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Multifunctional Hybrid Nano-Platform for Co-Delivery of Erlotinib and Silibinin: A Nanotherapeutic Strategy Against Triple-Negative Breast Cancer

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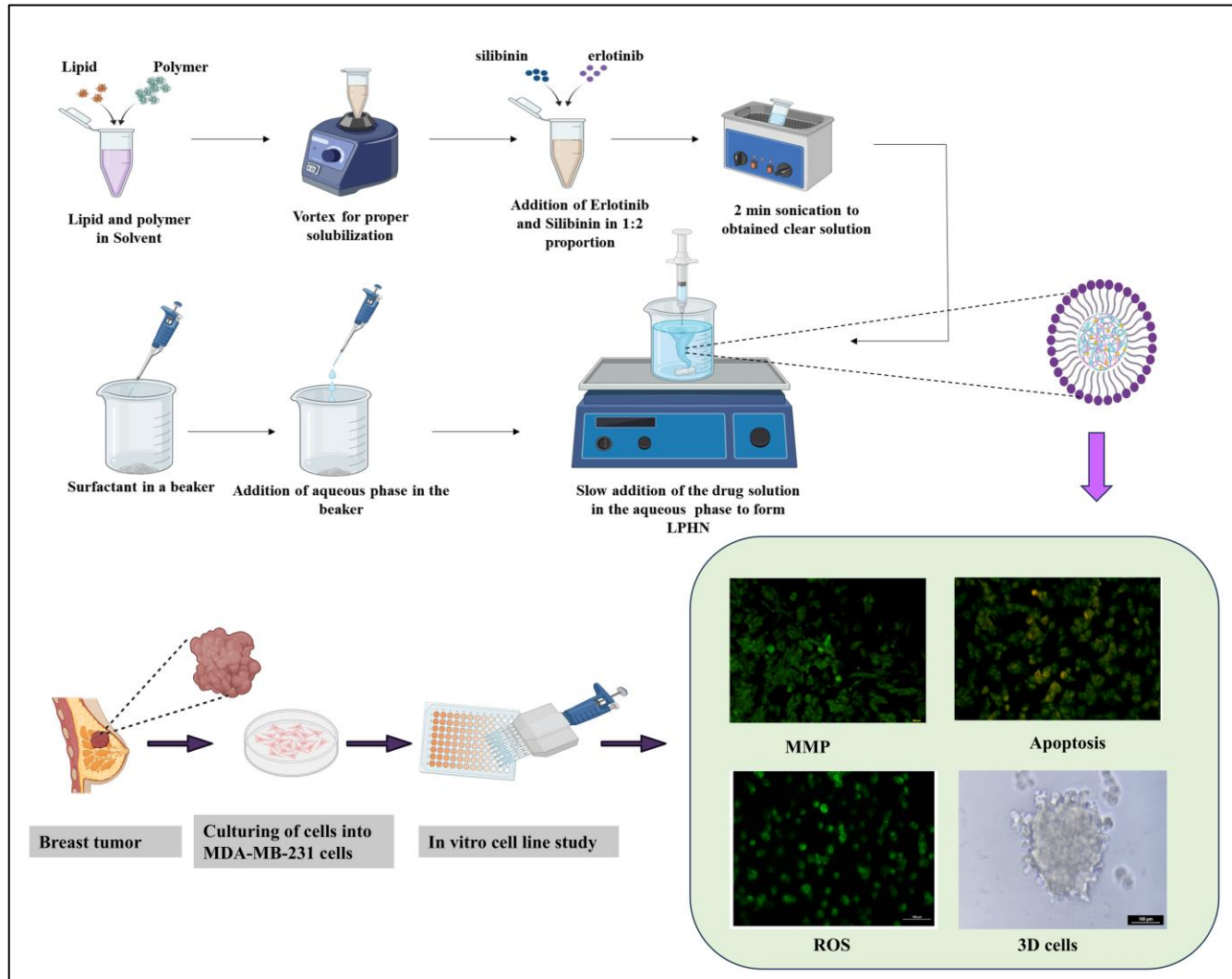
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Abstract

Triple-negative breast cancer (TNBC) is associated with high mortality due to the lack of hormonal receptors necessary for targeted therapies. However, the prevalent overexpression of basal biomarkers, such as EGFR, provides an opportunity to explore alternative treatment options. In this research investigation, we designed and fabricated erlotinib (ERL), an EGFR tyrosine kinase inhibitor, alongside silibinin (SLB), a phytoconstituent known for its additional anticancer as well as its antioxidant, anti-inflammatory, and antimetastatic effects. These agents were encapsulated in lecithin-PLGA hybrid nanoparticles (ERL-SLB-LPHN) to enhance therapeutic outcomes in TNBC. The fabrication of ERL-SLB-LPHN was optimized using the Box-Behnken design, resulting in a formulation with a particle size of 160.13 ± 0.90 nm, a PDI of 0.13 ± 0.03 , and percent encapsulation rates of ERL at $63.18 \pm 8.2\%$ and SLB at $98.21 \pm 0.12\%$, respectively. The *in vitro* release study showed controlled release behaviour of both drugs from the nanoparticles, and the stability data indicated that the ERL-SLB-LPHN is stable at 2-8°C. Further hemocompatibility studies of the formulation were conducted, indicating good biocompatibility. Furthermore, an *in vitro* cell culture study found that ERL-SLB-LPHN had an IC_{50} of 7.99 $\mu\text{g/ml}$ and exhibited higher cellular uptake than the free ERL and SLB. MMP, Apoptosis, and DCFDA staining analysis revealed that, as compared to the free drug, ERL-SLB-LPHN showed higher apoptosis and ROS production. An *in vivo* pharmacokinetic study revealed 1.49- fold and 1.35-fold enhancement in AUC_{0-t} with ERL-LPHN and SLB-LPHN, compared to free ERL and SLB, respectively. The toxicological study suggested that ERL-SLB-LPHN exhibits a safe toxicological profile. To summarize, the hybrid nanoparticle enhanced the drug's therapeutic potential and anticancer efficacy.

Keywords: Erlotinib hydrochloride, Lipid-polymer hybrid nanoparticles, Silibinin, Box-Behnken design, Pharmacokinetics, Toxicities.

Graphical abstract



Abbreviations

Human growth factor receptor 2: HER2

Epidermal growth factor receptor: EGFR

Poly (lactic-co-glycolic acid): PLGA

Lipid-polymeric hybrid nanoparticles: LPHN

Erlotinib: ERL

Silibinin: SLB

Box-Behnken design: BBD

Polydispersity index: PDI

Percent entrapment efficiency: %EE

Fourier transform infrared spectroscopy: FTIR

Powder X-ray diffraction: PXRD

ERL-SLB-physical mixture: ERL-SLB-PM

High-performance liquid chromatography: HPLC

2,2-diphenyl-1-picrylhydrazyl: DPPH

3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide: MTT

Inhibitory concentration: IC₅₀

Mitochondrial membrane potential: MMP

Acridine orange: AO

Ethidium bromide: EtBr

4',5-Diamidino-2-phenylindole dihydrochloride: DAPI

1. Introduction

Cancer, the second leading cause of mortality worldwide, is projected to reach 13.1 million by 2030, as per the WHO [1, 2]. Cancer cells can grow abnormally in any organ and may migrate to other organs, making the cancer highly challenging to treat [3]. Furthermore, cancer can affect both men and women. Amongst women, breast cancer has been the most prevalent malignancy to date, and it also exhibits considerable heterogeneity in its properties [4, 5]. Based on the expression of biomarkers, breast cancer can be categorised into hormone receptor-positive, HER2-positive, and triple-negative breast cancer (TNBC). TNBC is particularly aggressive, exhibits a lack of biomarkers, and is associated with poor survival outcomes [6, 7]. The most commonly available treatments for breast cancer are surgery, chemotherapy, and radiation therapy [8]. However, traditional therapies have certain limitations, such as destruction of cells in bone marrow, compromised immune function, gastrointestinal ulceration, and hair loss[9]. TNBC does not respond to hormonal therapy and therapies used to target the hormone and HER2 receptors, and is considered a highly aggressive and metastatic type of cancer [10]. To address these challenges, researchers are finding advanced treatments such as targeted drug delivery involving nanoparticles to avoid the interaction of the drug with other healthy tissues. For the delivery of chemotherapeutic payload, drug delivery systems like liposomes, micelles, and polymeric nanoparticles are used for tumor targeting, increasing efficacy, and reducing the side effects [11]. TNBC exhibits basal biomarkers, such as cytokeratin and epidermal growth factor receptor (EGFR) [12]. EGFR, a regulator of key signalling pathways such as RAS/MAPK, PI3K/AKT, JAK/STAT, and PLC- γ , is a member of the c-erbB tyrosine kinase receptor family. These pathways regulate cell proliferation, survival, invasion, and migration [13]. EGFR consists of three main part extracellular domain, the hydrophobic transmembrane part, and the intracellular domain [14, 15]. When ligand EGF binds to the extracellular domain of the EGFR, conformational changes lead to dimerization of the receptor, which in turn allows the attachment of a phosphate group to the intracellular domain, leading to phosphorylation. After phosphorylation, the dimer serves as a docking site for the various effector proteins, triggering multiple intracellular signalling pathways [16].

Lipidic and polymeric nanoparticles are considered two important drug delivery approaches in tumor-targeted therapy [17]. Polymeric nanoparticles and liposomes each offer distinct advantages of their own. Polymeric nanoparticles are widely used due to their greater structural integrity, reduced drug leakage, and controlled release. Polymeric nanoparticles can be synthesised by using natural as well as synthetic biocompatible and biodegradable polymers.

Chitosan is a natural polymer, while poly(lactic-co-glycolic acid) (PLGA) is an example of a synthetic polymer [18]. Polymeric nanoparticles show certain limitations, such as lower encapsulation efficiency for hydrophilic drugs, biocompatibility issues, and toxicity issues related to the degradation of polymers. Lipidic nanoparticles are considered the most effective drug delivery system compared to polymeric nanoparticles. Lipidic formulation closely resembles the biological membrane and is made up of either natural or synthetic phospholipids. The lipidic system possesses certain limitations related to structural integrity, resulting in drug leakage and stability issues during storage [19]. In recent times, lipid-polymeric hybrid nanoparticles (LPHN) have gained significant popularity in the nano drug delivery system. LPHN nanoparticles consist of an internal polymeric core surrounded by a lipidic layer. The hydrophobic polymeric core is used to incorporate the poorly water-soluble drug. Control drug release due to the presence of a polymeric matrix and provides structural integrity to the formulation. The lipid layer enhances cellular uptake by endocytosis and biocompatibility [20]. EGFR receptor-targeting drug erlotinib (ERL) has been approved by the FDA for the treatment of non-small cell lung cancer and pancreatic cancer under the brand name Tarceva® [21]. Prior research has revealed that ERL possesses notable efficacy in inhibiting TNBC [22, 23]. Like most anticancer drugs, ERL exhibits hepatotoxic effects in this study. In this study, ERL was used with the hepatoprotectant silibinin (SLB) obtained from *Silybum marianum* (milk thistle plant). Along with hepatoprotection, SLB possesses antioxidant, anti-inflammatory, and antimetastatic effects as well [24, 25]. The polymer used for the fabrication of the carrier system, i.e., PLGA, is a biodegradable polymer that hydrolyses into lactic and glycolic acid monomers. These metabolites are endogenous and easily metabolized in the body [26]. Egg lecithin is used as an outer layer to cover the PLGA polymer. It contains a phospholipid that mimics the biological membrane, facilitating the easy internalization of the nanoparticles through the cell membrane [27]. The primary aim of this study is to fabricate lipid-polymer hybrid nanoparticles and encapsulate ERL and SLB (ERL-SLB-LPHN) within them to enhance receptor targeting, reduce dose and side effects, and improve bioavailability. The formulation of ERL-SLB-LPHN was optimized using the Quality by Design (QbD) framework, which emphasizes a systematic approach to drug development. This optimization process involved meticulous evaluation of several critical parameters, including particle size, zeta potential, morphological characteristics, encapsulation or entrapment efficiency, and drug release profiles in vitro.

Furthermore, we conducted comprehensive anti-cancer studies, examining not only the apoptotic effects of the LPHNs but also their influence on MMPs assay and DCFDA assay, which are important in tumor progression and metastasis. The efficacy of the ERL-SLB-LPHN formulation was thoroughly assessed using three-dimensional (3D) cell-based assays, specifically TNBC cell lines, such as MDA-MB-231. These investigations aimed to establish a deeper understanding of the therapeutic potential of ERL and SLB when delivered via LPHNs, providing insights into their mechanisms of action and overall effectiveness in combating TNBC.

2. Materials and methods

2.1. Chemicals and solvents

Erlotinib was supplied as a gift sample from Zydus (Hyderabad, India). SLB, Egg Lecithin (L- α -Phosphatidylcholine), and dialysis membrane (mol. Wt. cut-off 10kDa) were purchased from Sigma-Aldrich (USA). Tween 80 was obtained from HiMedia Laboratories (India), and Acetonitrile (HPLC grade) and methanol were purchased from Actylis Lab Solution (India). PLGA was procured from Ashland India Pvt. Ltd (India). Ethidium bromide (EtBr) and acridine orange (AO) were sourced from Sisco Research Laboratories Pvt. Ltd., India. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 4',5-Diamidino-2-phenylindole dihydrochloride (DAPI HCl) were purchased from HiMedia Laboratories Pvt. Ltd, India.

2.2. Preparation of ERL-SLB-LPHN

ERL-SLB-LPHN nanoparticles were prepared using a single-step nanoprecipitation method, following a previously reported protocol with minor modifications [28]. In brief, 40 mg of PLGA polymer and 12 mg of egg lecithin (phospholipid) were dissolved in a mixture of acetonitrile and methanol (1:1) to create the organic phase. The drugs, ERL and SLB, were added to this organic phase in a 1:2 ratio, followed by sonication to obtain a clear solution. For the aqueous phase, Tween 80 (0.5-1%) was solubilized in Milli-Q water. The organic phase was then added dropwise using a 2 mL syringe into the aqueous phase, maintaining a constant addition rate under continuous stirring at 1000 rpm and a temperature of 25-30 °C. The mixture was further stirred continuously at 1000 rpm at room temperature for 4 h to allow for the evaporation of organic solvents. Similarly, coumarin-6 (C-6) loaded nanoparticles were formulated following the same protocol, with the substitution of 1 mg of C-6 in place of ERL and SLB.

2.3. Optimization of ERL-SLB-LPHN

2.3.1. Taguchi design for formulation risk evaluation

To screen the process variables affecting the critical quality attribute of ERL-SLB-LPHN, a Taguchi design was utilized. The batches were formulated by varying seven different factors at two levels each to distinguish the parameters that are critical for the properties of the dosage form. The impact of these factors on the responses was interpreted using half-normal probability plots and Pareto charts.

2.3.2. Box-Behnken design (BBD)

Optimization was performed through a statistical approach using Design-Expert® (Version 10.0.1, State-Ease Inc., Minneapolis, USA). For the optimization of the ERL-SLB-LPHN, a four-factor, three-level Box-Behnken experimental design was utilized along with response surface methodology. In comparison with the central composite design, BBD requires fewer experimental runs and offers an effective way to estimate both linear and quadratic effects. The early screening phase highlighted that the change in particle size, polydispersity index (PDI), and entrapment efficiency of dual drug-loaded nanoparticles was mainly influenced by the four factors, such as organic phase, total drug content, surfactant concentration, and lipid content. Each independent variable was coded as A (organic phase), B (Total drug content), C (surfactant concentration), and D (lipid), and each was assessed with three levels: -1, 0, and 1. The values selected for the experimental design were A = 2.25 ml, B = 3.75 mg, C = 0.75 %, and D = 7.5 mg. **Table 1** represents low, medium, and high levels of independent variables, dependent variables, and desired outcomes of the experiment. The regression models were used to independently analyse each formulation trial's result, and analysis of variance ANOVA was used to assess each factor and model terms' statistical significance, which helped in identifying the main variables affecting the crucial quality attributes [29].

Table 1: Independent and dependent variables for optimization of ERL-SLB-LPHN

Independent variables	LEVELS		
	Low (-1)	Medium (0)	High (+1)
Organic Phase ml (acetonitrile: methanol) (1:1)	1.5	2.25	3
Total drug content (mg)	1.5	3.75	6
Percentage surfactant concentration	0.5	0.75	1
Lipid (mg)	3.75	7.5	11.25
Dependant variables	Desired Outcome		

Mean particle size (nm)	Minimize
PDI	Minimize
Percentage of ERL entrapment	Maximize
Percentage of SLB entrapment	Maximize

2.4. Characterization of ERL-SLB-LPHN

2.4.1. Mean particle size and PDI

Mean particle size of the carrier system plays an important role in cellular uptake, biodistribution, and tumor accumulation. Smaller particles tend to aid cellular internalization, whereas larger particles are cleared rapidly and have poor tissue penetration. PDI is an indicator of the distribution of particles around the mean particle size, with lower PDI indicating homogeneity of the system. The mean particle size and PDI of the prepared ERL-SLB-LPHN were evaluated by using the Zetasizer Nano-ZS 90 (Malvern Instruments, England, UK), which operates on the principle of dynamic light scattering. Before analysis, samples were diluted with Milli-Q water and transferred to disposable polystyrene cuvettes for analysis. The scattered light intensity was measured at a 90° angle, with all measurements implemented at 25°C, followed by a 120-s equilibration period. Each sample was subjected to three analyses, and the mean diameter was reported as the outcome [30].

2.4.2. Percent entrapment efficiency (%EE)

The indirect method was used to measure the amount of unencapsulated drug via ultrafiltration in order to evaluate the entrapment efficiency of nanoparticles. The ultrafiltration method was employed for the separation of the free drug from the nanoparticle based on the principle of permeation of free drug through the ultrafiltration membrane Amicon® Ultra-4 centrifugal filter tubes (Millipore Corp., USA). In contrast, nanoparticles will remain in the upper compartment. The 10 kDa molecular weight membrane was used for filtration, and materials were centrifuged for 15 min at 12,000 RPM. The obtained ERL and SLB were subjected to analysis to determine the quantity of unencapsulated drug, using reverse-phase high-performance liquid chromatography (RP-HPLC) (Shimadzu Corp, Tokyo, Japan). The ERL and SLB drugs were quantified using a UV-Vis detector at wavelengths of 289 nm for SLB and 246 nm for ERL.

The following equation calculates the percentage EE and percentage drug loading

$$\text{Percent EE} = \frac{\text{Amount of Entrapped drug}}{\text{Total amount of drug added}} \times 100$$

$$\text{Percent drug loading} = \frac{\text{Amount of entrapped drug}}{\text{Amount of total solid content}} \times 100$$

2.4.3. Surface morphological characterization

Characterization of the surface morphology of an LPHNP aids in confirming the structural features such as core-shell structure, shape, surface smoothness, stability, and aggregation behaviour. These features greatly influence the biological interaction of the carrier system within the body. Surface morphology and structural integrity of ERL-SLB-LPHN were examined by using transmission electron microscopy. JEM-1400 (JEOL, Tokyo, Japan) transmission electron microscope was utilized for analysis at an accelerating voltage of 100 kV with $5,00,000 \times$ magnification. A copper grid covered with carbon was meticulously deposited with 10 μ l of nanoparticle dispersion. Phosphotungstic acid was used to negatively stain the samples to improve visibility and promote particle adhesion. The grids underwent imaging after a quick air-drying to enable the development of a homogeneous layer [31].

2.4.4. Fourier transform infrared spectroscopy analysis (FTIR)

The FTIR analysis of ERL, SLB, ERL-SLB-physical mixture (ERL-SLB-PM), ERL-SLB-LPHN, PLGA, and lecithin was performed to gain insight into any possible drug-polymer interaction and encapsulation of the drug in nanoparticles. The FTIR (Spectrum 2, Perkin Elmer, USA) instrument was utilized for this analysis. The scanning was conducted over the wavenumber range of $4000\text{--}400\text{ cm}^{-1}$, and the collected spectra were subsequently processed using Microlab software [32].

2.4.5. Powder X-ray diffraction analysis (PXRD)

The physical state of ERL-SLB-LPHN was examined to determine its crystalline or amorphous nature and compared with the free drugs ERL and SLB to confirm their successful incorporation in nanoparticles. ERL, SLB, ERL-SLB-PM, ERL-SLB-LPHN, and PLGA were analyzed using a Bruker D8 Advance diffractometer (Bruker AXS GmbH, Germany). Cu-K α radiation ($\lambda = 1.5406\text{ \AA}$) was used for the analysis, which was conducted at a voltage of 30–40 kV and a current of 40 mA. The scan was performed at room temperature throughout a 2θ range of 4° to 40° . Where applicable, alumina powder was used for calibration, and the powdered material phase distribution was analysed by the Bragg-Brentano geometry [33, 34].

2.4.6. *In vitro* Drug release

The *in vitro* drug release study was conducted to check the release profile of the drug from the nanoparticles by using the 12 kDa dialysis membrane (HiMedia, India). The release profile of

free drugs ERL, SLB, and ERL-SLB-LPHN, which is equivalent to 1mg of ERL and 2mg of SLB, respectively, was evaluated. In brief, the sealed dialysis bag, which is a dialysis membrane loaded with either free drug solutions or a drug-loaded formulation, was submerged in release media (phosphate buffer pH 7.4 with 1% Tween 80) and placed in the orbital shaker operating at 100 rpm [35]. The temperature was maintained at 37 ± 0.5 °C throughout the experiment. When 1 mL of the sample was withdrawn from the media at various time intervals (0, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h), an equal volume of fresh media was added to it to maintain the sink condition. The withdrawn samples were then analysed using HPLC, which provided precise quantification of drug release of free drug and ERL-SLB-LPHN over time. The drug release data were investigated using multiple kinetic models such as first-order, Higuchi's, Korsmeyer Peppas, and zero-order models. A linear analysis was performed to select the model that most accurately fits the release profile.

2.4.7. Hemolysis study

The haemolysis study was conducted to assess the impact of the nanoformulation on red blood cells, thereby predicting the biocompatibility of both the drug and the polymer. Blood samples were collected from the mice in a trisodium citrate tube as an anticoagulant. These collected blood samples were then centrifuged to obtain a pellet of red blood cells, which were then washed thrice with phosphate buffer and again re-suspended in the phosphate buffer at pH 7.4. Then, the prepared red blood cell suspension was incubated with free ERL, free SLB, a physical mixture of both drugs, and ERL-SLB-LPHN at varying concentrations. Red blood cell suspension treated with Triton X-100 (0.1%) served as a positive control, whereas those treated with phosphate buffer only were considered as a negative control. Incubation was done for two separate time intervals, i.e., 2 and 4 h. After the incubation period, the intact red blood cells were separated by centrifugation at 1500 rpm for 10 min at 4°C, and the supernatant containing the released haemoglobin due to lysis from red blood cells was analysed at 570 nm using EnVision Xcite Multimode Plate Reader (Waltham, USA) to estimate percent hemolysis [36].

$$\text{Percent hemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control} - \text{Absorbance of negative control}} \times 100$$

2.4.8. Antioxidant assay

The antioxidant potential assay of SLB, a phytoconstituent used in the formulation, was performed to check its radical scavenging property. The procedure was followed as reported in the literature, with minor modifications [37]. In the presence of an antioxidant, DPPH converts to DPPH-H by taking a hydrogen atom from the antioxidant. This reaction leads to a change in

the deep purple colour of DPPH to a light-yellow colour. This results in a decrease in absorbance, indicating the free radical scavenging activity of the nanoparticles.

$$\text{Percent radical scavenging activity} = 1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.4.9. Stability studies

2.4.9.1. Storage stability

The stability assessment study of ERL-SLB-LPHN was conducted under varying temperature conditions to determine the optimum storage condition for the formulation. The samples were stored at $25 \pm 2^\circ\text{C}$ and $5 \pm 2^\circ\text{C}$, and periodic analysis was conducted at set time points to identify variations in parameters, including particle size, PDI, and entrapment. The obtained results were compared with the initial values.

2.4.9.2. Stability in 10% fetal bovine serum (FBS)

To imitate *in vivo* conditions, a serum stability analysis was performed on the developed ERL-SLB-LPHN intended for parenteral administration. The study looked at how serum elements such as protein, growth factors, hormones, and vitamins affect the structural integrity of nanoparticles. ERL-SLB-LPHN nanoparticles were incubated with 10 % FBS at $37 \pm 0.5^\circ\text{C}$ for 24 h. Particle size and PDI were measured at predefined intervals to detect changes caused by nanoparticle aggregation.

2.4.9.3. Antidilution study

Nanoparticles undergo substantial dilution in the bloodstream after being administered intravenously. An antidilution test was performed to determine the robustness of nanoparticles under such settings. The ERL-SLB-LPHN were diluted in phosphate buffer (pH 7.4) at different levels, ranging from (10-50)-fold dilutions. The change in average particle size and PDI was determined. This provided insights into the potential for aggregation or destabilization when exposed to a large-volume fluid.

2.4.10. *In vitro* cell culture study

2.4.10.1. Cytotoxicity study

To detect cell viability, the enzymatic metabolic activity of cells was measured by their ability to convert MTT (3,4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide) Into formazan. The MTT assay was used to determine the cytotoxicity of ERL, SLB, and ERL-SLB-LPHN.

Cancer cells were plated in a 96-well plate at a density of 5000 to 10000 cells per well and incubated overnight at $37^{\circ}\pm 0.5$ °C with 5 % CO₂ to promote cell adhesion. The cell culture media were replenished the next day with different concentrations (3.125-50 µg/ml) of ERL, (6.25-200 µg/ml) of SLB, and (4.68-150 µg/ml) of ERL-SLB-PM and ERL-SLB-LPHN. The cells were subsequently incubated for 24-48 h. After the treatment period, the media was removed, and MTT solution was added to each well (0.5 mg/ml in RPMI). The formed formazan crystals from MTT after a 4 h incubation period were dissolved in 100-200 µl of dimethyl sulfoxide. The absorbance was taken at 570 nm by using a multimode plate reader (Agilent, Biotek, USA). Cell viability was compared to that of the untreated control and the inhibitory concentration. IC₅₀ values were calculated from the equation mentioned below. Results were reported as mean $\pm$ standard deviation [38].

$$\text{Percent cell inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.4.10.2. Cellular uptake study

A fluorescence-based uptake analysis was performed on MDA-MB-231 cells to assess the efficiency of ERL-SLB-LPHN internalization. Cells were seeded into 6-well plates at a density of 5×10^4 cells per well and incubated overnight at 37 °C to ensure adherence. Subsequent incubation, cells were subjected to either C-6-loaded LPHN or free coumarin-6 (equal to 2 µg/ml dye) for 2 h. After treatment, the wells were gently rinsed with phosphate buffer saline to remove the remaining formulation. Cells were then treated with 4 % paraformaldehyde to maintain their morphology. Fluorescence microscopy was used to visualize uptake, which allowed a qualitative examination of intracellular fluorescence as an output of nanoparticle penetration into cells.

2.4.10.3. Scratch test

A wound-healing test was used to assess how ERL, SLB, and ERL-SLB-LPHN affect MDA-MB-231 cell migration. After being seeded in a 6-well plate at a density of 1×10^5 cells per well, the cells were left to adhere for 12 h. The following day, a linear scratch was made using a sterile 200 µl pipette tip, guided by a reference mark. Then, cells were treated with ERL, SLB, and ERL-SLB-LPHN. After 24 h of incubation, cell migration into the wound area was observed under an inverted fluorescence microscope (T 12-S-HU, Nikon, Japan) and compared with the reference mark [39].

2.4.10.4. Mechanism of cellular uptake

The process of the endocytic pathway and receptor-mediated endocytosis of the formulated ERL-SLB-LPHN nanoparticles was examined in MDA-MB-231 cells. MDA-MB-231 cells were cultured on cover slips that were placed in a 6-well plate for 24 h. Following this initial treatment, the cells were exposed to various inhibitors that target distinct endocytic pathways. A concentration of 20 µg/mL of chlorpromazine was used to inhibit clathrin-mediated endocytosis. Colchicine, at a concentration of 8 µg/mL, was applied to obstruct macropinocytosis. Indomethacin was introduced at a concentration of 6 µg/mL to interfere with caveolae-mediated endocytosis, while quercetin, also at 6 µg/mL, acted as an inhibitor for both caveolae- and clathrin-independent endocytosis. Moreover, sodium azide was added at a concentration of 10 mM to inhibit the electron transport chain. The cells were treated with these inhibitors for 2 h, providing adequate time for the inhibitors to impact the endocytic pathways. After the incubation period, the cells were rinsed twice with PBS to eliminate any remaining inhibitors from the culture medium. The cells were then treated with C₆-LPHN nanoparticles for 4 h at 37°C to promote the uptake of nanoparticles through the various endocytic pathways. After incubation with the nanoparticles, all cells were washed again with PBS to remove uninternalized particles and then fixed with 4% paraformaldehyde to maintain cellular structure. The fluorescence of the internalized nanoparticles was later examined using a fluorescence microscope, capturing images under C₆ fluorescence channels to evaluate the uptake and localization of the nanoparticles within the cells.

2.4.10.5. Mitochondrial membrane potential assay (MMP)

The MMP assay is an evaluation of overall mitochondrial function revolving around the electrochemical gradient across the inner mitochondrial membrane. This assay is used to determine whether a treatment induces cell death via intrinsic mitochondrial pathways or not. In simple terms, it is used to assess anticancer activity by utilizing early indicators of apoptosis. The MMP assay was performed to assess the effect of ERL-SLB-LPHN on MMP in the MDA-MB-231 cell line, as per previously reported literature, with slight modifications [40]. JC-1, a lipophilic cationic dye, was used in this study due to its sensitivity to alterations in mitochondrial polarization. In cells with undamaged and functional mitochondria, JC-1 accumulates within the organelles, forming aggregates that emit red fluorescence. In contrast, the JC-1 dye remains monomeric in the cytoplasm of defective mitochondrial cells and emits green fluorescence. In brief, MDA-MB-231 cells were plated at a density of 0.1×10^6 cells per well in a 12-well plate and adhered overnight. On the subsequent day, they were subjected to

treatments such as ERL, SLB, ERL-SLB-LPHN, and untreated control, all at their respective IC_{50} values, for 24 h. Cells were treated with JC-1 DYE (10 μ g/ml in phosphate buffer) for 30 min in the dark. Following the staining, fluorescence was examined using an inverted fluorescent microscope (T 12-S-HU, Nikon, Japan) to detect a change in MMP.

2.4.10.6. Apoptosis assay

AO and EtBr staining were used to detect apoptotic alterations in MDA-MB-231 cells, discriminating between living and apoptotic cells based on fluorescence. AO stains both live and dead cells and emits green fluorescence, while EtBr emits red fluorescence after being selectively stained to damaged cells by reacting with the phosphatidylserine marker for ruptured membranes. Cells were seeded in a 12-well plate at a density of 0.3×10^6 cells per well and incubated overnight to promote adherence. The next day, cells were treated with ERL, SLB, and ERL-SLB-LPHN, and the respective IC_{50} values were determined, with a control group left untreated. After treatment, the wells were washed twice with phosphate buffer to remove residue, and then 10 μ l of AO/EtBr solution (10 mg/ml in phosphate buffer) was applied. Cells were immediately observed using an inverted fluorescence microscope (T 12-S-HU, Nikon, Japan) with a 480/30 nm excitation filter. The red-to-green fluorescence ratio was calculated to assess the amount of apoptosis induced by treatment [41].

2.4.10.7. DAPI staining assay

To assess nuclear alteration indicative of apoptosis, MDA-MB-231 cells were subjected to DAPI staining following treatment with free drugs and nanoparticles. In each well of a 6-well plate, about 1×10^6 cells were seeded and incubated to adhere. Then, cells were treated with the free drug formulation at the IC_{50} dose. After the following treatment, cells were fixed with a methanol-acetic acid mixture at 4 °C for 30 min. After being rinsed with phosphate buffer, fixed cells were stained with DAPI 1 μ g/ml. Then, the excess stain was removed with PBS and examined for chromatin condensation and nuclear fragmentation using a 40 $\times$ objective and DAPI filter under an inverted fluorescence microscope

2.4.10.8. DCFDA staining assay

Levels of intracellular ROS were measured in the MDA-MB-231 cell line using the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay. MDA-MB-231 cells were plated into a 12-well plate at a density of 1×10^5 cells per well and incubated overnight under standard culture conditions (37 °C, 5% CO₂). After attachment, the cells were treated with the following: control (untreated), ERL, SLB, ERL-SLB-PM, and LPHNP. Cells were treated at the predetermined

concentrations and incubated for 24 h. After treatment, the medium was discarded, and the cells were washed twice with PBS to remove any remaining drug or formulation. Cells were then incubated with 10 μ M DCFH-DA diluted in serum-free medium for 30 min at 37 °C in the dark, followed by two washes with PBS to reduce background fluorescence. Intracellular ROS generation was visualized using a fluorescence microscope. Images from at least 10 random fields per well were captured at the same exposure settings for quantitative analysis of fluorescence intensity using ImageJ, and the mean fluorescence intensity (MFI) was calculated and expressed as a fold change relative to the control group. All experiments were conducted in triplicate, and data were expressed as mean $\pm$ SD.

2.4.10.9. 3D Tumoroid test

Compared to 2D cultures, 3D tumoroids are more accurate in simulating the in vivo tumor environment. 3D tumoroids allow for a more accurate evaluation of the antitumor activity of the synthesized nanoparticles. In this study, the MDA-MB-231 cell line was seeded in a 96-well plate about 5×10^3 cells per plate by using DMEM/F-12 medium. Along with 10 % FBS, antibiotic solution, and MEM non-essential amino acids, the culture media were provided. To obtain 300-400 μ m diameter, the tumoroids were incubated for a complete 72 h. After the complete growth, tumoroids were treated with the different concentrations of ERL (100, 50, 25, 12.5, 6.25 μ g/ml), SLB (200, 100, 50, 25, 12.5 μ g/ml), and ERL-SLB (equivalent to ERL concentrations). The morphological assessment was done at 0 h and 48 h after the treatment to demonstrate the effect of free drugs and ERL-SLB-LPHN on the tumoroid. In addition, the AO/ EtBr staining was performed on the formed spheroid for the detection of cellular apoptosis caused by the free drugs and nanoparticles.

2.4.11. Animal studied

The entire set of experiments was performed in accordance with CPCSCA regulations, and the research protocol (NIP/12/2024/PE/675) was formally reviewed and approved by the institutional Animal Ethics Committee (IAEC) at NIPER, Hyderabad.

2.4.11.1. Pharmacokinetic assessment

To determine how free drugs ERL, SLB, and ERL-SLB-LPHN behave in the body following intravenous administration, a pharmacokinetic analysis was performed. Before the experiment, the female Balb/c mice weighing 20-25 grams each were habituated in a facility for 7 days period. The mice were randomly segregated into 3 groups, each group consisting of 12 animals. They were provided with complete availability of feed and drinking water throughout the

course of study. The mice received an intravenous dose of ERL, SLB, and ERL-SLB-LPHN through a tail vein, with each injection containing 10 mg/kg of ERL and 20 mg/kg of SLB. For pharmacokinetic evaluation, blood samples (0.2 mL) were collected from retro-orbital puncture at different time points, including 0.5, 1, 2, 4, 8, 12, 24, and 48 h under anaesthesia. After collecting the blood in EDTA-containing tubes, the plasma was separated by centrifugation for 10 min at 3000 rpm. The drug from the plasma was extracted using acetonitrile: methanol (1:1) by mixing 100 µl of plasma with 400 µl of solvent. The formed precipitate was then centrifuged at 3000 rpm for 5 min, and thereafter, the supernatant was separated. The obtained supernatant was analysed for quantification of the drug present in plasma using the developed bioanalytical method. The mobile phase used for this method was ACN: MeOH (1:1) as the organic phase and ammonium acetate as the aqueous phase. The ratio of organic to aqueous was maintained at 70:30 % v/v, and the flow rate was kept at 0.7 ml/min. The oven temperature was maintained at 50 °C throughout the analysis, and detection was done at the wavelengths of 246 and 289 nm.

2.4.11.2. Toxicity analysis

Toxicity analysis is done to evaluate the systemic safety of the fabricated nano drug delivery system and its biocompatibility across major organs. This analysis gives an insight into biodistribution and organ-specific toxicity detection. Female Balb/c mice weighing between 20 and 25 grams were used in a study to evaluate possible toxicity. Four groups of mice were randomly selected, each with 6 animals. All doses were given intravenously through the tail vein. One group served as the control and was given sterile saline to create physiological baseline parameters. The remaining groups received ERL, SLB, and ERL-SLB-LPHN for three alternative days with a dose of 10 mg/kg of ERL and 20 mg/kg of SLB. The animals were closely watched for any indication of toxicity over 14 days of observation period. After 14 days, all animals were euthanized, blood samples were collected in EDTA-containing tubes for further analysis, and all vital organs were collected in 10 % v/v neutral buffered formalin solution. The plasma obtained from blood samples was subjected to the biomarker assessment to evaluate the level of biomarkers such as alanine transferase, aspartate aminotransferase, and creatinine by using commercially available kits (Accurex Biomedical Pvt. Ltd, Mumbai, India). The collected organs were processed and embedded in paraffin wax. Then these organs were sectioned at a thickness of 5 µm by using a microtome (Leica, Wetzlar, Germany). The organ tissue was then subjected to histological analysis by staining with haematoxylin and eosin,

followed by examination under a microscope, to investigate the samples for any evidence of toxicity.

2.4.12. Statistical analysis

The results are presented as mean values with standard deviations ($\pm$ SD), and all experiments were carried out in triplicate. GraphPad Prism software 8.0.2 (GraphPad Software, San Diego, CA) was used to perform statistical analysis. ANOVA and Bonferroni post hoc tests were used to evaluate group differences. The following symbols were used to indicate statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

3. Results and discussion

3.1. Screening design

The Taguchi Orthogonal Array is a method of experimental design that reduces the number of experiments required for the analysis, while systematically screening and identifying the major parameters impacting the quality of the target product profile [42]. Here, Design Expert software (version 13) was used to screen the independent variables that significantly affect the dependent variable through a Taguchi Orthogonal array screening design. As shown in **Table 2**, seven different factors were examined at two levels each. Pareto and half-normal plots were created using Design Expert software in order to evaluate the influence of each factor on the product **Fig 1**. Among them, Total drug content, amount of organic phase, surfactant concentration, and lipid amount were found to be key variables that significantly impact selected critical quality attributes such as particle size and PDI.

Table 2: Screening study by Taguchi Orthogonal array method.

Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Response 1	Response 2
Aqueous (15ml): Organic	Total Drug Loading (mg)	Surfactant Concentration (%)	Stirring Speed (rpm)	Polymer (15mg): Lipid	Stirring Time (hours)	Addition rate	Size (nm)	PDI
1:0.2	1.5	1	1000	1:0.25	4	Low	147.7 ± 1.63	0.212 ± 0.024
1:0.1	6	1	1000	1:0.75	2	Low	199.9 ± 2.38	0.3379 ± 0.013
1:0.2	6	0.5	1000	1:0.25	2	High	421.5 ± 1.22	0.5654 ± 0.031
1:0.2	1.5	1	500	1:0.75	2	High	155 ± 2.81	0.1202 ± 0.009
1:0.2	6	0.5	500	1:0.75	4	Low	449.9 ± 3.34	0.5192 ± 0.039
1:0.1	1.5	0.5	1000	1:0.75	4	High	146.1 ± 1.35	0.1511 ± 0.016
1:0.1	6	1	500	1:0.25	4	High	212.2 ± 2.77	0.3451 ± 0.024
1:0.1	1.5	0.5	500	1:0.25	2	Low	302.7 ± 2.14	0.2559 ± 0.027

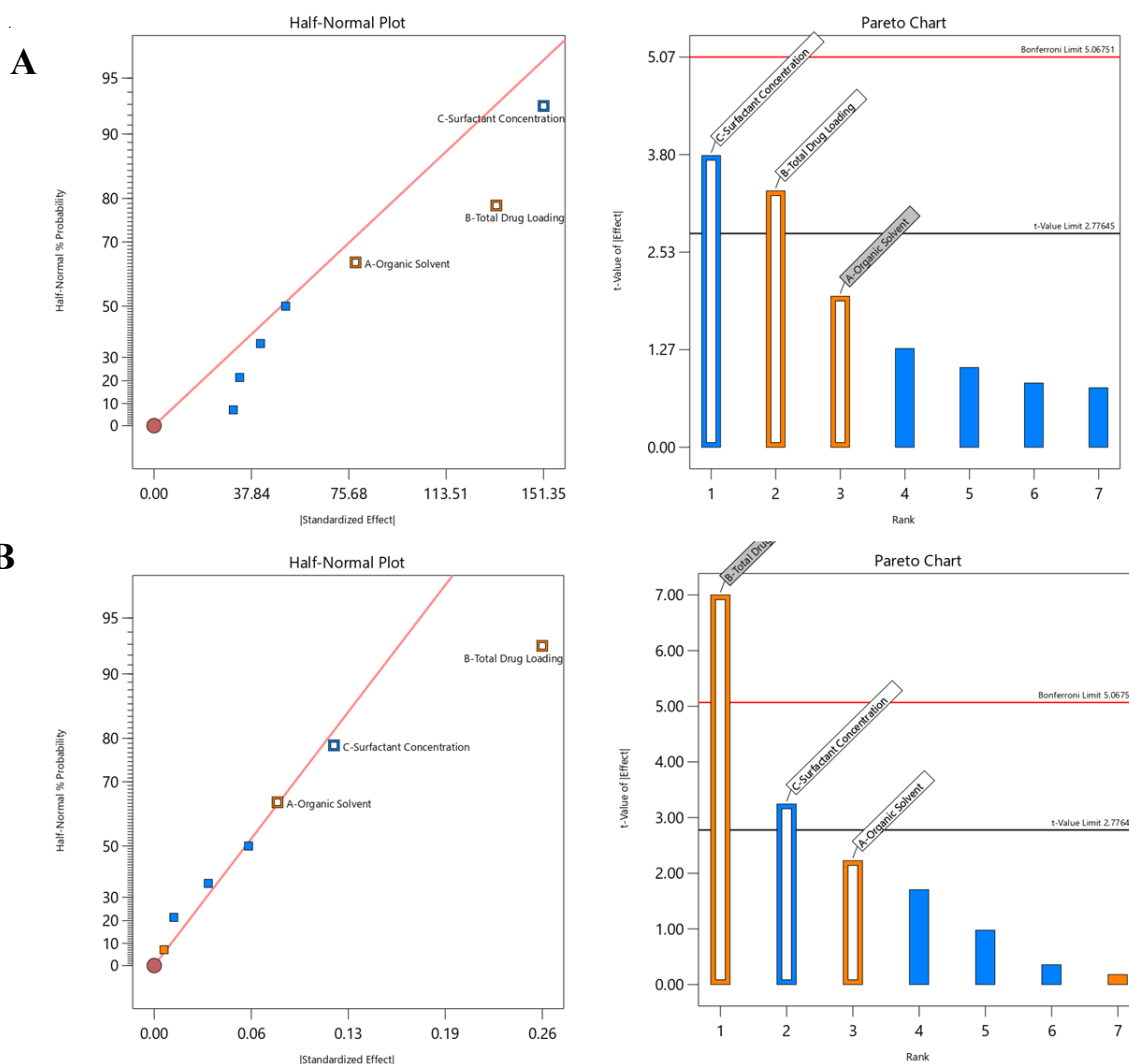


Figure 1. The figure represents a Half-Normal plot and Pareto chart; **A.** size, **B.** PDI.

3.2. BBD surface methodology

After investigating the independent variables that mostly affect the size and PDI of the nanoparticles, these factors were further studied by using response surface methodology. Four-factor three-level BBD was utilized for this analysis, using Design Expert software. **Table 3** summarizes the results of 29 experiments that were conducted as part of the study to investigate the interactions between outcomes and these variables at three distinct levels. The independent variables, such as the amount of organic phase (ml) (A), total drug content (B), surfactant concentration (C), and lipid amount (D), were examined. A response surface plot was used for evaluating their impact on mean particle size (Y1), PDI (Y2), percentage EE of SLB (Y3), and percentage EE of ERL (Y4). Statistical analyses like ANOVA were used to select the most suitable models for each response, considering the F-value, lack of fit, and correlation coefficients (R^2) mentioned in **Table 4**.

Table 3: Optimization of nanoparticles by Box- Behnken model

Run	Organic (ml)	Total drug content (mg)	Surfactant Concentration (%)	Lipid (mg)	Size (nm)	PDI	%EE SLB	%EE ERL
1	2.25	375	1	3.75	200.3	0.232	95.12 ± 4.21	60.15 ± 2.52
2	2.25	6	0.75	3.75	401	0.471	97.75 ± 6.05	66.94 ± 4.07
3	3	3.75	0.75	3.75	199.5	0.443	96.63 ± 2.87	64.74 ± 2.43
4	2.25	3.75	0.75	7.5	171.2	0.223	95.44 ± 4.82	59.85 ± 2.76
5	2.25	1.5	0.5	7.5	202.3	0.287	87.69 ± 3.99	47.97 ± 3.41
6	3	3.75	1	7.5	203.8	0.282	89.51 ± 3.59	40.64 ± 4.67
7	1.5	3.75	0.75	3.75	213.2	0.382	96.67 ± 6.32	51.17 ± 2.31
8	1.5	3.75	1	7.5	210	0.175	98.25 ± 2.49	49.66 ± 2.07
9	3	3.75	0.5	7.5	225.1	0.445	97.66 ± 3.84	47.33 ± 1.64
10	2.25	3.75	1	11.25	201.5	0.285	95.66 ± 3.29	32.93 ± 2.46
11	2.25	1.5	1	7.5	244	0.214	94.51 ± 5.21	49.51 ± 5.12
12	2.25	6	1	7.5	241.1	0.359	95.29 ± 4.88	48.78 ± 2.75
13	1.5	3.75	0.5	7.5	212.9	0.376	95.92 ± 5.02	34.62 ± 1.89
14	1.5	3.75	0.75	11.25	258.5	0.235	96.15 ± 2.37	42.90 ± 3.11
15	2.25	3.75	0.5	3.75	279.3	0.450	95.07 ± 4.37	35.62 ± 2.56
16	3	6	0.75	7.5	325.4	0.476	97.92 ± 2.75	46.93 ± 2.02
17	3	3.75	0.75	11.25	243.4	0.425	98.36 ± 3.09	52.06 ± 1.43
18	2.25	3.75	0.75	7.5	259.1	0.334	95.01 ± 2.98	49.39 ± 2.45
19	2.25	1.5	0.75	3.75	184	0.0888	76.15 ± 3.32	51.28 ± 2.75
20	3	1.5	0.75	7.5	154.5	0.154	89.67 ± 2.02	50.15 ± 5.32
21	1.5	6	0.75	7.5	247	0.448	98.18 ± 3.47	60.24 ± 3.64
22	2.25	3.75	0.75	7.5	205.1	0.3943	96.75 ± 3.62	49.10 ± 2.43
23	2.25	6	0.75	11.25	287.6	0.4349	96.06 ± 2.86	34.03 ± 2.83
24	2.25	3.75	0.5	11.25	220.3	0.353	95.06 ± 1.95	55.89 ± 3.63
25	2.25	6	0.5	7.5	303.5	0.506	99.14 ± 3.75	76.18 ± 2.32
26	2.25	1.5	0.75	11.25	188.8	0.255	86.91 ± 3.65	50.05 ± 1.69
27	2.25	3.75	0.75	7.5	187.6	0.275	84.90 ± 2,43	48.40 ± 4.07
28	1.5	1.5	0.75	7.5	210.5	0.2481	86.57 ± 1.96	43.72 ± 2.47
29	2.25	3.75	0.75	7.5	250.1	0.361	95.68 ± 2.36	51.64 ± 4.44

3.2.1. Impact on particle size

Particle sizes less than 200 nm have better circulation and tumor penetration by the EPR effect, whereas larger particles have limited access and are quickly cleared from the body. The Box-Behnken model was used for the optimization of a smaller particle size of ERL-SLB-LPHN. The particle size obtained was in the range of 154.5 ± 7.27 to 401 ± 9.72 nm after 29 runs of the experiment. Based on the observed data, a linear model was the most suitable approach suggested for the particle size (Y1). The significant model was suggested based on the non-significant lack of fit with an F value of 5.32. Low predictive accuracy is shown by a significant lack of fit in the model, which shows poor alignment with the data. The model's favourable predictive capacity was validated by a small difference of less than 0.2 between the adjusted and predicted R^2 values. The coded equation representing the model is,

$$\text{Mean particle size (Y1): } +232.09 + -0.0333 *A + 51.79 *B + -11.89 *C + -6.43 *D$$

Based on the model, the factor that most significantly increases the particle size is the total drug content. An increase in the particle size may be due to an increase in the total solid content of nanoparticles, which has also been observed in the given research references [43, 44]. Lipid amount and organic phase showed a slight negative impact on particle size. Reduction in particle size with increasing surfactant concentration could be due to the surfactant stabilizing the nanoparticles by preventing aggregation [45].

3.2.2. Impact on PDI

A formulation's particle size uniformity is evaluated by the PDI. A PDI less than 0.3 represents a homogeneous system with a narrow size distribution and is monodispersed. The variability in particle size may lead to inconsistent cellular uptake, and it also affects the pharmacokinetics and stability of the nanoparticles. For optimization of the PDI of the ERL-SLB-LPHN Box-Behnken model, a method was implemented. After 29 experimental runs, the PDI values were observed within the range of 0.0888 ± 0.001 to 0.5062 ± 0.004 . The linear model has been demonstrated to be the most appropriate for characterizing PDI (Y2) among the models that were examined by Design Expert. An F value of 17.22 indicates adequate model fidelity with the experimental data, with only a 0.01% chance of noise, demonstrating a non-significant lack of fit, supporting the suitability of the chosen model. The adjusted and predicted R^2 values deviating by less than 0.2 demonstrated the suitability of the model's predictive dependability. The coded equation representing the model is,

$$\text{PDI (Y2): } +0.3319 + 0.0302 *A + 0.1207 *B + -0.0724 *C + -0.0065 *D$$

The equation demonstrates that the amount of drug and organic solvent has a positive impact on the PDI, which can correlate with previously reported literature [46]. The increase in PDI due to an increase in the amount of drug may be due to a lack of excipient availability and high solid drug content, which might have resulted in less uniform particle formation and a wider size distribution [47]. The PDI was found to be reduced with increasing surfactant concentration, as surfactant produces steric hindrance on the particle surface, which prevents particle aggregation [48]. **Fig 2** represents the 3D surface plots and perturbation plot of factors affecting particle size and PDI.

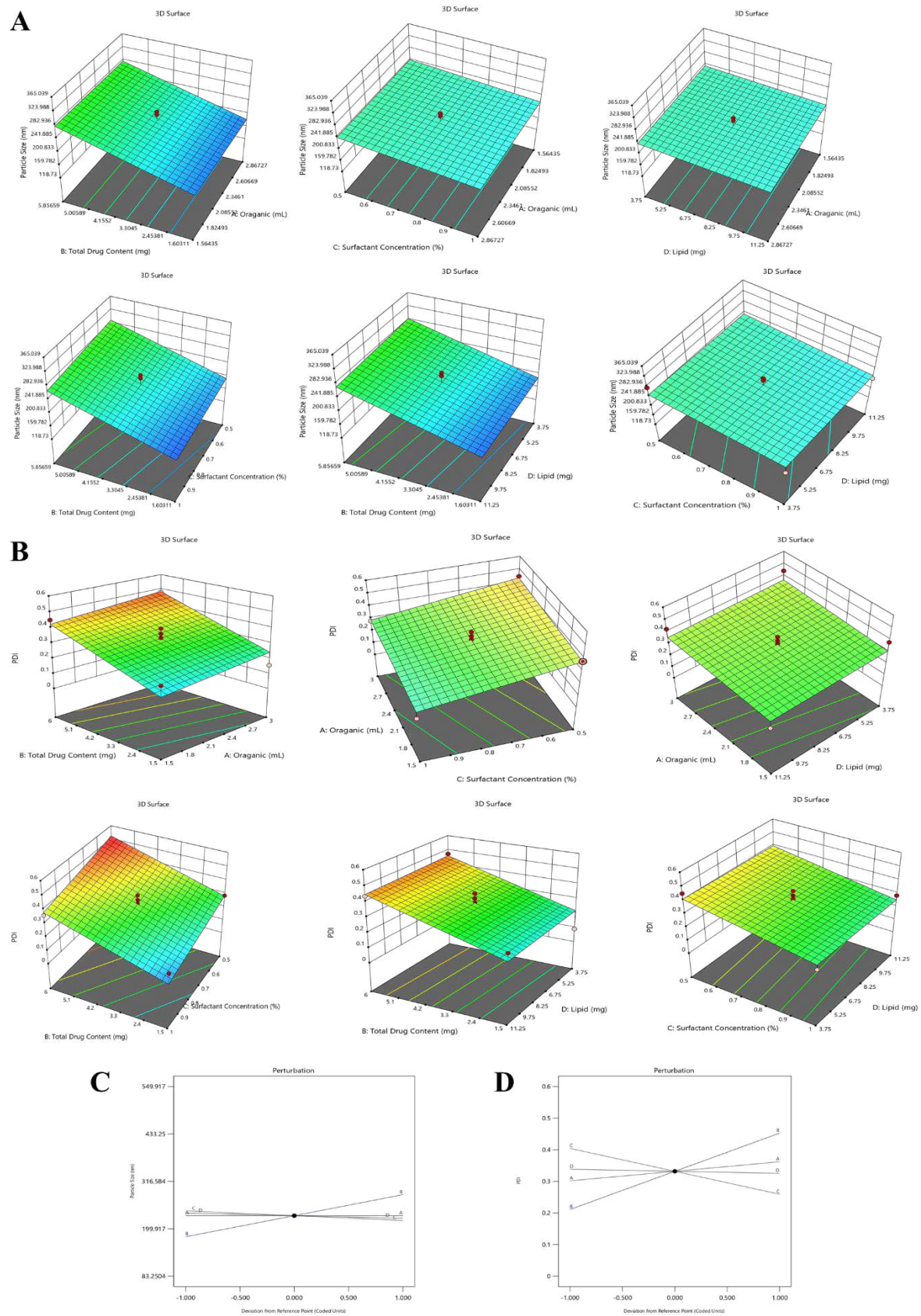


Figure 2. (A) 3D surface plots of factors affecting mean particle size. (B) 3D surface plots of factors affecting PDI. (C) Particle size perturbation plot. (D) PDI perturbation plot.

3.2.3. Impact on entrapment of SLB and ERL

The percentage EE entrapment of SLB was found within the range of 76.14 ± 3.32 % to 99.14 ± 3.75 % after the 29 runs suggested by the Design Expert software. Entrapment efficacy is a key consideration when evaluating nanoparticles' effectiveness. Higher entrapment allows for the targeted delivery of a higher dosage of the drug to malignant tissues, potentially improving the therapeutic outcome. The linear model suggested a significant percentage of EE of SLB. With a non-significant lack of fit, the F value was 4.87. The discrepancy of less than 0.2 between the adjusted and predicted R^2 values suggests that the model is a good fit for forecasting results and has dependable predictability. % EE of ERL was observed in the range of 32.93 ± 2.46 to 76.18 ± 2.32 . The 2FI model was recommended and chosen, which was found significant with an F value of 3.23, with no significant lack of fit. The coded equations representing both models are

$$\text{Percentage EE SLB (Y3): } +93.92 + -0.1668 *A + 5.24 *B + -0.1834 *C + 0.9006 *D$$

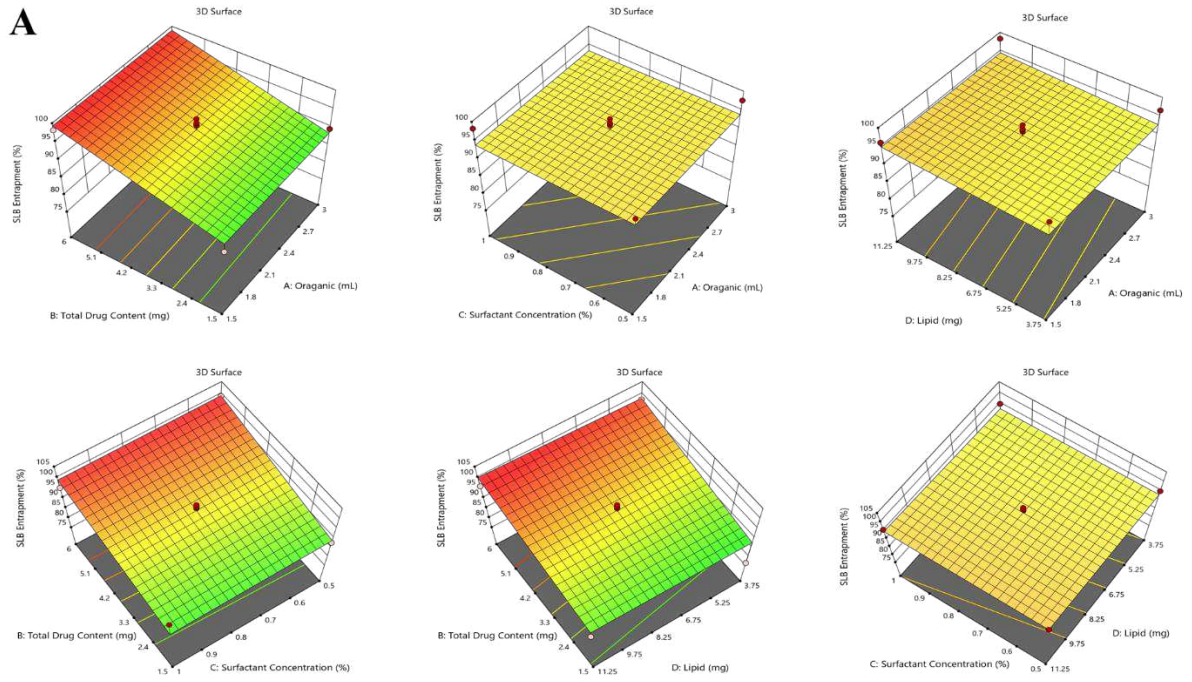
$$\text{Percentage EE ERL (Y4): } +50.07 + 1.63 *A + 3.37 *B + -1.33 *C + -5.17 *D + -4.94 *AB + -5.43 *AC + -1.10 *AD + -7.23 *BC + -7.92 *BD + -11.87 *CD$$

Increasing the amount of organic phase causes an increase in entrapment, which may be due to the organic phase decreasing the amount of drug dissolved in the aqueous phase; therefore, lowering the drug loss from the organic phase leads to improved entrapment efficiency [49]. **Fig 3** represents the 3D surface plots and perturbation plot of factors affecting the percentage EE of SLB and ERL.

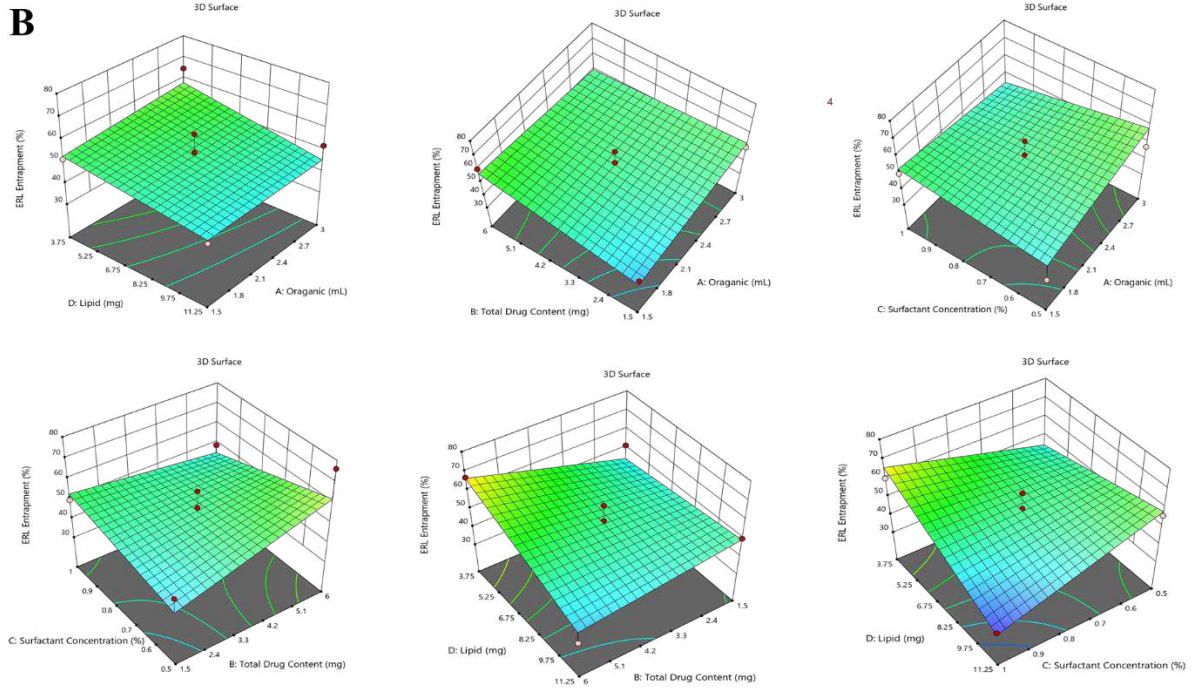
Table 4. Regression modelling and validation of adjusted regression and predicted regression

Responses	Regression R^2	Adjusted R^2	Predictive R^2
Mean particle Size (nm)	0.4700	0.3816	0.2199
PDI	0.7416	0.6986	0.6242
% EE SLB	0.5481	0.3561	0.1978
% EE ERL	0.8421	0.7232	0.5523

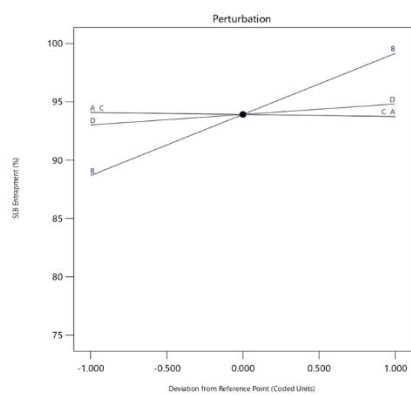
A



B



C



D

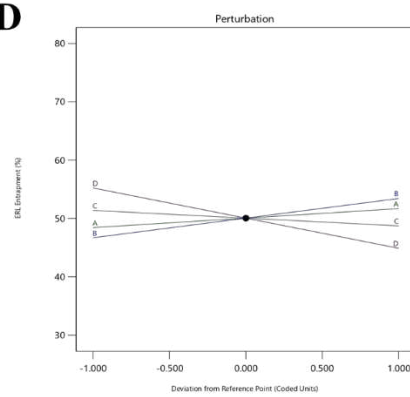


Figure 3. A. 3D surface plots of factors affecting percentage EE SLB B. 3D surface plots of factors affecting percentage EE ERL. C. Percentage EE SLB perturbation plot. D. %EE ERL perturbation plot.

3.2.4. QbD-based optimized formulation and design space

An overlay plot was created after the optimal formulation of ERL-SLB-LPHN was established using a combination of numerical and graphical methods. The design space plot provided a framework for choosing a suitable factor level. For the optimized region, maximum %EE, minimum particle size, and PDI were selected. The composition of the optimized formulation includes 1.5 ml of organic, 3.6 mg of drug amount, 1 % surfactant, and 3.75 mg of lipid content, which results in a particle size of 160.13 ± 0.90 nm (**Fig 4A**), PDI 0.13 ± 0.03 , % EE of SLB 98.21 ± 0.12 %, and % EE of ERL 49.74 ± 8.2 %.

3.3. Characterization of ERL-SLB-LPHN

3.3.1. Surface morphological characterization

A transmission electron microscope (TEM) was used to examine the morphological properties of ERL-SLB-LPHN, as shown in **Fig 4B**. TEM pictures showed that there was no aggregation, and nanoparticles were evenly dispersed with a definite spherical shape. Furthermore, the particle size observed through TEM correlated well with data obtained from dynamic light scattering.

3.3.2. FTIR study

The FTIR study was carried out to gain insight into any possible drug-polymer interaction and encapsulation of the drug in LPHN. The FTIR spectra of ERL, SLB, PLGA, lecithin, ERL-SLB-PM, and ERL-SLB-LPHN were evaluated, and the results are outlined in **Fig 4C**. ERL exhibited the characteristic absorption peaks at 3271.3 cm^{-1} due to N-H stretching and at 2709.8 cm^{-1} , potentially related to C-H stretching. Distinct peaks at 1626.1 cm^{-1} , 1505.8 cm^{-1} , and 1235.6 cm^{-1} ascribed to N-H bending, aromatic C=C stretching, and C-N bending, respectively [50]. SLB showed a prominent peak at 3456.1 cm^{-1} of O-H stretching and 1634.7 cm^{-1} , suggesting aromatic-related C=O stretching. Lecithin displayed characteristic absorption peaks at 2922 cm^{-1} and 2853 cm^{-1} , corresponding to the C-H stretching vibration of methylene groups. An additional prominent peak was observed at 1744 cm^{-1} and 1240 cm^{-1} , which is attributed to ester carbonyl (C=O) stretching and P-O-C bond vibrations. In the case of PLGA, a strong peak near 1745 cm^{-1} indicated ester carbonyl stretching, typical of its polymeric backbone. In ERL-SLB-PM, the characteristic peaks of both drugs were retained, including 3456.1 cm^{-1} of SLB

and 3272.3 cm^{-1} of ERL. However, in the ERL-SLB-LPHN, the distinctive peaks N-H and C-H of ERL at 3272.3 cm^{-1} and 2709.8 cm^{-1} vanished, while other characteristic peaks were reduced in intensity. Similarly, O-H stretching of SLB was not found. Peaks associated with lecithin (2922.3 cm^{-1} and 2855.1 cm^{-1}) and PLGA (1749.9 cm^{-1}) remained unchanged. These spectral changes confirm successful encapsulation of ERL and SLB within the hybrid nanoparticles.

3.3.3. PXRD analysis

To determine the crystalline or amorphous nature of ERL-SLB-LPHN and correlate it with the free drugs ERL and SLB to confirm their successful incorporation in nanoparticles, PXRD analysis was performed. The PXRD diffraction pattern of pure ERL, SLB, mannitol, ERL-SLB-PM, and ERL-SLB-LPHN is shown in **Fig 4D**. ERL and SLB showed sharp and intense diffraction peaks at 2θ values of 11.04° , 18.6° , 22.5° , 23.3° , $34.3 \pm 0.2^\circ$, and 13.5° , 14.4° , 19.5° , 24.3° , $26.7^\circ \pm 0.2^\circ$ confirming their crystalline nature. These characteristic peaks of both drugs were retained in the physical mixture with less intensity, which may be due to the presence of an amorphous excipient like PLGA in a higher concentration than the drug, as previously reported in the literature [51]. Although in the ERL-SLB-LPHN diffractogram, the complete absence of the ERL and SLB sharp peaks was observed. This suggested that both drugs were successfully encapsulated and converted into amorphous or less ordered form within the nanoparticle matrix. These changes confirm the incorporation of ERL and SLB into the hybrid nanoparticle.

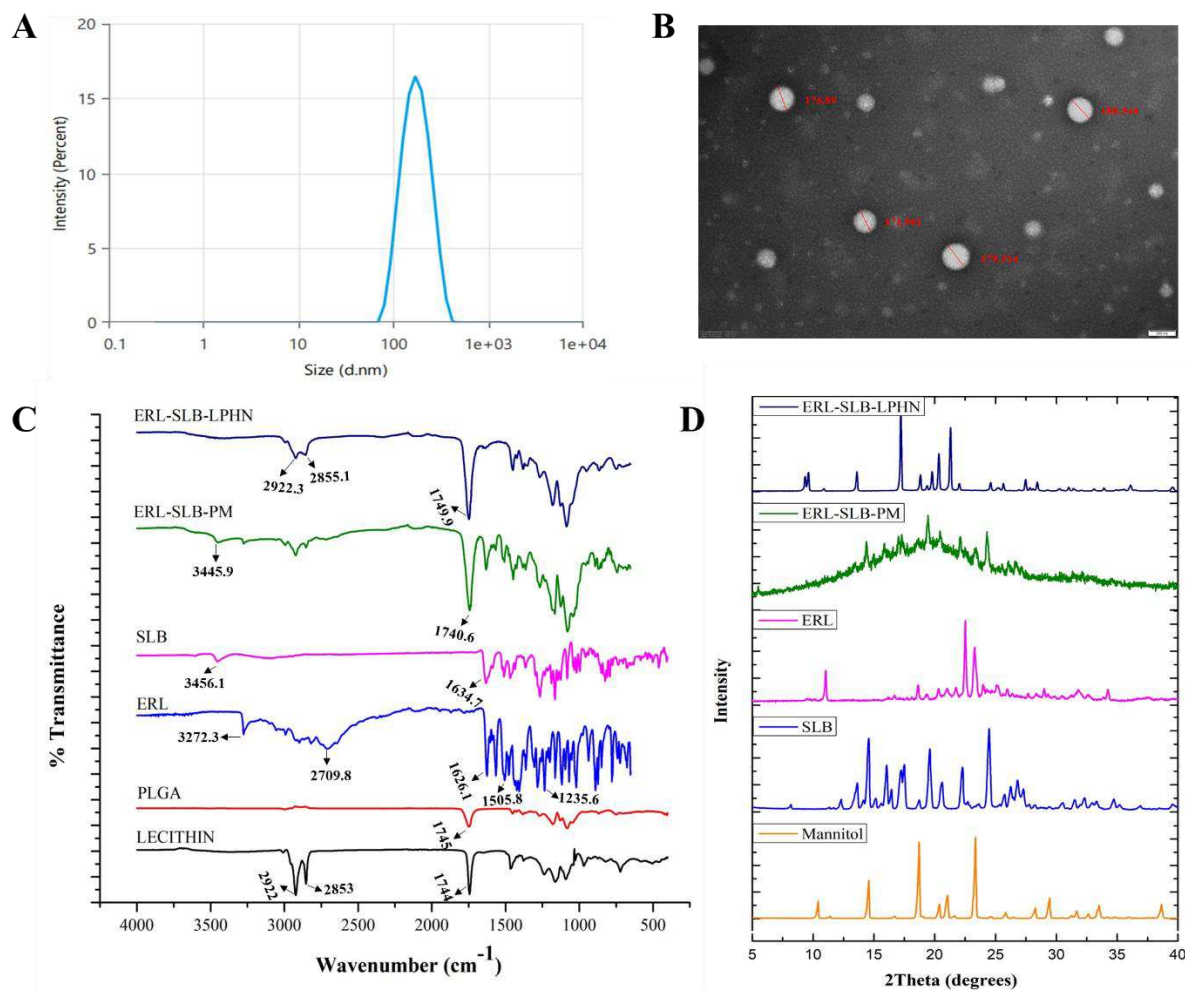


Figure 4. (A) Dynamic light scattering size distribution by intensity graph. (B) Morphological characterization by TEM (Scale 200nm). (C) FTIR spectra of Lecithin, PLGA, ERL, SLB, ERL-SLB-PM, and ERL-SLB-LPHN. (D) PXRD diffractogram of mannitol, ERL, SLB, ERL-SLB-PM, and ERL-SLB-LPHN.

3.3.4. In vitro drug release

To demonstrate how nanoparticles modified the release of the free drug, an in vitro release study was performed. The drug release behaviour of the free form of ERL and SLB, as well as ERL-SLB-LPHN, was carried out by using the dialysis bag method. **Fig. 5(A and B)** depicted the release patterns of ERL, SLB, and ERL-SLB-LPHN. In the case of ERL-SLB-LPHN, ERL released $8.74 \pm 4.3\%$, while SLB released $2.89 \pm 1.5\%$ within the initial 0.5 h. Concurrently, the free drug solution of ERL and SLB demonstrated substantially higher drug release of $28.52 \pm 2.1 \%$ and $10.78 \pm 0.6 \%$, respectively. Moreover, ERL and SLB in their free form achieved nearly complete release, reaching $94.30 \pm 1.5 \%$ and $92.98 \pm 1.4 \%$ at 4 and 12 h, respectively. In contrast, the sustained release pattern was found in ERL-SLB-LPHN with $49.65 \pm 1.3 \%$

release of ERL and 34.93 ± 1.2 % of SLB. The lipid-polymeric nanostructure of ERL-SLB-LPHN is responsible for the prolonged release of ERL and SLB, as the lipidic layer around the polymeric matrix core efficiently delays the drug diffusion. This results in a more extended and controlled release profile compared to the free drug form [52]. The various kinetic models were used to determine the release kinetics of the ERL-SLP-LPHN: first-order release model, Higuchi model, Korsmeyer-Peppas model, and zero-order model. The release kinetics of ERL and SLB in ERL-SLB-LPHN followed the Korsmeyer-Peppas and Higuchi models, respectively, as evidenced by the corresponding regression coefficients shown in **Table 5**.

Table 5. In vitro drug release kinetic models

	First order $\ln Q_t = \ln Q_0 + K_1 t$	Higuchi $Q_t = K H t^{1/2}$	Korsemeyer-peppas $(M_t/M_\infty = K_t n$	Zero order $Q_t = Q_0 + K_0 t$
ERL	0.5317	0.893	0.9418	0.7122
SLB	0.4762	0.9203	0.9083	0.7122

3.3.5. In vitro Hemolysis study

The amount of haemoglobin in the surrounding medium is used to quantitatively measure the degree of hemolysis, as it indicates the extent of red blood cell membrane disruption. The possibility of hemolysis, which results from direct contact between the drug and red blood cells, is one of the main drawbacks of intravenous formulations. The RBC treated with the positive control Triton X-100 showed complete hemolysis. Whereas normal cellular morphology was observed in the negative control group, with no hemolysis, as shown in **Fig 5C**. After 2 h incubation, more than 10 % hemolysis was observed in both free ERL and free SLB-treated cells, which further increased after 4 h incubation. The ERL-SLB-LPHN demonstrated significantly lower hemolysis ($P < 0.0001$) than the free ERL and SLB at 50 ppm, with 6.5 ± 0.3 % after 2 h and 7.6 ± 1.4 % after 4 h of incubation, as presented in **Fig 5(D&E)**.

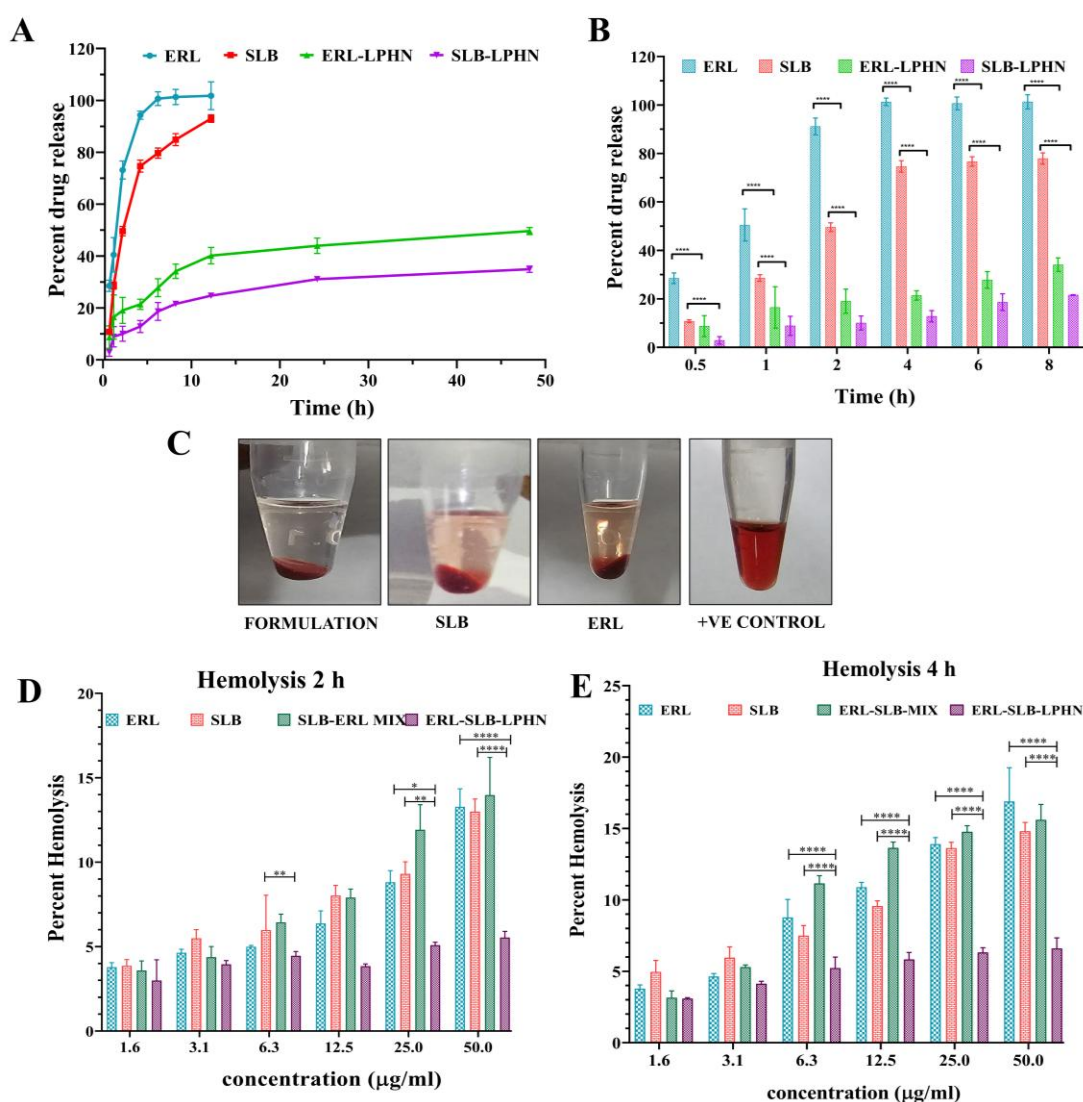


Figure 5. (A and B) Drug release profile of ERL and SLB in free form and in LPHN. (C and D) Percent hemolysis of Free-ERL, Free-SLB, ERL-SLB-PM, and ERL-SLB-LPHN after 2 and 4 h. (E) Comparative hemolysis images in different groups. Asterisk marks denote the statistically significant difference (**** $P < 0.0001$), (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$).

3.3.6. Antioxidant assay

The DPPH assay was used to evaluate the antioxidant potential of ERL-SLB-LPHN and SLB, with ascorbic acid acting as the positive control (**Fig 6A**). Ascorbic acid exhibited remarkable free radical scavenging, approximately 95%. For both SLB and ERL-SLB-LPHN, antioxidant activity increased with increasing concentration. At 10 $\mu\text{g/ml}$, ERL-SLB-LPHN demonstrated 51.69 ± 1.65 % scavenging activity, which escalated to 72.66 ± 0.89 % at 100 $\mu\text{g/ml}$, following

a pattern similar to that of free SLB. These results suggest that LPHN effectively retains the bioactivity of the phytoconstituent, as reported by [52], and that PLGA nanoparticles maintain the antioxidant activity of the polyphenolic compound after entrapment. Subsequently, strengthening its potential functions in reducing oxidative stress in anticancer treatment.

3.3.7. Stability studies

3.3.7.1. Storage stability

The stability of the formulation ERL-SLB-LPHN was rigorously assessed over 90 days at two different temperature conditions: 25 ± 2 °C (room temperature) and 4 ± 2 °C (refrigerated conditions). During this study, several critical parameters were meticulously evaluated, including particle size, PDI, and percent EE. At the elevated temperature of 25 ± 2 °C, the results indicated a marked increase in both particle size and PDI, while the EE demonstrated a significant decline. These changes suggested a loss of formulation stability under room temperature conditions, which could potentially compromise the effectiveness of the product. In contrast, when the formulation was stored under controlled refrigerated conditions at 4 ± 2 °C, the stability parameters remained consistent and showed no significant variations over the first 60 days of the study. This lack of deterioration highlights the favourable stability profile of ERL-SLB-LPHN at lower temperatures, as shown in **Table 6**. Based on these findings, it is recommended that the ERL-SLB-LPHN formulation be stored at a temperature of 4 ± 2 °C for up to 90 days to ensure optimal stability and maintain its intended efficacy.

Table 6. Storage stability data at $25 \pm 2^\circ\text{C}$ and $4 \pm 2^\circ\text{C}$

Storage condition	Particle size (nm)	PDI	% EE SLB	%EE ERL
0 days	159.2 ± 1.63	0.1243 ± 0.0163	98.1364 ± 5.21	61.4811 ± 3.62
$4 \pm 2^\circ\text{C}$				
15 days	160.3 ± 4.19	0.1241 ± 0.0092	98.0763 ± 3.89	60.5727 ± 4.47
30 days	163.1 ± 1.89	0.1949 ± 0.0153	96.9635 ± 4.22	58.5624 ± 2.54
60 days	171.2 ± 2.63	0.2232 ± 0.0280	91.0106 ± 7.35	54.5206 ± 4.81
90 days	196.9 ± 6.01	0.2426 ± 0.0251	84.4109 ± 4.21	52.6543 ± 7.22
$25 \pm 2^\circ\text{C}$				
15 days	175.3 ± 1.23	0.1976 ± 0.0321	93.9534 ± 5.68	56.5413 ± 2.81
30 days	201.1 ± 3.83	0.2255 ± 0.0496	84.1008 ± 3.66	51.2971 ± 4.58
60 days	327.2 ± 4.62	0.3424 ± 0.0261	57.7591 ± 5.19	36.8932 ± 2.94
90 days	303.7 ± 2.13	0.5228 ± 0.0391	49.8321 ± 7.42	33.4632 ± 3.23

3.3.7.2. Stability in 10% FBS

The ability of nanoparticles to bind to the blood plasma proteins could lead to nanoparticles enlarging and being eliminated from the body more quickly. In order to verify this process, the stability of ERL-SLB-LPHN was examined in 10% FBS. To evaluate whether the nanoparticles tend to agglomerate or undergo protein adsorption in FBS. After incubating with FBS, the size was measured at different time points from 0 to 24 h, observed to increase from 159.25 ± 1.2 nm to 177.7 ± 2.76 nm, and no significant change was observed in PDI, as shown in **Fig 6B**. The increase in the particle size observed after 24 h is in the range to undergo the EPR effect at the tumor site [53]. As per findings, ERL-SLB-LPHN exhibits significant stability in serum over a 24 h period, with size increment remaining within the range for efficient tumor targeting through the EPR effect.

3.3.7.3. Antidilution study

When the nanoparticles are administered in the body, it undergoes the multiple fold dilutions. The antidilution study was performed to evaluate the effect of multiple-fold dilution on the stability of nanoparticles. After the multiple-fold dilution with phosphate buffer solution, the size of ERL-SLB-LPHN decreased from 168.8 ± 2.57 nm to 141.1 ± 5.57 nm, with no substantial change in PDI, as shown in **Fig 6C**. The reduction in mean particle size could be

due to a decrease in the concentration of nanoparticles, which may lead to agglomerate dispersion and a narrow size distribution [54]. These outcomes demonstrate that the nanoparticles maintain their stability and structural integrity even after being diluted multiple-fold in phosphate buffer, indicating that ERL-SLB-LPHN are ideal for retaining stability in *in vivo* conditions when exposed to large fluid volumes.

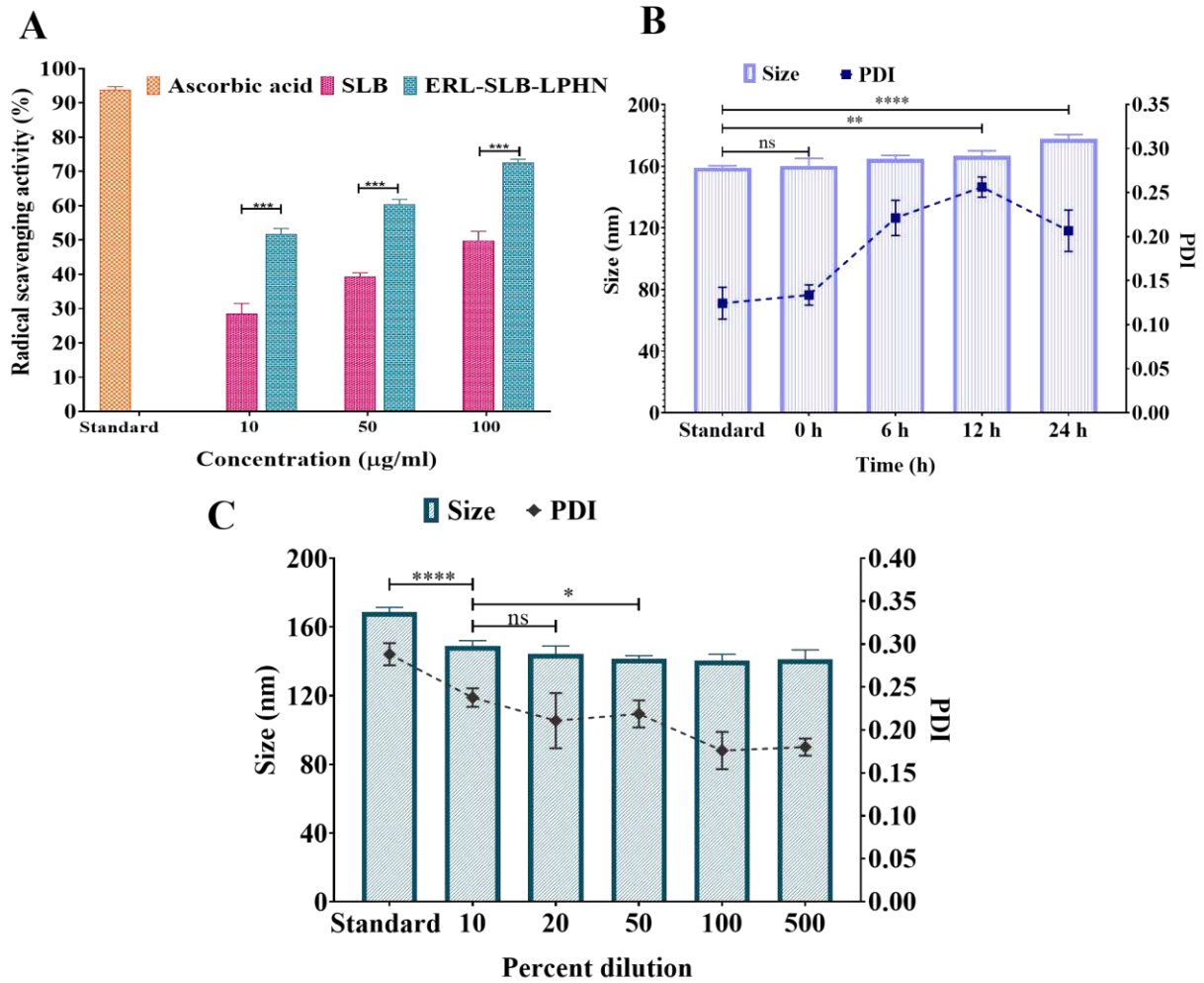


Figure 6. A. Comparison of the coagulation time of standard ascorbic acid with Free SLB and SLB extracted from LPHN. B. Stability of ERL-SLB-LPHN IN 10% FBS from 0 h to 24 h. C. Antidilution study of ERL-SLB-LPHN, particle size, and PDI after multiple-fold dilutions.

3.3.8. Cell culture study

3.3.8.1. Cytotoxicity study

The MTT assay was performed to investigate the relative cytotoxicity of the ERL, SLB, ERL-SLB-PM, and ERL-SLB-LPHN. The cytotoxicity study was carried out in the MDA-MB-231 cell line and demonstrated that ERL-SLB-LPHN exhibits substantially higher cellular toxicity

compared to ERL and SLB after 48 h of treatment, as represented in **Fig 7A & B**. Furthermore, the IC_{50} of ERL and SLB were found to be 23.75 $\mu\text{g/ml}$ and 42.65 $\mu\text{g/ml}$, respectively. The IC_{50} value of ERL-SLB-PM and ERL-SLB-LPHN was reduced by about 2.36 and 2.97-folds that the ERL. The IC_{50} of ERL-SLB-PM and ERL-SLB-LPHN was obtained as 10.04 and 7.99 $\mu\text{g/ml}$, respectively. The reduced IC_{50} value of ERL-SLB-LPHN may be attributed to the enhanced cellular uptake of the formulation, as observed in the uptake study, which also correlated with previously reported findings [55]. Additionally, it has been reported that the co-delivery of the SLB with an anticancer drug is more effective in inhibiting cell growth than monotherapy [56].

3.3.8.2. Cellular uptake

Cellular uptake study demonstrates whether the drug delivery system has been taken up by cancer cells or not, and aids in understanding the mode of entry. The fluorescence images of the qualitative cellular uptake of coumarin-6 (C_6) loaded LPHN and free coumarin-6 carried out in the MDA-MB-231 cell line are represented in **Fig 7C**. Coumarin-6-loaded LPHN demonstrated improved cellular penetration relative to the free coumarin-6. The fluorescence intensity of Coumarin-6-loaded LPHN was observed to significantly increase by 4.7 folds than the hydrophobic free coumarin-6 dye ($p < 0.0001$), as shown in **Fig 7D**. The uptake could have increased due to nano-sized particles, which promote endocytosis and make it easier to pass through the biological membrane [57]. According to the earlier research, it has been stated that the presence of a lipidic layer improves the nanoparticles' interaction with the cell membrane and promotes endocytosis, which enhances the uptake [58].

3.3.8.3. Scratch test

Breast cancer cells exhibit a high propensity to detach from the primary tumor and invade surrounding tissues and organs, contributing to metastasis. To determine the antimigratory effect of ERL-SLB-LPHN, a scratch test assay was performed. Cells treated with ERL, SLB, and ERL-SLB-PM demonstrated significant cellular migration into the scratch area. In contrast, cells treated with ERL-SLB-LPHN showed minimal to no migration in the scratch area represented in **Fig 7E**, confirming a strong inhibition of cellular motility. These results demonstrate the potential of ERL-SLB-LPHN to regulate the cancer spread and lower the risk of metastasis by effectively limiting the migration of breast cancer cells.

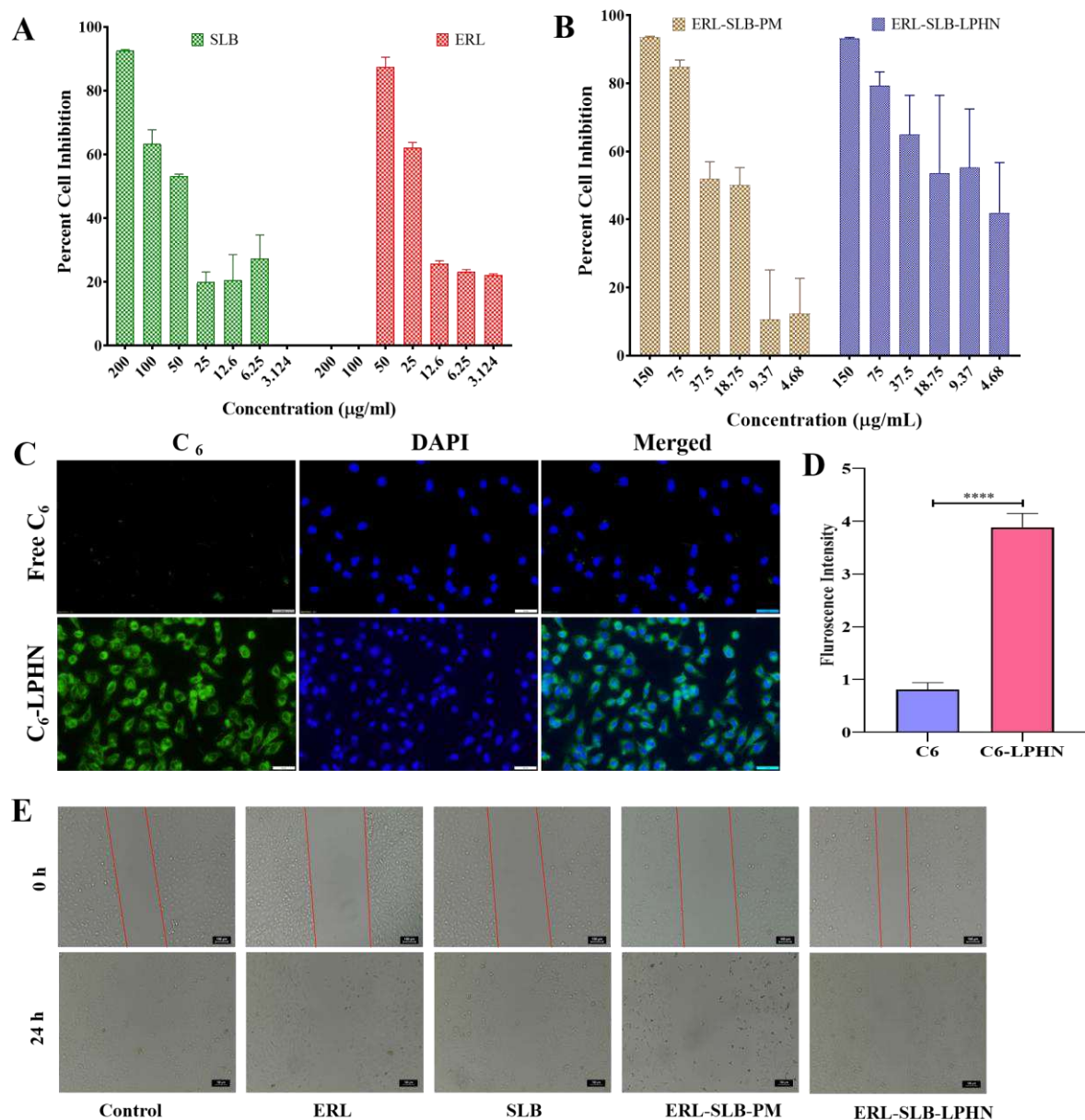


Figure 7. (A & B) Cytotoxicity study by MTT assay after 48 h treatment. (C) Fluorescence images of qualitative uptake of C₆ and C₆-LPHN. (D) Intensity of fluorescence of C₆ and C₆-LPHN. (E) Comparison of migration between the MDA-MB-231 cells treated with ERL, SLB, ERL-SLB-PM, and ERL-SLB-LPHN at 0 h and 24 h.

3.3.8.4. Mechanisms of uptake

A comprehensive experimental approach was employed, utilizing a set of specific inhibitors to investigate the various endocytosis pathways followed by C₆-LPHA nanoparticles for complete internalization. This study focused on the impact of these inhibitors on the uptake of nanoparticles by MDA-MB-231 breast cancer cells. A detailed analysis of the internalization pathway for C₆-LPHA was primarily achieved through fluorescence imaging, as illustrated in

Fig 8. Among the inhibitors tested, chlorpromazine emerged as the most effective in significantly reducing the green fluorescence signal compared to the others. The substantial decrease in fluorescence intensity indicates that the uptake of C6-LPHN nanoparticles into the cells is a critical process occurring predominantly via the clathrin-mediated endocytosis pathway. Additionally, sodium azide was employed as an inhibitor of energy-dependent endocytosis to provide further insight into the cellular mechanisms involved. As a common metabolic inhibitor, sodium azide can partially deplete ATP in the cell, leading to a notable reduction in nanoparticle uptake. The marked decrease in green fluorescence following sodium azide treatment underscores the importance of an energy-dependent endocytic process for the efficient internalization of these nanoparticles. Collectively, the findings of this study confirm the intricate processes of cellular uptake likely involved in the internalization of C₆-LPHN nanoparticles and their association with the clathrin-mediated endocytosis pathway.

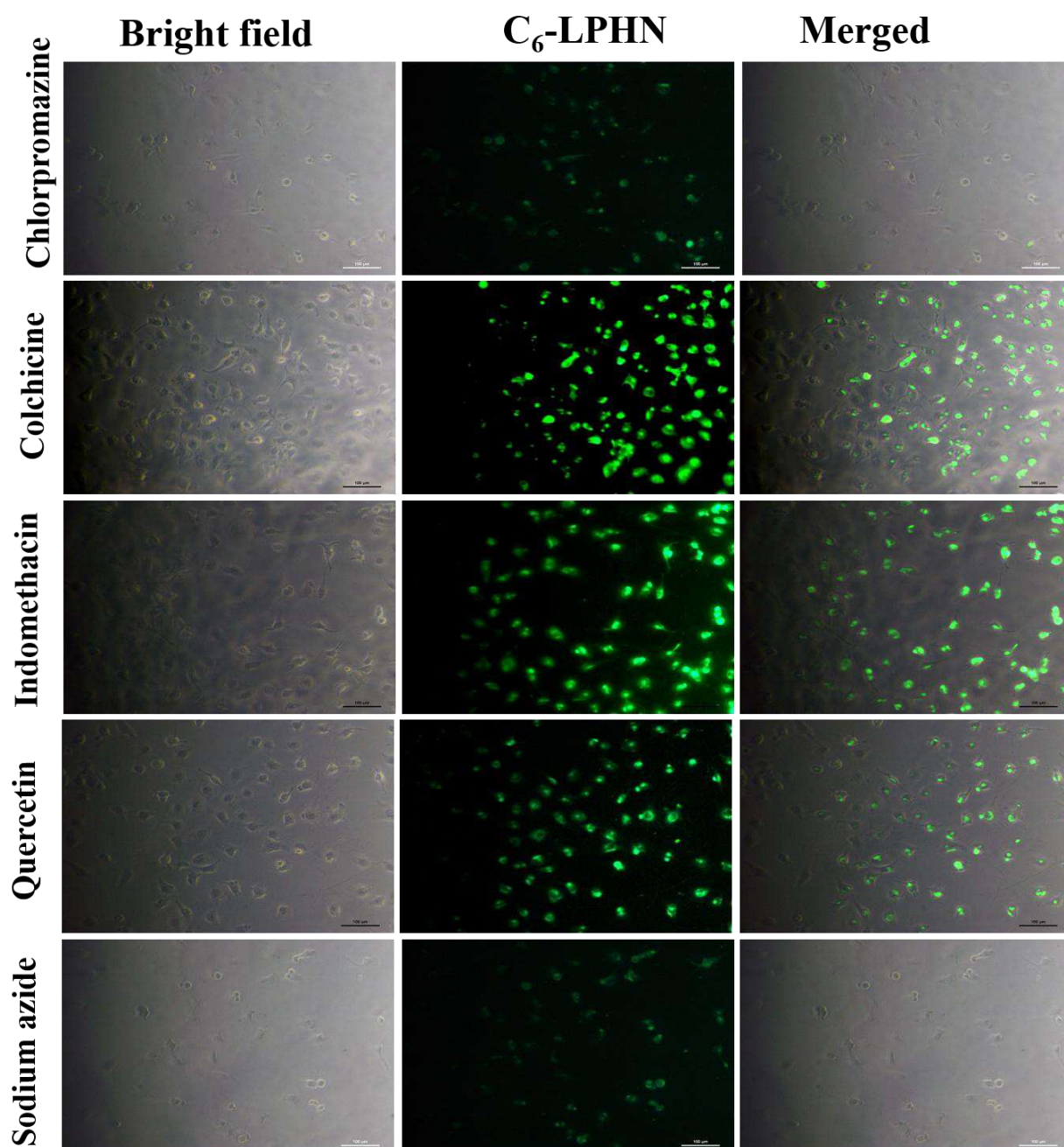


Figure 8: Mechanism of NPs uptake in MDA-MB-231 cells: Impact of different inhibitors on the uptake of C₆-LPHN.

3.3.8.5. MMP assay

Early stages of apoptosis are often characterized by mitochondrial membrane depolarization. JC-1 dye is commonly used to assess the MMP, as it accumulates in the polarized mitochondria of healthy cells, forming aggregates that emit red fluorescence. In contrast, in apoptotic cells, JC-1 remains in its monomeric form in the cytoplasm, emitting green fluorescence. The fluorescence images presented in **Fig 9A** illustrate the MMP of cells treated with ERL, SLB,

ERL-SLB-PM, and ERL-SLB-LPHN. Among these groups, the cells treated with ERL-SLB-LPHN exhibited a significantly lower red to green fluorescence ratio, as shown in **Fig 9C**. These findings revealed that the ERL-SLB-LPHN triggers mitochondria-related cell death more effectively than the free drugs. Which further confirmed by the high apoptotic potential and cytotoxicity of ERL-SLB-LPHN.

3.3.8.6. Apoptosis assay

The AO/EtBr dual staining technique was employed to assess the apoptotic potential of the formulation in the MDA-MB-231 breast cancer cell line. In this method, both viable and apoptotic cells present a distinct green fluorescence following the uptake of AO, which permeates intact cell membranes. In contrast, cells that have undergone necrosis exhibit red fluorescence due to the action of EtBr, which specifically binds to phosphatidylserine on the inner leaflet of the cell membrane once it is compromised. In our study, the cells treated with the novel formulation ERL-SLB-LPHN displayed a significantly more intense red fluorescence compared to those treated with the other formulations: ERL, SLB, and ERL-SLB-PM, as illustrated in **Fig 9B**. This observation suggests a pronounced induction of apoptosis in the ERL-SLB-LPHN-treated group. Additionally, the red-to-green fluorescence ratio was markedly elevated in cells exposed to ERL-SLB-LPHN, as shown in **Fig 9D**. This enhanced ratio not only underscores an increase in apoptotic activity but also implies that the nanoparticles facilitate greater cellular uptake and contribute to a more substantial disruption of MMP. This correlation indicates that the enhanced apoptotic potential observed with the ERL-SLB-LPHN formulation is likely attributable to its improved cellular interactions and membrane-targeting capabilities.

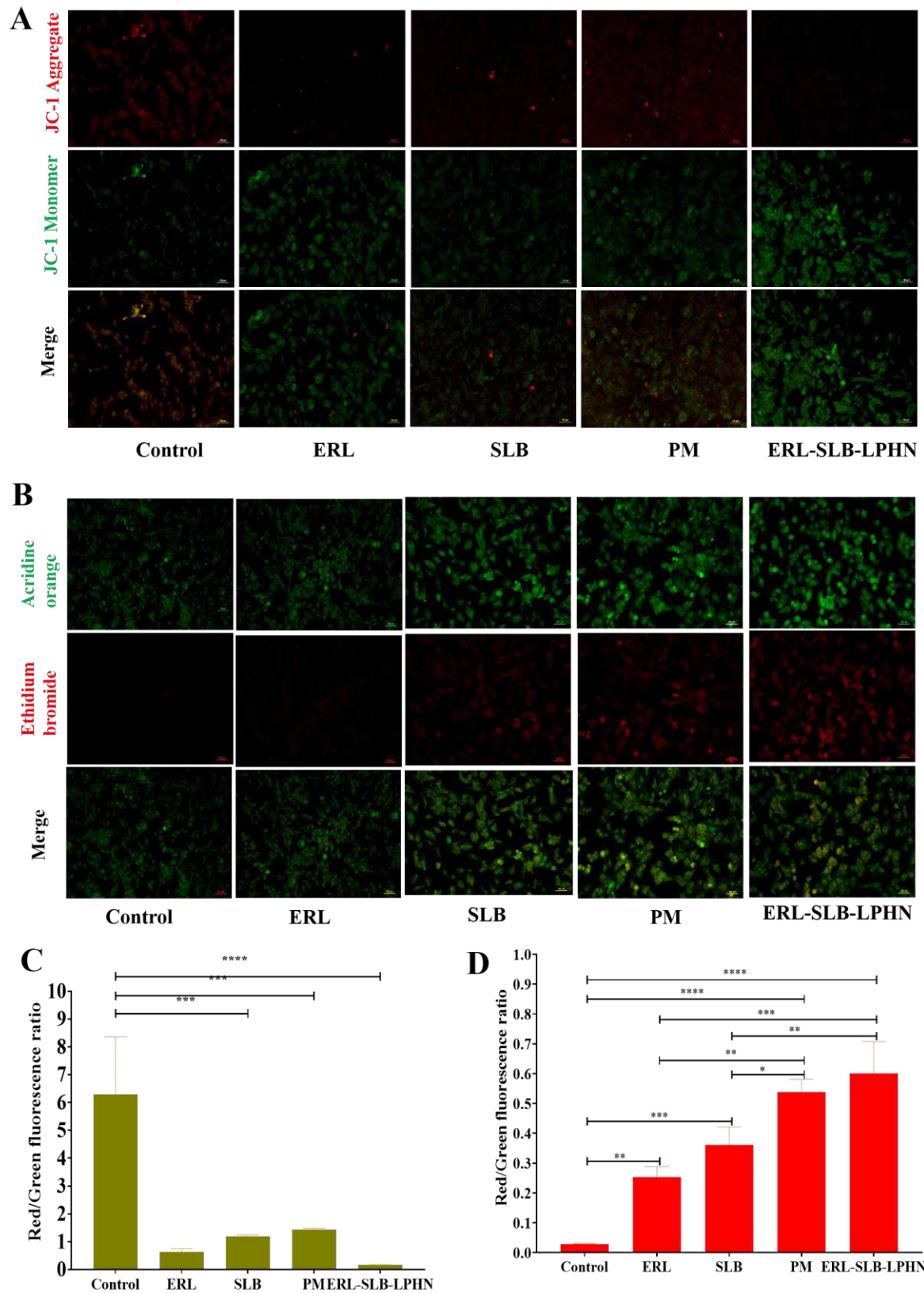


Figure 9: The figure represents JC-1, acridine orange, and ethidium bromide staining assay of MDA-MB-231 cells. (A) Fluorescence images of mitochondrial membrane depolarization in control, ERL, SLB, ERL-SLB-PM, and ERL-SLB-LPHN-treated MDA-MB-231 cells. (B) The

fluorescence microscope images were used to observe intracellular apoptosis in different groups, such as control, ERL, SLB, PM, and ERL-SLB-LPHN. (C) Red/green fluorescence intensity of JC-1 dye. (D) Graphical representation of apoptosis.

3.3.8.7. DAPI nucleic acid staining

DAPI is a blue fluorescent dye widely utilized in cell biology for staining the nuclei of MDA-MB-231 TNBC cells. This particular dye plays a crucial role in the selective identification of apoptotic cells by illuminating distinct nuclear alterations, which include condensed chromatin, fragmented nuclei, and the emergence of bright, horseshoe-shaped nuclei. As depicted in **Fig. 10A**, the results from DAPI staining revealed significant differences between the control and treatment groups. The control group exhibited uniformly round and intact nuclei, indicative of healthy and non-apoptotic cells. Notably, the most dramatic level of nuclear fragmentation was observed in the group treated with the ERL-SLB-LPHN formulation, highlighting its effectiveness in promoting apoptosis. The alterations in nuclear morphology serve as critical indicators of apoptosis, a form of programmed cell death that is essential for the elimination of damaged or unwanted cells. Additionally, the ERL-SLB-LPHN treatment exhibited the most remarkable apoptotic characteristics when compared to the other two treatments, ERL and SLB alone. Specifically, the cells treated with the ERL-SLB-LPHN formulation displayed significant nuclear shrinkage, aggressive fragmentation, and the formation of horseshoe-shaped configurations. These findings suggested that the co-delivery of SLB and ERL via a lipid-polymer hybrid nanocarrier not only enhances the induction of apoptosis but also amplifies nuclear damage in MDA-MB-231 cells, indicating a promising strategy for cancer therapy

3.3.8.8. Reactive oxygen species generation

The chemotherapy treatment elevates the ROS production, which plays a crucial role in decreasing tumor growth and promoting the death of tumor cells. This increase in ROS production was effectively measured by the conversion of the nonfluorescent compound dichlorofluorescein diacetate (DCFH) into the fluorescent compound DCF within cells treated with the ERL-SLB-LPHN formulation, particularly in comparison to the control group and treatments involving free drug administration. The fluorescence dye was utilized as a reliable indicator to quantify oxidative stress and measure ROS levels within the cellular environment. The ROS level of ERL-SLB-LPHN was comparatively higher than that of ERL, SLB, and ERL-SLB-PM in MDA-MB-231 cells, as shown in **Fig. 10 (B and C)**. The fluorescence images

exhibited that in the control group, there was negligible green intensity, which indicated less ROS production. On the other hand, ERL, SLB, ERL-SLB-PM, and ERL-SLB-LPHN showed more green intensity than the control group. When a direct comparison was made with that of ERL and SLB, the ERL-SLB-LPHN formulation yielded fluorescence signals that were approximately 2.16 times and 2.60 times greater in intensity than those of free ERL and SLB, respectively, in MDA-MB-231 cells. These results may be attributed to the improved intracellular concentration of ERL-SLB-LPHN, which facilitated the greater release of the ERL-SLB combination, thereby enhancing the overall production of ROS within the tumor cells. This suggests that the modified formulation not only improves drug delivery but also amplifies oxidative stress, contributing to its therapeutic efficacy against breast cancer.

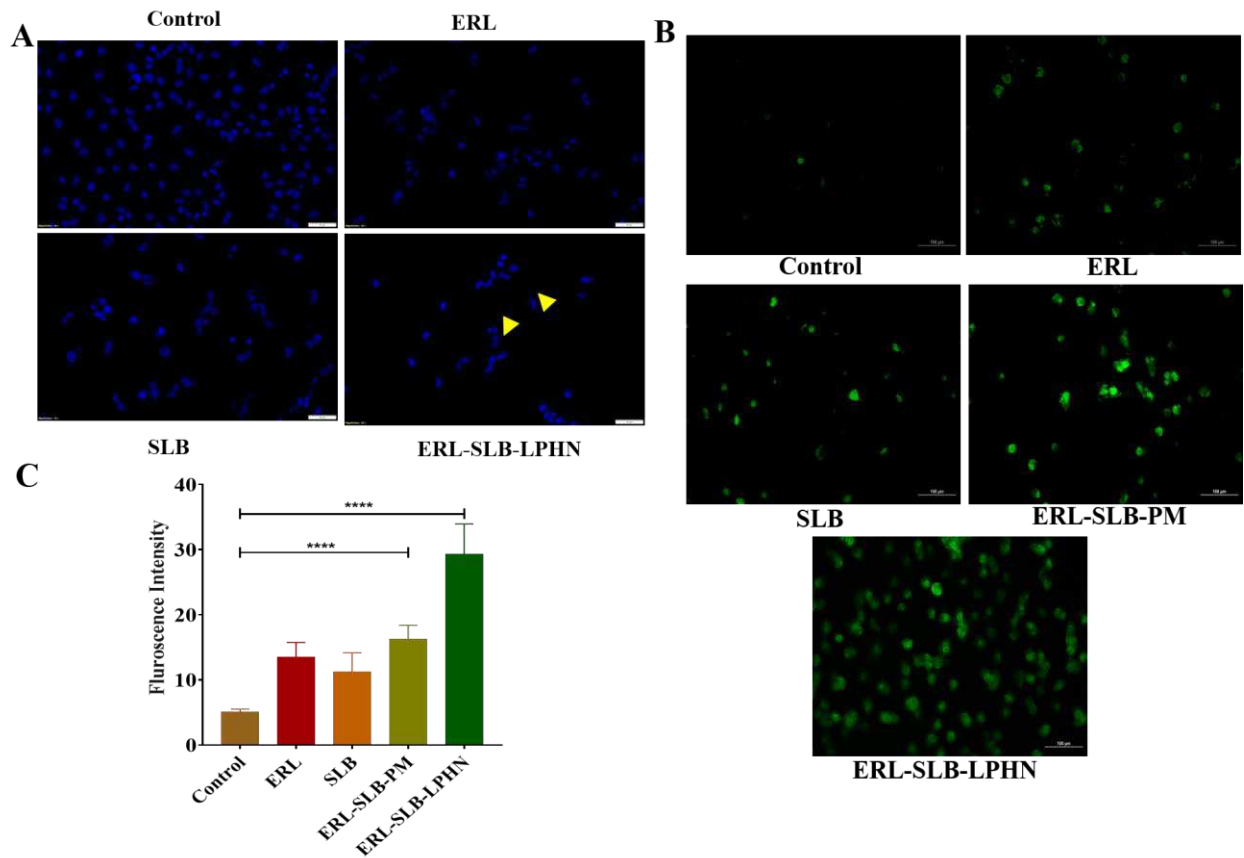


Figure 10: (A) DAPI staining images of treatment with ERL, SLB, and ERL-SLB-LPHN at the IC₅₀ dose on MDA-MB-231 cells. The ROS assay in MDA-MB-231 cells. (B) Fluorescence images of DCFDA staining showing ROS generation after treatment with ERL, SLB, ERL-SLB-PM, and ERL-SLB-LPHN. (C) Graphical representation of ROS assay.

3.3.8.9. 3D tumoroid test

The 3D tumoroid test was performed using the MDA-MB-231 cell line. The cells were treated with the various concentrations of ERL, SLB, and ERL-SLB-LPHN, and morphological changes were evaluated after treatment. As illustrated in **Fig 11A**, morphological evaluation demonstrates that the cells treated with the low concentrations of ERL and SLB remain in a compact mass, with noticeable detachment occurring only at higher concentrations. Conversely, in the ERL-SLB-LPHN-treated spheroid core, cells detached from the compact mass at lower concentrations, demonstrating higher cell death at lower nanoparticle concentrations than with free drugs. Further confirmation was performed using AO/EtBr staining, and a higher red colour intensity was observed in the spheroid treated with ERL-SLB-LPHN, as shown in **Fig 11 B**. Thus, ERL-SLB-LPHN induced higher apoptosis in tumor cells than the free ERL and SLB. The results obtained from this study suggest that ERL-SLB-LPHN has potential for the targeting and treatment of breast cancer cells.

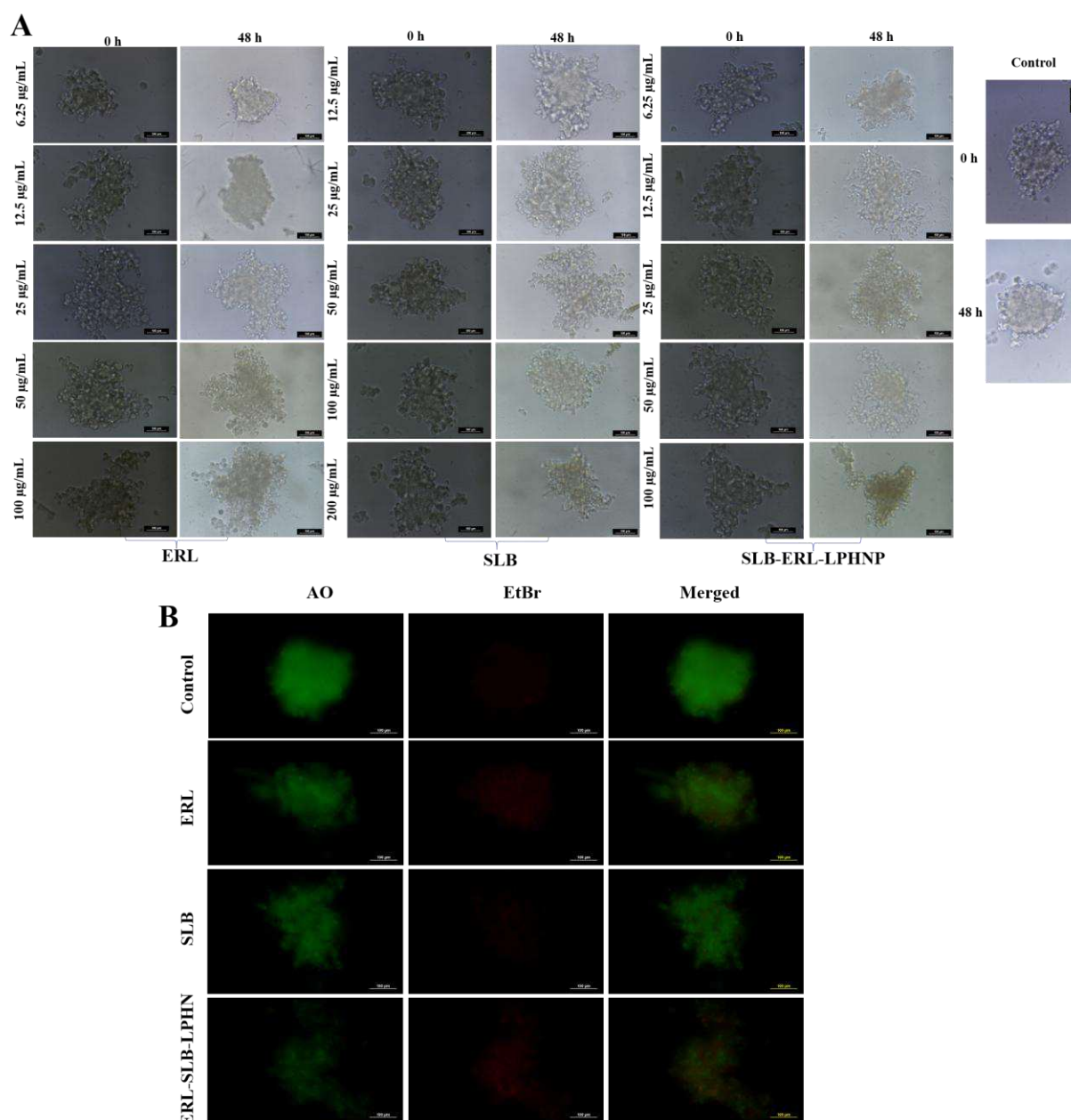


Figure 11. A. Morphological assessment of MDA-MB-231 cells 3D tumoroid treated with 6.25 to 100 µg/ml of ERL, 12.5 to 200 µg/ml of SLB, and ERL-SLB-LPHN concentration equivalent to ERL. B. AO/EtBr staining images of 3D tumoroid for apoptosis detection.

3.3.9. In vivo animal studies

3.3.9.1. Pharmacokinetics study

A pharmacokinetic (PK) study was performed on Balb/c female mice to examine how ERL-SLB-LPHN affects the systemic exposure and clearance of nanoparticles when administered intravenously, in comparison to the free drug. The plasma drug concentration profiles for ERL,

SLB, SLB-LPHN, and ERL-LPHN are depicted in **Fig 12A**, while the various pharmacokinetic parameters are detailed in **Table 7**. The nanoparticle formulation demonstrated improved PK properties over the free forms of ERL and SLB. The $C_{\max}$ for ERL was 2.26 ± 0.24 ppm, surpassing that of ERL-LPHN post-intravenous administration. The AUC_{0-t} and MRT for ERL-LPHN were 14.82 ± 1.95 ppm*h and 24.82 ± 2.96 h, respectively, which were 1.49 and 5.19 times greater than those of the free ERL. Conversely, the AUC_{0-t} and MRT for SLB-LPHN were 4.94 ± 0.69 ppm*h and 6.76 ± 1.65 h, exceeding those of the free SLB. The increased AUC suggests enhanced systemic exposure to the nanoparticles compared to the free drug. Additionally, the half-life of SLB-LPHN and ERL-LPHN indicated a more sustained release than the free ERL and SLB. The $t_{1/2}$ for SLB-LPHN was 3.37 times longer than that of the free ERL, and ERL-LPHN exhibited a 3.78-fold increase in $t_{1/2}$ compared to the free SLB. The sustained release of ERL and SLB from SLB-LPHN and ERL-LPHN led to prolonged $t_{1/2}$, increased AUC_{0-t} , and extended MRT. The analysed pharmacokinetic parameters suggest that SLB-LPHN and ERL-LPHN have a longer circulation time in the bloodstream compared to free ERL and SLB, which are rapidly cleared from the blood. This prolonged circulation is likely attributed to the slow diffusion and sustained release properties of ERL and SLB from the lipid-polymer matrix of the nanoparticles.

Table 7: Pharmacokinetic parameters of ERL, SLB and ERL-SLB-LPHN.

Pharmacokinetics Parameter	ERL	SLB	SLB-LPHN	ERL-LPHN
Half-life ($t_{1/2}$) (h)	5.97 ± 1.25	1.97 ± 0.18	7.45 ± 1.3	20.12 ± 3.22
$C_{\max}$ (ppm)	2.26 ± 0.24	1.11 ± 0.09	1.2 ± 0.12	1.32 ± 0.06
AUC (0-t) (ppm*h)	9.94 ± 1.11	3.65 ± 0.43	4.94 ± 0.69	14.82 ± 1.95
AUC (0-inf_obs)(ppm*h)	10.36 ± 2.11	3.85 ± 0.29	6.22 ± 0.96	17.55 ± 2.26
MRT (0-inf_obs) (h)	4.19 ± 0.52	1.8 ± 0.01	6.76 ± 1.65	24.82 ± 2.96

3.3.9.2. Toxicity analysis

To assess the effects of ERL, SLB, and ERL-SLB-LPHN on vital organs, such as the kidneys and liver, following repeated dosing, an acute toxicity study was performed. Also, the levels of biomarkers such as AST, ALT, and creatinine were evaluated to provide details on possible systemic toxicity, as shown in **Fig 12B, C & D**. The group treated with ERL demonstrated significantly higher levels of liver and kidney biomarkers such as AST, ALT, and creatinine than the group treated with saline vehicle. Whereas the group treated with ERL-SLB-LPHN showed no substantial increase in the level of these biomarkers compared with saline. The histological study was performed using haematoxylin and eosin staining through microscopic imaging, depicted in **Fig 12E**. ERL-SLB-LPHN showed no morphological difference compared to saline, with intact tubules in the kidney and no clear vacuolation in live. Conversely, the group treated with ERL showed damage to tubules in the kidney and irregular damage in the central hepatic vein in the liver section. The results obtained from this study suggest that ERL-SLB-LPHN shows a protective effect on the toxicity caused by the free ERL on the liver and kidney.

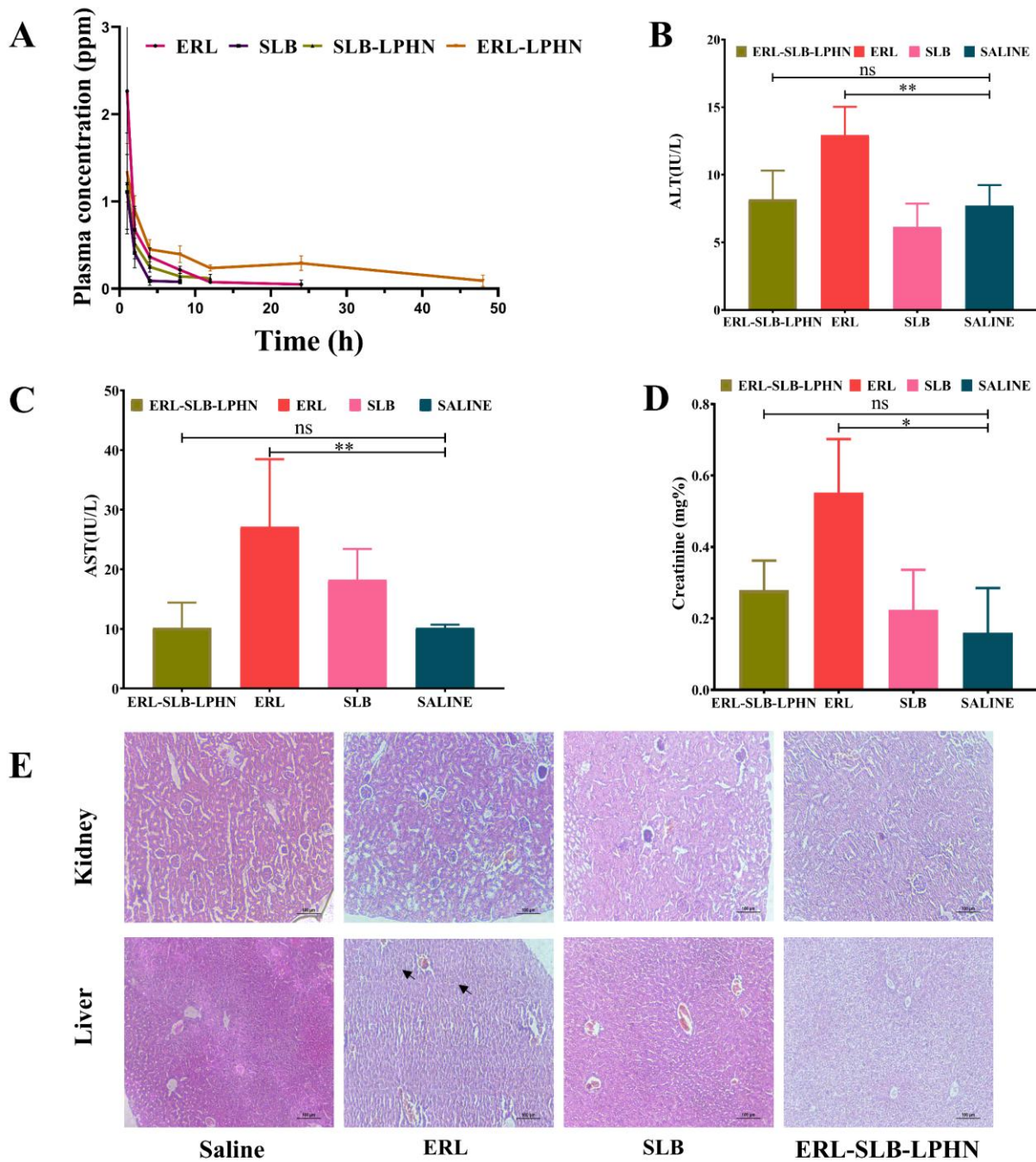


Figure 12. (A) Pharmacokinetic profile of ERL, SLB, SLB-LPHN, and ERL-LPHN. (B, C, and D) Investigation of the level of biomarkers and comparison with saline. (E) Microscopic histological images of the kidney and liver after treatment with ERL, SLB, and ERL-SLB-LPHN.

4. Conclusion

ERL-SLB-LPHN was successfully fabricated by the nanoprecipitation method for the treatment of triple-negative breast cancer. The formulation was optimized by screening design (Taguchi) and the Box-Behnken method to determine the effect of independent variables on dependent variables. The average particle size obtained was 160.1 ± 1.28 nm with PDI 0.1243 ± 0.012 . The release study showed the sustained drug release of ERL and SLB, 49.65 ± 1.3 and 34.93 ± 1.2 %, respectively, from the nano formulation up to 48 h. The formulation showed low haemolytic activity and no change in prothrombin time, implying the hemocompatibility of the formulation. The antioxidant property of SLB is present in formulation $62.66 \pm 0.89\%$. The stability study showed that the formulation is stable for 1 month at 2-8 °C and unstable at room temperature. Cell culture studies revealed that ERL-SLB-LPHN significantly enhances cellular toxicity and cellular uptake in the MDA-MB-231 cell line. The pharmacokinetics data showed that SLB-LPHN and ERL-LPHN showed prolonged $t_{1/2}$, increased AUC_{0-t} , and extended MRT compared to the free ERL and SLB. Further, ERL-SLB-LPHN showed favourable biocompatibility and safety in a toxicity study.

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Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Bhavana Gajare: Conceptualization, Execution, Analysis, Methodology, Interpretation, Data collection, **Tanmoy Kanp:** Conceptualization, Execution, Analysis, Methodology, Interpretation, Data collection; **Mayur Aalhate:** Conceptualization, Contribution to cell culture studies, **Anish Dhuri:** Writing – original draft; **Neeraj Kumar Ravi:** Review and editing; **Bharath Manoharan & Khushi Rode:** Review & editing; **Rahul Nair, Priti Paul & Yadav Hira Kamlesh:** Review & editing, **Pankaj Kumar Singh:** Conceptualization, Supervision, Resources, Review & editing, Visualization.

Data availability

Data will be made available on request

Declaration

Ethics approval: The Institutional Animal Ethics Committee (IAEC) at NIPER Hyderabad (NIP/12/2024/PE/675) ethically approved all animal procedures.

Consent for publication: Informed consent was not required as no human participants were involved.

Competing Interests: The authors declare no competing interests.

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