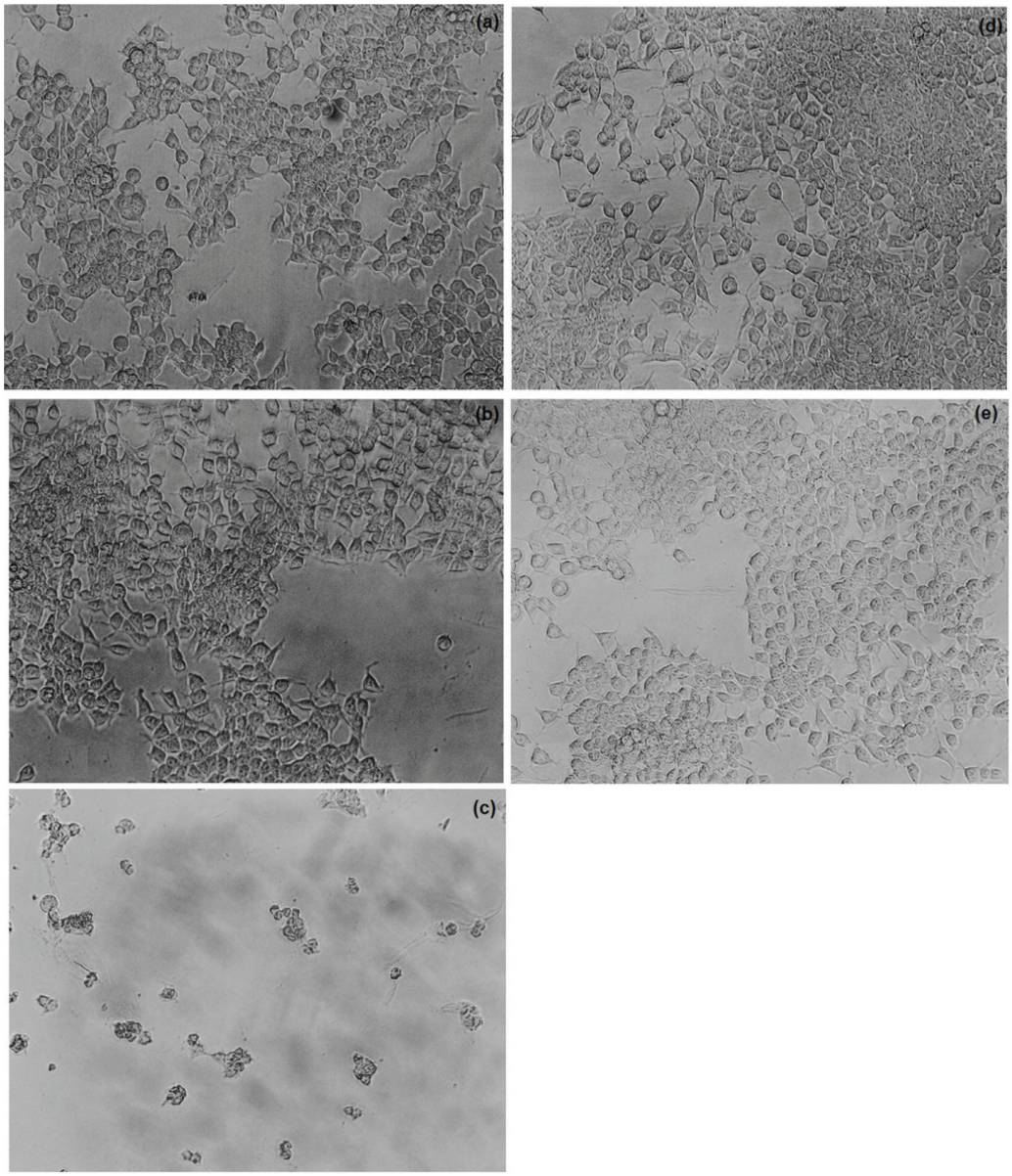

Figure 14. Olympus Inverted Microscope Images of HEK cell growth after treatment with **(a)** Untreated cells, **(b)** 1.5 % DLX-loaded EUD-CHT nanoparticles, **(c)** DMSO 3 %, **(d)** Plain duloxetine drug, **(e)** 1.5 l % blank nanoparticles.

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