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Ezetimibe-Loaded Nanostructured Lipid Carrier for Oral Delivery: Response Surface Methodology; In Vitro Characterization and Assessing the Antihyperlipidemic Effect in Rats

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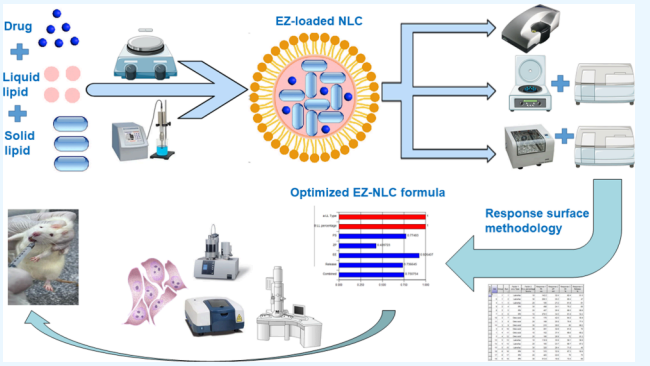
ABSTRACT: Among the independent risk factors for the occurrence of cardiovascular diseases like atherosclerosis is hyperlipidemia. To decrease cardiovascular events and patient mortality, antihyperlipidemia therapy is crucial. Our study aimed to enhance the solubility of the poorly soluble lipid-lowering agent ezetimibe (EZ), a member of class II as per the Biopharmaceutics Classification System (BCS). The drug was formulated as a nanostructured lipid carrier (NLC) employing the ultrasonication technique. A response surface D-optimal design was employed to study the effect of changing the liquid lipid type and the percentage of liquid lipid with respect to total lipid amount on the particle size, zeta potential, percentage entrapment efficiency, and percentage of drug released after 24 h. Nine NLC formulations were prepared and pharmaceutically evaluated, and the optimized NLC formulation was selected, further characterized, and evaluated as well. Optimized EZ-NLC was assessed in the high-fat diet model to induce hyperlipidemia in rats in comparison with the EZ suspension. The results of the optimized formulation showed that the prepared NLCs were spherical with no aggregation having a particle size of 204.3 ± 19.17 nm, zeta potential equal to −32 ± 7.59 mV, and entrapment efficiency of 81.5 ± 3.58% and 72.15 ± 4.58% drug released after 24 h. EZ-NLC significantly decreased the elevated serum lipid parameters, including total cholesterol, triglycerides, and LDL-C, but significantly normalized serum HDL-C levels of rats kept on a high-fat diet. The results demonstrated the improved efficacy of EZ-NLC in ameliorating the elevated serum lipid parameters compared to EZ.



1. INTRODUCTION

High plasma cholesterol levels known as hypercholesterolemia (hyperlipidemia) are responsible for the death of about 2.6 million people all over the world, which represents 4.5% of the total mortality. This condition is defined by a decrease in high-density lipoprotein (HDL) levels (cholesterol <40 mg/dL) besides an increase in low-density lipoprotein (LDL) levels (cholesterol >190 mg/dL) as well as triglycerides (TG) in plasma (>200 mg/dL), which ultimately leads to atherosclerosis.[1] Hypercholesterolemia increases the risk of developing several cardiovascular diseases. Standard antihyperlipidemic medications, such as statins, fibrates, and bile acid-binding resins, are frequently used to treat those risk factors. Unfortunately, these substances are frequently related to a variety of adverse medication reactions including gastrointestinal problems, muscular illnesses, and enhanced liver enzyme activity. Statin intolerance along with family diseases, treatment resistance, noncompliance, and optimization of lipid-lowering therapy, especially in severe hypercholesterolemia, continues to be therapeutic challenges in addition to adverse responses. Thus, there is a need for brand-new, potent, and secure therapeutic substances that decrease cholesterol levels while reducing the serious side effects of existing lipid-lowering medications.[2] Moreover, a novel approach for combining nonstatin agents with low-intensity statins instead of high-intensity statins has gained attention to minimize statin intolerance and discourage therapy discontinuation.[3]

Ezetimibe (EZ), a novel agent developed with exceptional cholesterol-lowering effects, is the latest successful treatment for hypercholesterolemia after statins.[4] EZ (synthetic 2-azetidinone) can be used as a monotherapy or combined with statins for reducing high cholesterol levels and treating primary hypercholesterolemia. It has great characteristics, with respect to its tolerance and side effects. It selectively inhibits biliary and dietary cholesterol absorption from the small intestine by

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blocking the Niemann-Pick C1-like l protein without affecting the fat-soluble vitamins and nutrients absorption.[5] Furthermore, there is little chance that EZ will have negative effects on muscles due to its reduced pharmacokinetic interactions with medications that undergo metabolism by cytochrome P450.[6]

Unfortunately, EZ is a member of class II as per the Biopharmaceutical Classification System (BCS) which is known for having poor water solubility besides high tissue permeability.[7] It is a highly lipophilic molecule with a log p of 4.5 and displays intersubject variability with oral bioavailability of (35−60%).[8] This variable bioavailability is attributed to its poor aqueous solubility (0.00846 mg/mL) and consequently, dissolution rate, besides that it has an extensive efflux by p-glycoprotein (P-Gp).[9] To increase the oral bioavailability of poorly soluble substances, various techniques have been developed over time. These methods have advantages and few disadvantages, according to Aukunuru et al.[10] Their main advantages are represented in significantly speeding up the dissolution rate of drugs and enhancing their oral bioavailability, besides reducing variability and minimizing the favorable food effects of therapeutic molecules administered orally as mentioned by Kesisoglou et al.[11]

New formulations based on nanotechnology showed the most potential among these strategies and had the fewest drawbacks as nonspecificity, toxicity, and high cost according to Talegaonkar and Rai.[12] These drug delivery systems include polymeric micelles, biodegradable polymeric nanoparticles, nanosuspensions, nanocrystals, and lipid colloidal nanoparticles. Lipid nanoparticles are composed of either natural or synthetic lipids. They have the good properties of controlling the release of drugs with great biocompatibility.[13]

Additionally, those NPs displayed excellent properties related to their good production scaleup, controlled drug release, avoiding the use of organic solvents throughout the preparation process, and minor cytotoxicity along with having various possible applications.[14]

The nanostructured lipid carriers (NLCs), the new generation of lipid NPs, are composed of a blend of diverse types of lipids; i.e., a solid lipid is mixed with a liquid one. The incorporation of liquid lipids disrupts perfect lipid crystal formation and therefore increases the loading capacity of the drugs. This in turn causes a decrease in the size of the produced particles and lowers the risk of drug leakage and gelation on storage.[13]

Due to the hydrophobic nature of the carrier, NLCs exhibit significant favorability for lipophilic medicines. In addition to the previously mentioned advantages, NLCs possess superiority in pH and enzyme degradation prevention, sensory masking, and most outstandingly P-glycoprotein (P-gp) efflux circumvention by passing through the cell membrane via endocytosis.[15−17]

To our best knowledge, few comparative studies considered the utilization of SLNs and NLCs in an attempt to enhance the solubility of EZ, for example, refs 7,18−21. However, none of them focused on optimizing the composition of different formulations to attain the maximum improvement in aqueous solubility and drug efficacy. Moreover, our study was carried out using different types (oleic acid, labrafac, and isopropyl myristate) and concentrations (10%, 20%, and 30%) of liquid lipids in order to select the most desired EZ-NLC formulation.

Generally speaking, to contribute to the better therapeutic management of hypercholesterolemia, this study was undertaken to improve the effectiveness of the poorly water-soluble drug (EZ) through its encapsulation into a nanostructured lipid carrier. The effects of various types and percentages of the liquid lipid used were investigated and optimized to select the most desired EZ-loaded NLC formula using response surface methodology and employing D-Optimal design by Design-Expert 10.1.1 software (Stat-Ease Inc., Minneapolis, MN). Nine formulations were prepared and in vitro-characterized for particle size, zeta potential, percentage entrapment efficiency, and percentage drug release after 24 h. An additional objective of this work was to assess the safety and clinical efficacy of the optimized EZ-NLC formula via cellular toxicity assessment as well as evaluating the antihyperlipidemic effect through lipid profile assessment in rats.

RESULTS AND DISCUSSION

2.1. Statistical Analysis of the Experimental Design.

The effects of liquid lipid (LL) type (a) and percentage of liquid lipid with respect to total lipid amount (LL %w/w) (B) as independent variables were evaluated on four dependent variables, the particle size (PS) of EZ-NLC (R1), zeta potential (ZP) (R2), entrapment efficiency (EE) (R3), and % drug release after 24 h (R4). Analysis of variance (ANOVA) was employed to choose the most suitable mathematical fitting model (linear, 2FI, cubic, and quadratic) by comparing various statistical parameters such as R^2, adjusted R^2, and sequential p-value using Design-Expert 10.1.1 software.[22,23]

It was found that the two factor interaction (2FI) was the best model for R1 and R3 while the quadratic model was the best fit for R2 and R4. Design-Expert 10.1.1 software decided on these models based on ANOVA study displaying a high F-value, high adjusted and predicted R^2 (difference <0.2), high correlation coefficient, and nonsignificant lack of fit. Each response's relationship to the two independent variables was observed independently.[24] Table 1 summarizes the results of the analysis for the four responses.

Table 1. ANOVA Analysis of Response Results Using Design Expert 10 Software

response	model	R^2	adjusted R^2	equation
R1	2FI	0.96	0.94	R1 = +270.69 − 86. 16 × a[1] − 67. 46 × a[2] + 71.44 × B − 48. 19 × a[1]B + 15. 36 × a[2]B
R2	quadratic	0.85	0.72	R2 = +25.85 + 3. 63 × a[1] − 1. 41 × a[2] − 5. 3 × B + 0. 68 × a[1]B − 0.13 × a[2]B + 4. 78 × B^2
R3	2FI	0.89	0.81	R3 = +69.09 + 4. 42 × a[1] − 4. 21 × a[2] + 5. 77 × B + 1. 96 × a[1]B − 0. 84 × a[2]B
R4	quadratic	0.95	0.9	R4 = +71.98 + 7. 33 × a[1] − 7. 95 × a[2] + 1. 91 × B + 4. 14 × a[1]B − 7. 23 × a[2]B − 15.18B^2

The coefficient of B, which refers to the percentage of liquid lipid with respect to total lipid amount, is linked to the influence of this independent variable on the response. A synergistic effect is indicated by a positive sign of the coefficient, whereas an antagonistic effect is indicated by a negative one upon the response. The greater value of the coefficient denotes the more powerful effect of the independent variable on the response.[22]

Concerning the type of liquid lipid a, the software considered one of the levels of this categorical factor as a dummy variable. Accordingly, a[1] and a[2] refer to oleic acid and labrafac, respectively, while IPM was assigned to be the dummy variable. A positive sign of a reflects a rise in the response in comparison

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Table 2. Composition of Different EZ-NLC Formulations along with Their Observed Values of Responses

formula no.[a]	liquid lipid type a	liquid lipid %w/w of total lipid B	(R1) P.S (nm) ± SD	(R2) Z.P (mV) ± SD	(R3) EE% ± SD	(R4) %drug release ± SD	PDI ± SD[c]
F_1	oleic acid	10	160.95 ± 19.31	−39.95 ± 2.26	66.85 ± 3.75	58.05 ± 4.22	1 ± 0.0305
F_2	labrafac	10	130.75 ± 16.97	−34.14 ± 3.6	60.25 ± 3.04	54.15 ± 3.35	1 ± 0.015
F_3	IPM[b]	10	297.85 ± 25.46	−33.7 ± 1.13	65.9 ± 3.79	52.5 ± 4.21	0.808 ± 0.006
F_4	oleic acid	30	207.5 ± 16.97	−30.7 ± 2.96	82.3 ± 0.98	70.15 ± 2.48	0.387 ± 0.017
F_5	labrafac	30	304.45 ± 22.63	−23.3 ± 4.38	70.1 ± 0.35	43.5 ± 3.8	0.564 ± 0.025
F_6	IPM	30	506.7 ± 11.29	−22.05 ± 2.96	73.85 ± 3.46	62.5 ± 5.18	0.334 ± 0.03
F_7	oleic acid	20	185 ± 29.7	−27.35 ± 2.19	71.4 ± 0.84	79.4 ± 2.12	0.647 ± 0.031
F_8	labrafac	20	175.85 ± 14.18	−25.45 ± 2.48	64.3 ± 3.39	64.1 ± 3.35	0.881 ± 0.021
F_9	IPM	20	470.75 ± 18.38	−24.75 ± 3.04	68.25 ± 2.47	72.45 ± 3.56	0.299 ± 0.009

[a]All NLC formulations contain 10 mg of EZ. [b]IPM: isopropyl myristate. [c]PDI: polydispersity index.

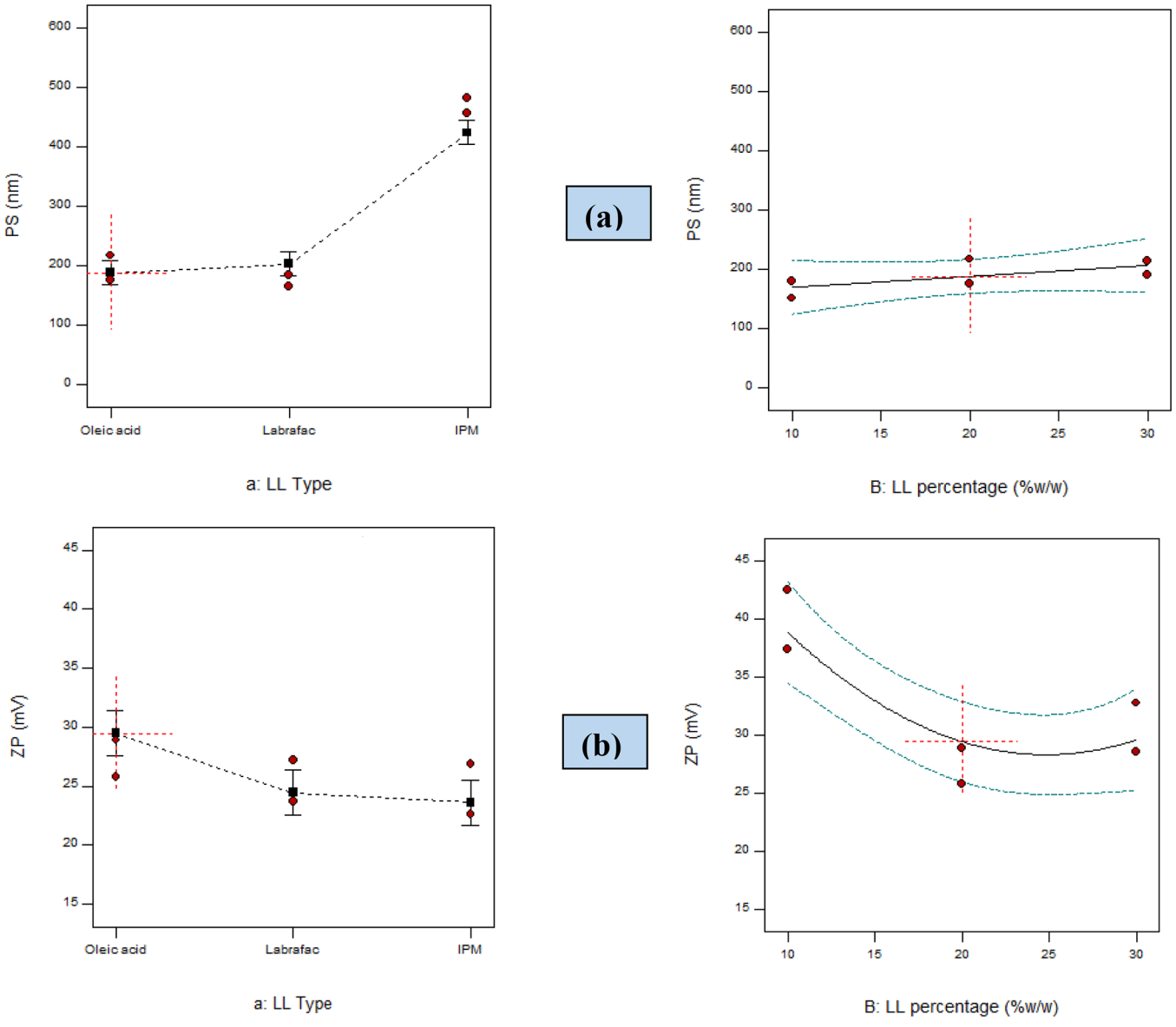

Figure 1. Effects of the liquid lipid type and liquid lipid percentage on the dependent variables of different EZ-NLC formulations: (a) particle size and (b) zeta potential.

with IPM, whereas the negative coefficient denotes a fall in the response as compared to IPM.[25,26]

2.1.1. Effect of Formulation and Process Variables on Particle Size (R1). Physicochemical characterization of different EZ-NLCs was estimated with regard to the mean particle size (PS), polydispersity index (PDI), and zeta potential (ZP). Results are recorded in Table 2 as the mean ± standard deviation (SD) of three investigations. It was observed that the particle size of EZ-NLC formulations ranged from 160.95 ± 19.31 to 506.7 ± 11.29 nm. Generally, the incorporation of two

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emulsifiers (tween 80 and poloxamer 188) with different HLB values played an important role in varying the particle size of different EZ-NLC formulations.[27] Tween 80 was chosen from a range of surfactants owing to its regulatory approval and ability to effectively prepare a variety of NLCs. Moreover, poloxamer 188 was selected as a second nonionic surfactant due to its capability to improve the NLC's mechanical stability, besides its ability as a hydrophilic polymer, to create a coating layer around lipid particles. Consequently, they significantly extend the residence period of therapeutic molecules in the systemic circulation.[28]

The statistical analysis of the particle size revealed significant effects of liquid lipid type and liquid lipid percentage on the response with (p-value <0.0001) for both independent variables. The positive coefficient of B in the equation displayed in Table 1 refers to the direct relationship between the percentage of liquid lipid with respect to total lipid amount (LL%w/w) and the PS as observed from Table 2 and Figure 1a. This means that higher liquid lipid percentages were accompanied by an increase in the PS values of the formed NLCs. This relation came in great agreement with previously carried out experiments and comparative investigations and can be elucidated on the basis of three theories. First, with increasing liquid lipid percentage, the process of droplet disruption would be challenging as a result of higher resistance of flow, and therefore, the rate of breaking up of the droplet is going to be greatly limited. Additionally, at constant emulsifier and high oil percentages, an imperfect coverage of emulsifier particles on the recently formed oil−water interface could probably cause an increase in the size of droplets. The last possible rationalization for this relation could be attributed to the high chance of droplet coalescence resulting from the elevated rates of collision frequency between the emulsion droplets.[29,30]

Concerning the liquid lipid type, the negative coefficients of both a[1] and a[2] show that the incorporation of oleic acid as well as labrafac in NLCs, affected the PS negatively compared to IPM. This means that EZ-NLC formulations prepared by IPM as liquid lipid displayed the largest values for PS and the switching to either oleic acid or labrafac caused the formation of particles with a smaller size range. The increase in the particles' size could be directly linked to the length of the carbon chain of liquid lipids incorporated in EZ-NLC formulations. For instance, labrafac is a medium chain triglyceride (MCT) whereas oleic acid and IPM belong to long chain triglycerides (LCT) class of lipids; that is why the size of particles prepared using labrafac displayed relatively smaller values than those of IPM. However, oleic acid despite being a LCT, produced carriers with small size of particles, this exception could possibly be due to the ionic interaction between the tertiary amine group in EZ and the carboxylic acid group of oleic acid which caused the high solubility of EZ in oleic acid and consequently the high entrapment of the drug within the formed NLC that in turn decreased the particle size significantly.[31,32] The effects of changing the liquid lipid type and liquid lipid percentage are listed in Figure 1a.

ANOVA analysis revealed that the interaction between the two independent variables was significant for the particle size, with a p-value of 0.0065. The negative coefficient of a[1]B in the equation displayed in Table 1 denotes the antagonistic effect of oleic acid on the percentage of liquid lipid; this means that a significant decrease in the particle size was observed at higher percentages of oleic acid. This effect may be due to the ionic interaction between the tertiary amine group in EZ and the carboxylic acid group of oleic acid that resulted in the high entrapment of EZ within the formed NLC and in turn the reduction in PS values,[31,32] unlike labrafac, which produced a synergistic effect on the percentage of liquid lipid indicated by the positive coefficient of a[2]B. To explain, higher labrafac percentage caused an increase in the particle size of NLCs and this follows the direct relationship observed between B and PS values. Additionally, it was found that increasing the IPM amount caused a significant increase in the values of particle size, which suggests the synergistic effect of this LCT on the percentage of liquid lipid with respect to the total lipid amount. To conclude, increasing labrafac and IPM percentages caused the formation of NLCs with higher PS values than oleic acid.

The polydispersity index (PDI) is a reflection of the homogeneity of nanoformulations. It was observed that the PDI of all prepared NLC formulations was less than or equal to 1, which indicates that they were sufficiently homogeneous.

2.1.2. Effect of Formulation and Process Variables on Zeta Potential (R2). The knowledge of zeta potential (ZP) values is required to consider the physical stability of all colloidal dispersions. ZP gives an image of the repulsion power between the charged molecules. Typically, in order to achieve optimum stability for nanoparticles stabilized electrostatically, at least a ZP of ±30 mV is necessary; however, for the stability of nanoparticles with both steric and electrostatic forces, a zeta potential of ±20 mV can be sufficient. This is because the stability of NLC can additionally be improved by coating with a hydrophilic surfactant through the hydration of the external layer.[33] By reducing the interfacial tension between the dispersed phase and the dispersion medium and therefore bypassing particle agglomeration and coalescence, the surfactants added in the prepared EZ-NLCs played an important role in their stabilization.[14]

It was observed that the zeta potential of different EZ-NLC formulations held negative charges and ranged from −22.05 ± 2.96 to −39.95 ± 2.26 mV as shown in Table 2. These negatively charged values were promising and provided good qualities of long-term stability and particle adhesion; besides, they might be related to the carboxylic group of free fatty acids (FFA) in the structure of lipids used in the formulations.[24,28]

The statistical analysis of ZP for different EZ-NLC formulations demonstrated significant impacts of a (p-value = 0.0121) and B (p-value = 0.0005) upon the response. It was observed from Figure 1b that the formulations prepared using oleic acid displayed larger values of ZP compared to IPM as indicated by the positive coefficient of a[1]. This might be accredited to the relatively smaller particle size of the formulations fabricated using oleic acid along with being the longest triglyceride (TG) among others used and according to ref 34, larger ZP values are produced by lengthening the TG's carbon chain. On the other hand, shifting to labrafac revealed a negligible effect with regard to ZP values in comparison with IPM as observed from Figure 1b.

To consider the influence of the liquid lipid percentage on observed zeta potential values, ANOVA analysis revealed a nonlinear relationship, indicated by the negative coefficient of B and the positive coefficient of B^2 in the equation shown in Table 1. To illustrate, it was observed that increasing the liquid lipid percentage from 10% to 20% of the total lipid content caused a decrease in the values of zeta potential including all types of liquid lipids. One possible justification for this observation might be credited to the disruption of the surfactant layer upon raising the percentage of liquid lipids which consequently resulted in

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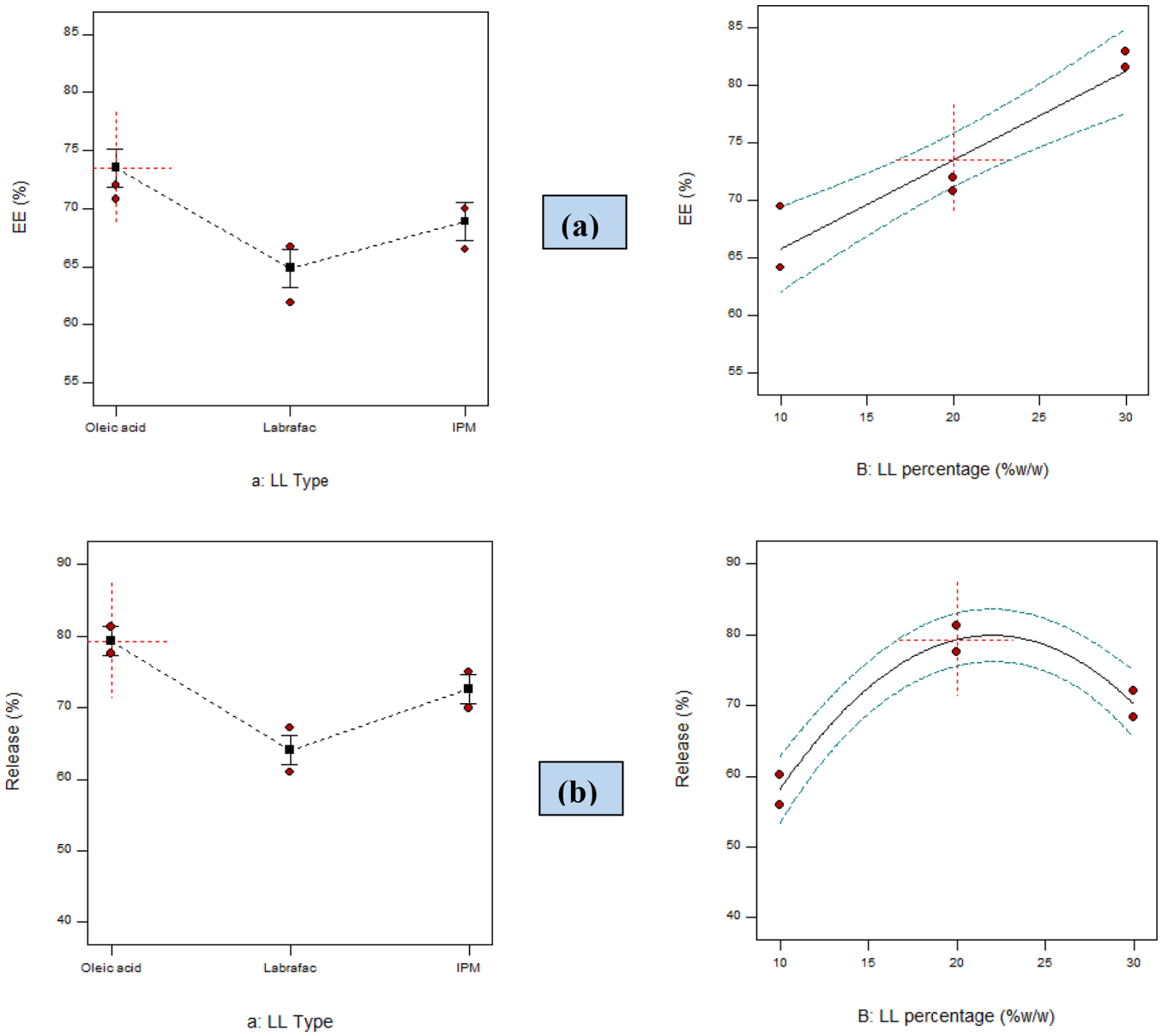

Figure 2. Effects of the liquid lipid type and liquid lipid percentage on the dependent variables of different EZ-NLC formulations: (a) entrapment efficiency percentage and (b) in vitro drug release percentage.

the rearrangement of charges on the surface and the reduction of ZP values.[35] Another theory claimed that increasing the percentage of lipophilic liquid lipids that are devoid of free hydroxyl groups caused a decrease in ZP values resulting in poor stability of NLC particles.[36]

However, it was noticed that raising the liquid lipid percentage from 20% to 30% resulted in a slight elevation of ZP value in the case of oleic acid only. This could be due to the relatively smaller PS of NLCs prepared using oleic acid or might be attributed to the increased number of ionizable (charged) carboxylic groups of FFA on the surface of the prepared NLCs producing more stable particles.[28,33] On the other hand, increasing labrafac and IPM percentages did not affect the relation and continued on reducing ZP values.

2.1.3. Effect of Formulation and Process Variables on Entrapment Efficiency % (R3). It was noticed that the entrapment efficiency percentage of EZ-NLC formulations ranged from 60.25 ± 3.04 to 82.3 ± 0.98% as shown in Table 2. ANOVA statistical analysis revealed that a and B affected EE% significantly with (p-value = 0.004) and (p-value <0.0001), respectively. The positive coefficient of B denotes the direct relationship between the percentage of liquid lipid with respect to the total lipid amount and the entrapment efficiency percentage of EZ in NLCs; this means that increasing the proportion of liquid lipid was associated with higher entrapment of drug molecules within the NLCs. It was reported from previous studies that the incorporation of liquid lipids into solid ones caused a huge disturbance in the crystal order, which permits enough space for the drug molecules to be accommodated and accordingly this space increases proportionally with the rise in liquid lipid content.[13,37]

For the type of liquid lipid used, it was observed from Figure 2a that oleic acid significantly raised the percentage entrapped of EZ in NLCs when compared to IPM as denoted by the positive coefficient of a[1]. However, the negative coefficient of a[2] refers to the fall in EE% when IPM is replaced with labrafac. The

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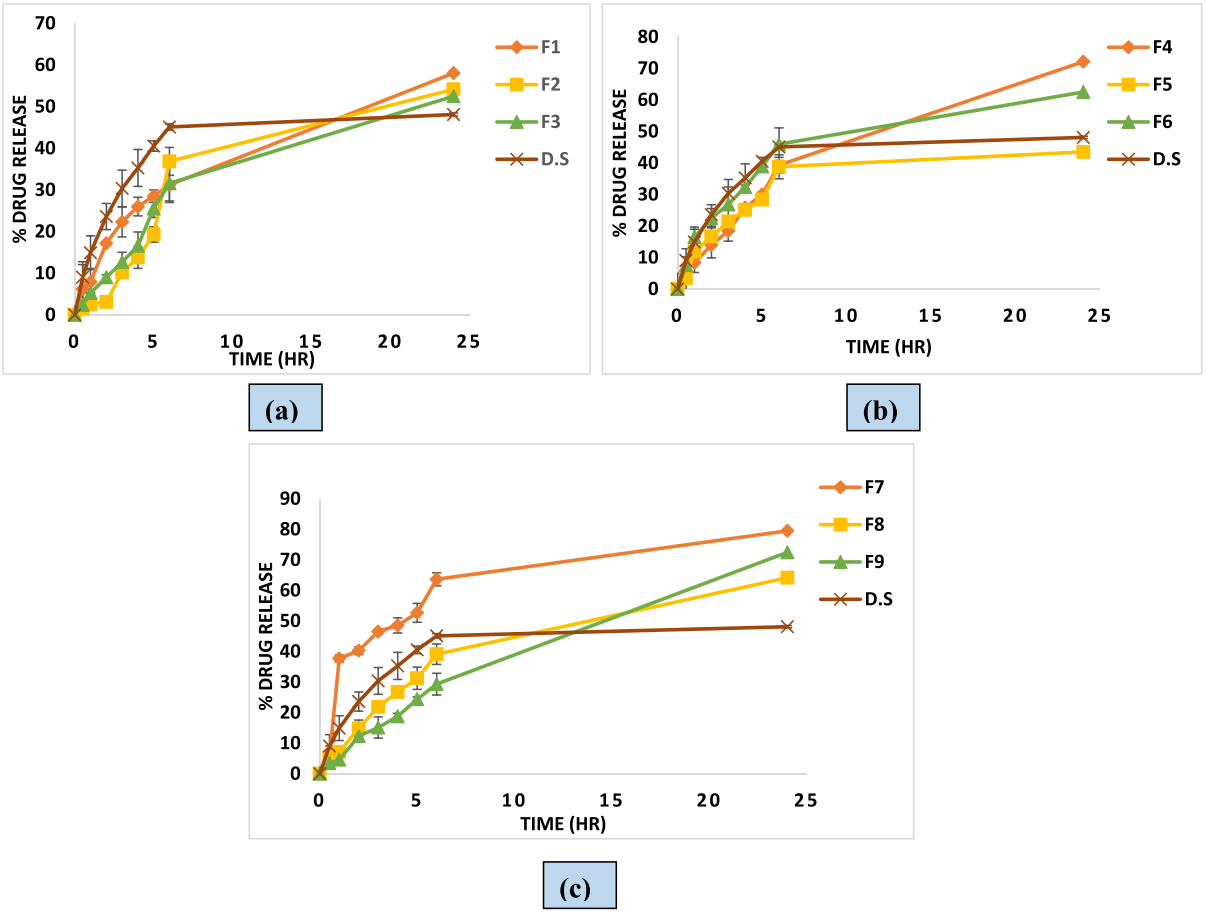

Figure 3. In vitro drug release profiles of different EZ-NLC formulations against EZ suspension: (a) EZ-NLCs with 10% LL, (b) EZ-NLCs with 30% LL, and (c) EZ-NLCs with 20% LL.

superiority of oleic acid regarding EE% might be attributed to the higher ability of liquid lipids with longer carbon chains, being more hydrophobic, to solubilize and encapsulate more drug molecules.[34]

Another possible justification for this observation, as explained with the particle size, is that it might be attributed to the higher solubility of EZ in oleic acid due to the interaction of the amine group of the drug with the carboxylic group of the acid.[31]

2.1.4. Effect of Formulation and Process Variables on In Vitro Drug Release (R4). In general, drug release is regarded as a routine quality control test for ensuring the final uniformity of the finished product and is a crucial step during the development stages of a new formulation. 24 h release profiles of the nine prepared EZ-NLC formulations and the drug suspension as well showed biphasic drug release patterns as observed in Figure 3. The first 6 h showed a faster drug release in comparison to the remaining profile. This rapid release may be related to either the localization of some unentrapped drug particles on the surface of NLCs or could be due to the presence of liquid lipids in the external layer carrying dissolved lipophilic drug causing faster release at the first stage.[38] Meanwhile, the sustained release phase of the drug after that could be attributed to the drug being deeply entrapped in the core matrix of EZ-NLC particles due to its lipophilic nature. These drug particles have a longer diffusion pathway to reach the superficial in comparison with those entrapped adjacent to the surface.[13]

Also, the incorporation of a long-chain fatty acid (LCFA) such as stearic acid in different EZ-NLC formulations contributed to this slow and sustained release due to its superior lipophilicity, which in turn led to an enhanced retaining capacity for the drug.[32,34]

It was observed that the drug release percentage ranged from 43.5 ± 3.8 to 79.4 ± 2.12% at the end of the 24 h. ANOVA demonstrated the significance of a and B on percentage drug release after 24 h (p-value <0.0001) for both variables. From Figure 2b, we can observe that there was a nonlinear relationship between the percentage of liquid lipid with respect to total lipid amount and the drug release percentage. Initially, the release percentage was increased with the rise in LL%. However, this increase was followed by a fall in the release after 20% liquid lipid incorporation. This was confirmed by the positive coefficient of B and the negative coefficient of B^2. This relationship could be described briefly as that the initial rise may be related to the small particle size, which gave a larger surface area existing for drug dissolution. However, accompanying the rise in the size of particles with higher liquid lipid percentages, the release rates declined.[13,20]

Concerning the type of liquid lipid used, the positive sign of a[1] indicates that the addition of oleic acid contributed to higher drug release rates than IPM. However, the negative coefficient of a[2] refers to a reduced percentage of drug release as compared to IPM upon labrafac incorporation. To consider the interaction, a significant effect of aB on the % of drug released was observed with a p-value of 0.0007. The positive

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Table 3. Predicted and Observed Values for the Optimized NLC Formula and the Prediction Error of Each Response

	PS (nm)	ZP (mV)	EE %	release %
predicted values	207.778	−29.59	81.169	70.42
observed values	204.3 ± 19.17	−32 ± 7.59	81.5 ± 3.58	72.15 ± 4.58
prediction error (%)	98.33	108.14	100.4	102.46

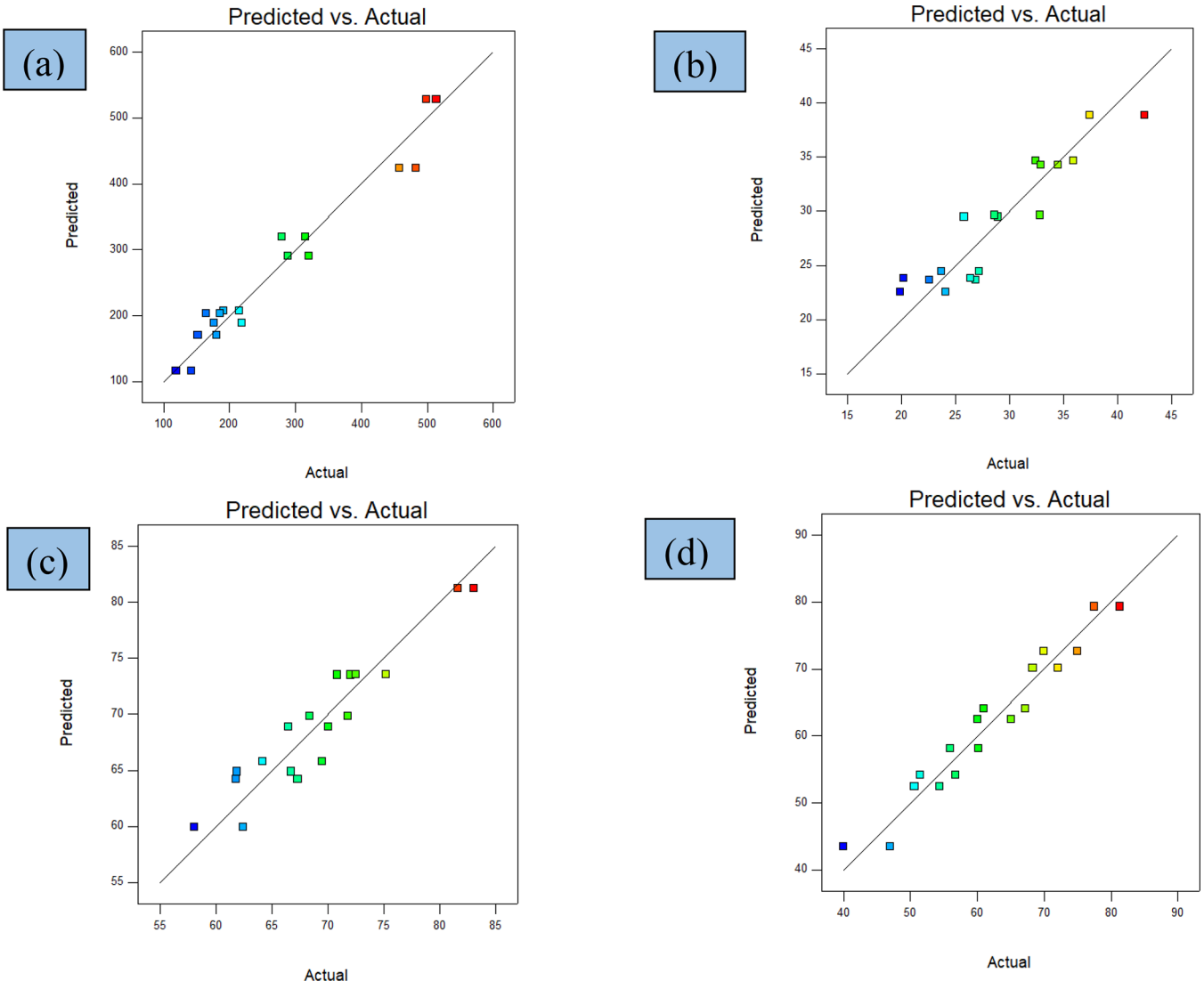

Figure 4. Linear correlation plots between predicted and actual values with the corresponding residual plots for the independent responses: (a) particle size, (b) zeta potential, (c) entrapment efficiency percentage, and (d) in vitro drug release percentage.

coefficient of a[1]B and the negative coefficient of a[2]B in Table 1, refer to the synergistic and antagonistic impacts of oleic acid and labrafac, respectively, on the percentage of liquid lipid with respect to the total lipid amount. This means that raised proportions of oleic acid demonstrated a higher % of drug release, unlike labrafac, which revealed a lower drug release % upon increasing its proportion (up to 20%). Moreover, It was noted that at higher IPM proportion (up to 20%), the % of drug release was raised, indicating the synergistic effect of IPM on the percentage of liquid lipid. The only possible explanation available is the length of the carbon chain and the interaction between EZ and oleic acid, as discussed in the previous sections.

2.2. Optimization of EZ-NLC Formulation by Desirability Function. The desirability function of Design-Expert 10.1.1 software was employed for the optimization of all investigated responses, where R1 was set to be minimized, while R2, R3, and R4 were set to their maximum, as displayed in Table 5. The independent variables of a in its low level (oleic acid) and B in its high level (30%), represented in EZ-NLC formula 4, were suggested by the program Design-Expert 10.1.1 as the optimized formula after calculation by combining all polynomial equations mentioned above, with a desirability value of 0.751. The predicted and observed values of the optimized NLC formula, along with the prediction error of each response, are displayed in Table 3.

The linear relationship between the results of experimental responses and those of the values predicted for all dependent variables is shown in Figure 4.

2.3. Characterization of the Optimized NLC Formula. *2.3.1. Transmission Electron Microscopy (TEM).* The results of

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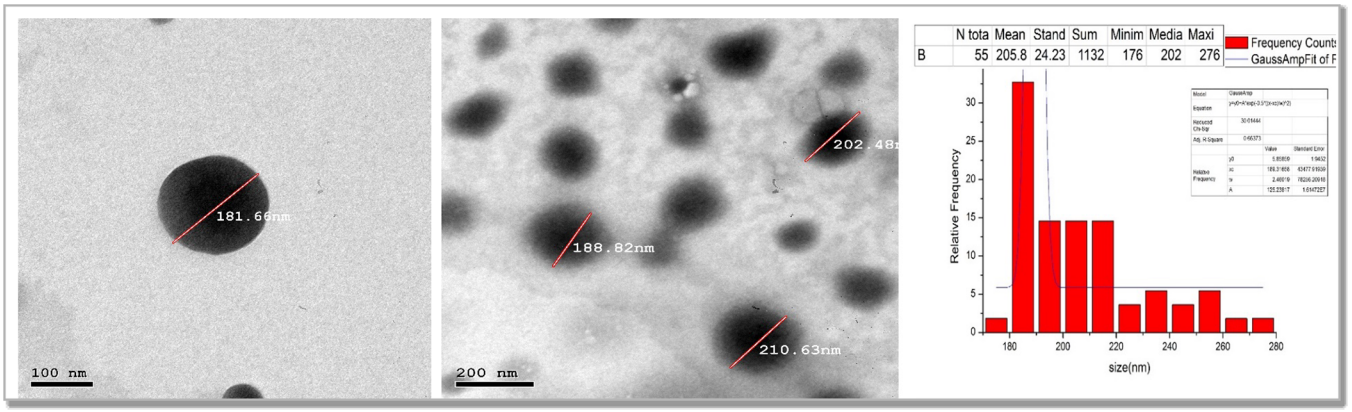

Figure 5. TEM image of the optimized NLC formula at different scales and a size distribution histogram.

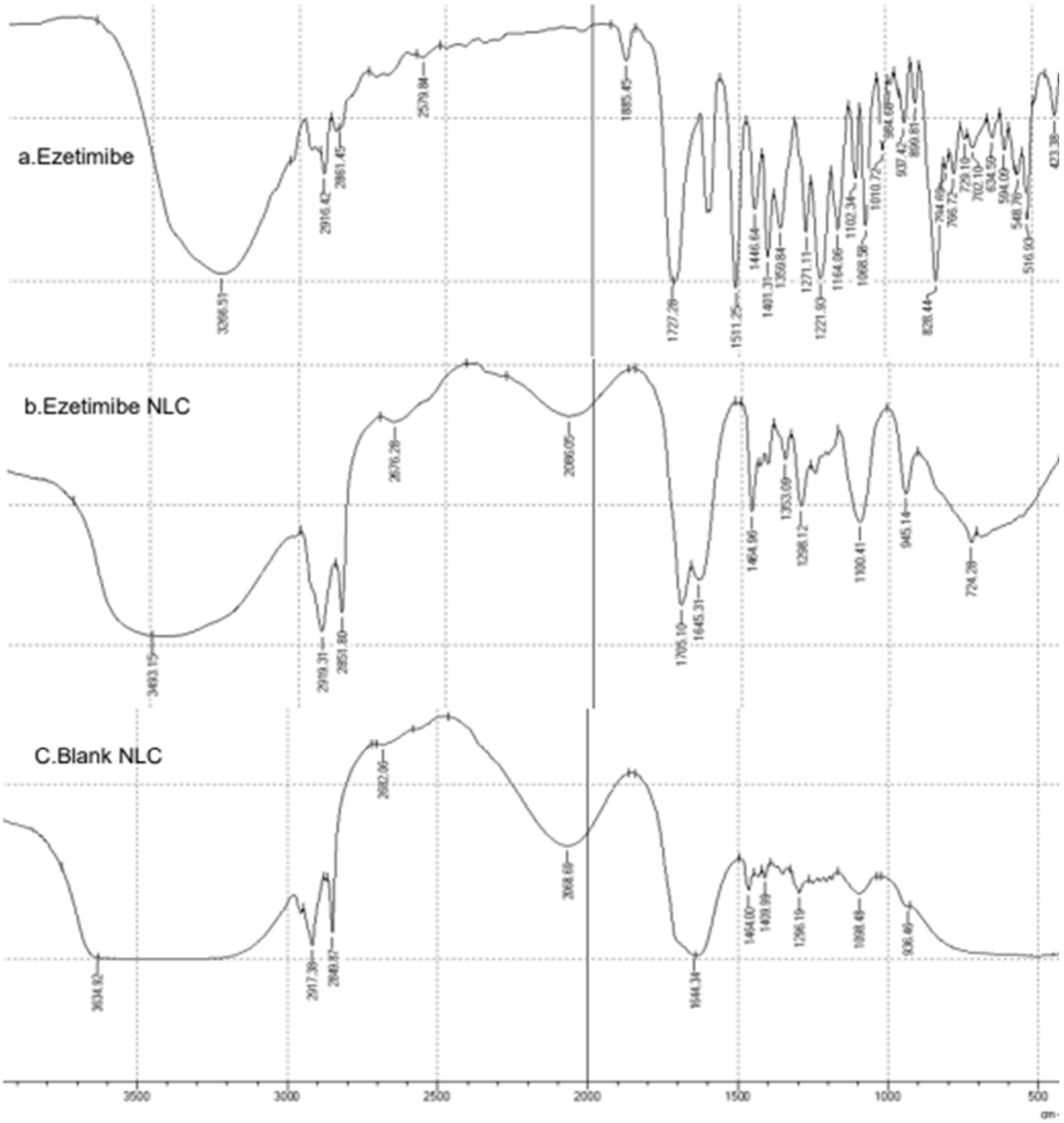

Figure 6. FTIR Spectra of (a) EZ, (b) optimized NLC formula, and (c) blank NLC.

the TEM study carried out in Figure 5 displayed that optimized EZ-NLC particles were spherical with no aggregation observed. A less ordered crystalline structure of the lipid matrices was ascertained by the spherical morphology of the particles due to the fact that an ordered structure (β modification) typically results in elongated crystals. Moreover, previous studies have

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reported the spherical or ovoid structure for NLCs. The absence of particles' aggregation denotes the high repulsion interaction among NLC particles.[35]

Figure 5 also shows that the optimized NLC formula demonstrated an average diameter of 180−220 nm at 25 °C, indicating a narrow size distribution and low polydispersity. The size of the particles obtained was found to correlate well with that obtained from the study performed by the Malvern zeta sizer.

2.3.2. Fourier Transform Infrared Spectroscopy (FTIR). FT-IR spectra of EZ (pure), optimized NLC formula, and blank NLC formulation are illustrated in Figure 6. We can observe from the FTIR spectra of EZ that it has characteristic peaks of O−H stretching in alcohols at 3266.51 cm^{-1}, C−F stretching in alkyl halide at 1359.84 cm^{-1}, C−N stretching in amine at 1102.34 cm^{-1}, C═O stretching of β-lactam ring at 1727.28 cm^{-1}, C−H stretching in alkanes at 2916.42 cm^{-1}, and C═C stretching in benzene ring at 1511.25 cm^{-1}.[9]

There was no major shifting of these peaks observed in the spectrum of the formula, which confirms the lack of interaction between the drug, lipids, and surfactants added. The most characteristic peak of stearic acid and oleic acid is the C═O stretching of carboxylic acids seen in the spectra of the formula and blank at 1645.31 and 1644.34 cm^{-1}, respectively. There was no interaction between this peak and that of β-lactam in EZ. To conclude, the excipients used in the formula were chemically compatible with the drug.

2.3.3. Differential Scanning Calorimetry (DSC). Generally, differential scanning calorimetry (DSC) is used for the characterization of raw materials to evaluate the compatibility of the drugs with the excipients used in lipid-based drug delivery systems.[28] In our study, DSC was carried out on pure EZ, optimized NLC formula, and blank NLC, and the results are displayed in Figure 7. A sharp endothermic peak corresponding

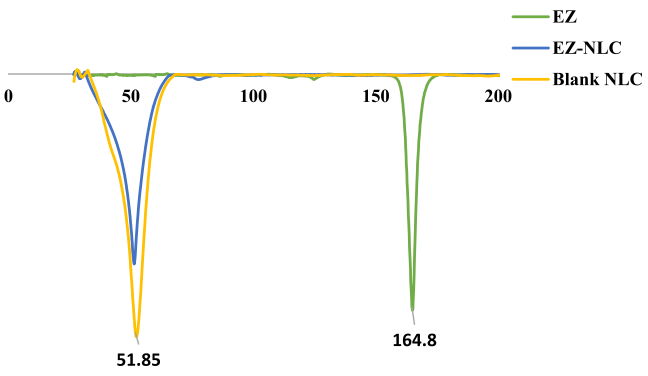

Figure 7. DSC of EZ, optimized NLC formula, and blank NLC.

to the melting point of EZ was observed at 164.8 °C, demonstrating its crystalline state. The alteration of the EZ state from crystalline to amorphous and its solubilization in the formed nanostructured lipid carrier enlightens the disappearance of this peak.[7,18]

The thermograms of EZ-NLC and blank NLC in Figure 7 reveal a clear peak at 51.85 °C that might be related to the melting point of poloxamer 188.

2.3.4. MTT Cytotoxicity Assay. To check the safety of EZ, optimized NLC formula, and blank NLC on the normal Vero cells, we treated the cells with tested materials for 72 h, and the cytotoxic effect of tested materials was evaluated using the MTT assay. As shown in Figure 8 and Table 4, the drug inhibited cell

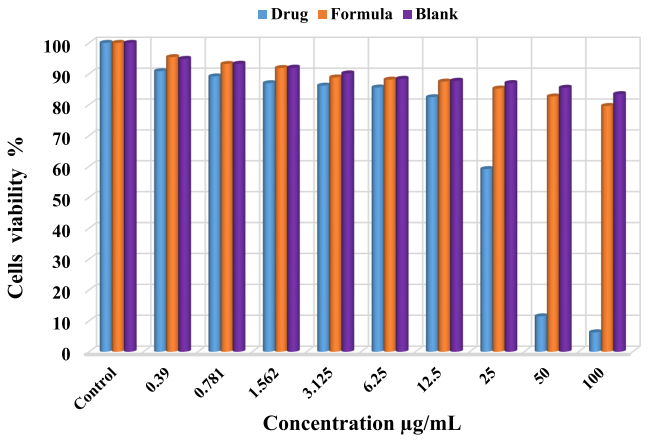

Figure 8. Effect of the drug, optimized NLC formula, and blank NLC on the normal Vero cells compared with untreated cells.

Table 4. IC_{50} Value of Tested Materials on a Normal Vero Cell Line

tested materials	IC_{50} (μg/mL)
drug	30.41
formula	251
blank	316.4

growth (decreased the number of viable cells) of Vero cells compared to the control cells in a concentration-dependent way with an IC_{50} value of 30.41 μg/mL. However, optimized NLC produced very minimal cell inhibition compared to the drug at high concentrations with an IC_{50} value of 251 μg/mL. Results concluded that EZ-NLC was far safer than the pure EZ on normal Vero cells.

To sum up the results of the previous pharmaceutical experiments, optimized EZ-NLC revealed superior in vitro characterization and cell viability investigation outcomes. Consequently, to shed light on the effect of EZ nanoformulation on lowering cholesterol levels, the optimized NLC formula was subjected to pharmacological investigations.

2.5. Assessment of the Antihyperlipidemic Effect in Rats. Significant changes in lipid profile parameters through cholesterol feeding and continuous ingestion of a high-fat diet have been used to investigate the effect of pathophysiological changes on blood homeostasis and the efficacy of EZ-NLC.

The aim of our study was achieved by induction of hyperlipidemia by oral administration of cholesterol-cholic acid, in addition to feeding a saturated fat diet. The administration of cholesterol directly increases the plasma level of cholesterol in the blood. Meanwhile, cholic acid increased the level of lipids and improved cholesterol absorption from the duodenal cells into systemic circulation.[9]

The hyperlipidemic responses induced by high-fat diet administration were manifested by assessment of the lipid profile markers, TC, TG, LDL, and HDL. According to our results, it was evident that the high-fat diet group showed a significant elevation in cholesterol levels by 2-fold compared to the negative control group. These findings concur with a previously proven hypothesis that an elevated TC is conferred by the deleterious effects of a hyperlipidemic regimen. On the other hand, the EZ-NLC-treated group showed a significant reduction in the level of total cholesterol as compared to the high-fat diet group as shown in Figure 9. In parallel, TG and LDL showed a significant rise by 2-fold in the HFD group as

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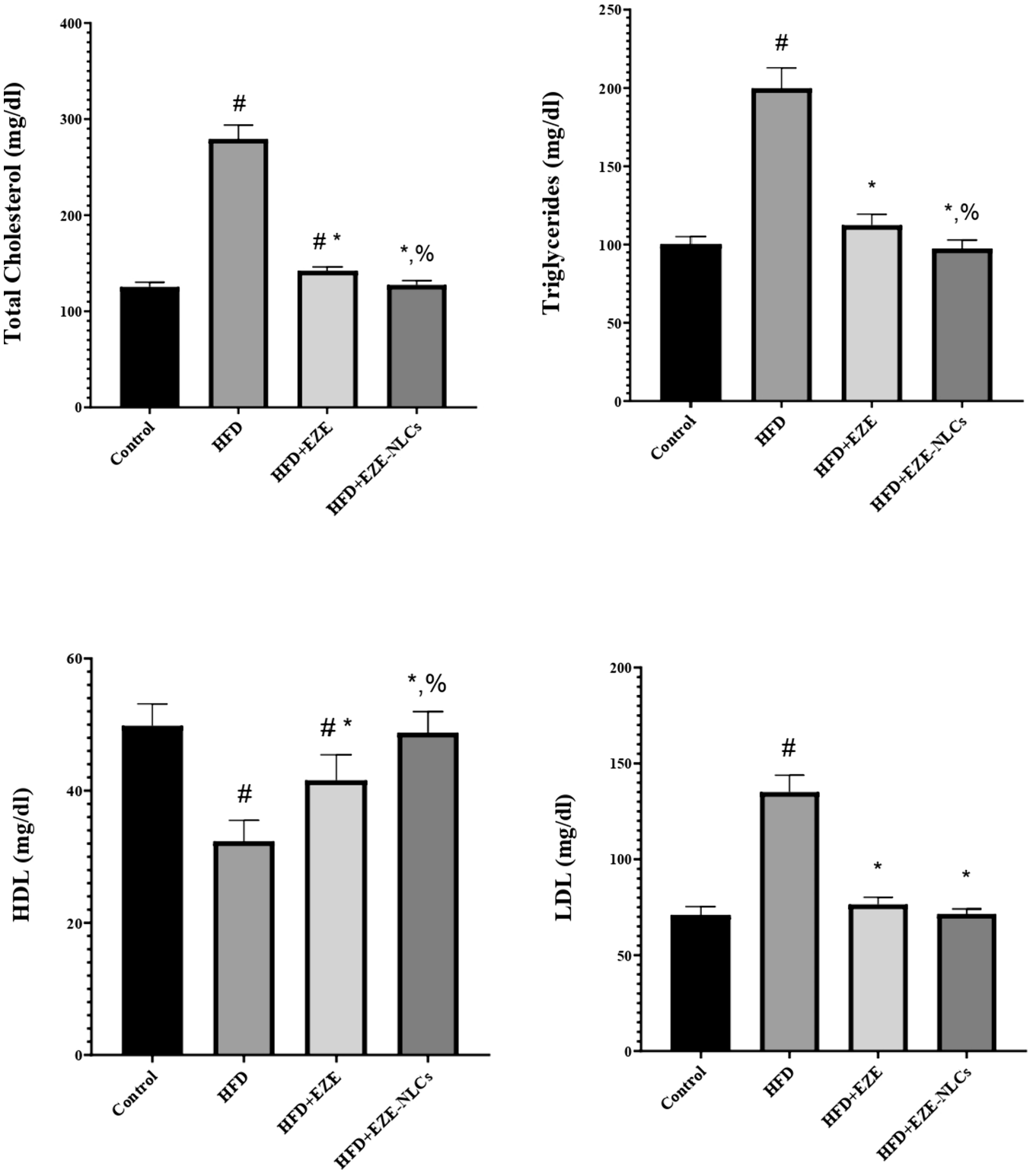

Figure 9. Effect of EZ-NLC on serum level of total cholesterol, triglyceride, low-density lipoprotein, and high-density lipoprotein. Values presented as means ± SD, ANOVA followed by Tukey's post hoc test (P value <0.001). #: As compared with control, *: as compared with HFD, and %: as compared to HFD+EZE.

compared to the negative control group. In the current study, EZ-NLC showed a significant amelioration in the level of both TG and LDL as compared with the high-fat diet group.

Interestingly, EZ-NLC-treated animals showed a significant reduction in cholesterol levels and triglycerides and a significant elevation in HDL levels as compared to animals treated with EZ suspension.

CONCLUSIONS

In the present study, different EZ-loaded NLC formulations were successfully prepared by an ultrasonication technique using

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various types and percentages of liquid lipids. All EZ-NLC formulations exhibited satisfactory characterization results regarding particle size, PDI, zeta potential, entrapment efficiency %, and drug release %. Different mathematical models were developed in order to correlate the significant formulation variables with the measured responses. The optimized NLC formula composed of 30% oleic acid as liquid lipid revealed significant improvement in the dissolution profile of EZ by about 1.6-fold in comparison with EZ suspension. The cellular cytotoxicity assessment performed proved that EZ-NLC was far safer than EZ with a difference of 221 μg/mL in the value of IC_{50}. Furthermore, EZ-NLC demonstrated significant enhancement in the efficacy of EZ denoted by the outcomes of the lipid profile assessment carried out in comparison to the EZ suspension. Accordingly, the employment of nanostructured lipid carriers is considered a promising approach to enhance the antihyperlipidemic effect of EZ.

3. MATERIALS AND METHODS

3.1. Materials. Ezetimibe (EZ) was gifted by the Marcyrl Pharmaceutical Company (Cairo, Egypt). Oleic acid was purchased from Alpha Chemicals (Cairo, Egypt). Labrafac lipophile WL 1349 was obtained as a gift from Gattefossé (Saint-Priest, France). Stearic acid, Tween 80, methanol, and sodium lauryl sulfate were purchased from El-Nasr Pharmaceutical Company (Cairo, Egypt). Poloxamer 188 and isopropyl myristate (IPM) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). All of the other reagents that were used are of analytical grade.

3.2. Methods. *3.2.1. Design of the Experiments.* A response surface D-Optimal design was employed to investigate the influence of the independent variables (liquid lipid type and percentage of liquid lipid with respect to total lipid amount) on the particle size, the zeta potential, the entrapment efficiency, and the percentage of drug released after 24 h of the prepared NLC formulations in order to choose the best formula for further characterization. These two independent variables and their three levels are summarized in Table 5. Design-Expert

Table 5. Response Surface D-Optimal Design for the Preparation of EZ-NLC Formulations

	normalized levels of independent variables		
independent variables	low level		high level
a: liquid lipid type	oleic acid (−1)	labrafac (0)	IPM (1)
B: liquid lipid %w/w	10 (−1)	20 (0)	30 (1)
dependent variables:		constraints	
R1: particle size (nm)		minimize	
R2: zeta potential (mV)		maximize	
R3: entrapment efficiency (%)		maximize	
R4: release after 24 h (%)		maximize	

10.1.1 software was utilized to analyze the experimental results. The concentration of the total lipid (10%) and solid lipid type (stearic acid) as well as the concentration of the two surfactants utilized tween 80 (1%w/v) and poloxamer 188 (2%w/v) were kept constant during the study. The formulations were prepared, and their results were fitted to various polynomial models. These models were selected by considering several statistical parameters provided by analysis of variance (ANOVA), such as coefficients of determination (adjusted and predicted R^2), sequential p-values, and adequate precision. After that, the desirability function was employed to choose the optimized formula in accordance with the responses' goal via numerical optimization. The EZ-NLC formula fulfilling the greatest desirability was prepared according to the chosen composition and then subjected to further characterization.[24,39,40]

3.2.2. Preparation of EZ-Loaded NLC Formulations. EZ-loaded NLC formulations were prepared by employing the ultrasonication method. Briefly, the oily phase was prepared by melting a mixture of stearic acid and the liquid lipid (oleic acid, labrafac, or isopropyl myristate) with different percentages (10, 20, and 30%) at 75 °C on a hot plate stirrer (MSH-20D, HP-20D/30D, and MS-20D Models). Simultaneously, the aqueous phase, which was prepared by dispersing the surfactant mixture of 1% Tween 80 and 2% poloxamer 188 in distilled water, was heated to the same temperature. Then, EZ was added to the molten lipid to form a uniform clear oil phase followed by dropwise addition of the aqueous phase to the oily one under magnetic stirring at 1400 rpm and then continuously stirred for 15 min. A pre-emulsion was obtained and then subjected to ultrasonication using a probe sonicator (SONICS Vibra cell VCX500, USA) at an amplitude of 100% for 5 min. The final dispersion obtained was cooled to (4 °C) to solidify the lipid matrix and produce NLCs.[28,32,41] The compositions of different EZ-NLC formulations are shown in Table 2.

3.2.3. Characterization of Different EZ-NLC Formulations.
3.2.3.1. Particle Size and Zeta Potential Determination. The mean particle size (PS), polydispersity index (PDI), and zeta potential (ZP) were determined using a Malvern particle size analyzer (Zeta sizer 4000S, Japan). Representative samples were taken from each NLC formula, diluted in a ratio of 1:10 with double distilled water, and measured at 25 °C.[40] The recorded results in Table 2 are the means ± the standard deviations (SD) of three determinations.

3.2.3.2. Determination of Drug Content and Entrapment Efficiency (%). Methanol was selected as a suitable solvent to break down the formed nanostructured lipid carriers and determine the total drug content (free + entrapped) of EZ in the dispersion. Briefly, 1 mL of the NLC dispersion was diluted to 25 mL with methanol and sonicated for 1 min, and then the UV absorbance of EZ was measured spectrophotometrically (UV-20000214-J-A34E13440−000809 SHIMADZU JAPAN) at λ_{max} of 231 nm.[7,9] The direct method was adopted in order to determine the drug entrapment efficiency. To illustrate, 1 mL of each formula was appropriately diluted with distilled water, and then NLCs were isolated from the unentrapped EZ by ultracentrifugation at 15,000 rpm for 1 h using a cooling centrifuge at 4 °C (S.N.: 159105, Model: 3−16 KL SIGMA).[42] The supernatant was discarded, the separated carriers were disrupted by sonication in methanol, and then the concentration of EZ entrapped within the carriers was calculated from the following equation after measuring its absorbance at the predetermined λ_{max}.[21,43] The test samples were carried out in triplicate.

$$\text{EE\%} = \frac{\text{amount of EZ entrapped}}{\text{total amount of EZ}} \times 100$$

3.2.3.3. In Vitro Drug Release Study. The percentage drug release test was carried out for 24 h on the nine prepared EZ-NLC formulations along with the drug suspension according to ref 7 by providing slight modifications. The study was carried out using the dialysis bag diffusion technique. The dialysis bags (molecular weight cutoff 12000−14000 Da) used in the experiment were soaked overnight in the release medium (1%

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w/v sodium lauryl sulfate (SLS) in phosphate buffer, pH 6.8). 1 mL of each formulation as well as the drug suspension were added within the dialysis bags and immersed in 100 mL of release medium mentioned before. The experiment was carried out in a shaking incubator (S.N.: XP0018667, Model: SI-64) maintained at 100 rpm and 37 ± 1 °C. After fixed time intervals (0.5, 1, 2, 3, 4, 5, 6, and 24 h), 5 mL aliquots were withdrawn and substituted with the same volume of freshly released medium to maintain the constant volume and sink condition. Samples were filtered and the amount of EZ released was measured spectrophotometrically after appropriate dilution.[32,44] Test samples were performed in triplicate.

3.2.4. Characterization of the Optimized Formula. 3.2.4.1. Transmission Electron Microscopy (TEM). The surface morphology of the optimized NLC formula was evaluated through a transmission electron microscope JEOL 1010 (JEOL Ltd., Tokyo, Japan) at 200 Kv. One drop from the optimized NLC formula was diluted 50 times with pure water and then dropped on Formvar/Carbon 230 Mesh copper grids (Zhongjingkeyi Technology Co. Ltd., Beijing, China). After that, phosphotungstic acid (1% w/v) was used to negatively stain the sample for about 20 min before observation.[28,45]

3.2.4.2. Fourier Transform Infrared Spectroscopy (FTIR). The interaction among the drug, lipids, and surfactants used in the formula was evaluated through Fourier transform infrared (IRAffinity-1S A219654 SHIMADZU 01660) studies. The FTIR spectrum of EZ, optimized NLC formula, and blank NLC were obtained by adding 10 mg weighed accurately of the sample on the glass window of FTIR. The FTIR spectra were measured with a resolution of 4 cm^{-1} over the range of 4000−400 cm^{-1} for 50 scans. Each data point was recorded in three replicates using absorbance mode to facilitate quantitative analysis.[20]

3.2.4.3. Differential Scanning Calorimetry (DSC). Differential scanning calorimetry (DSC) is a thermoanalytical technique used to identify many physical properties and thermal transitions of polymeric materials. DSC measurements were carried out for the pure drug along with the optimized NLC formula and blank NLC using a Shimadzu DSC-50 (Shimadzu Instruments, Japan). The NLC and the blank dispersions were lyophilized before the investigation. 3 mg from each sample was weighed accurately and sealed in standard aluminum pans. Then, the samples were subjected to a heating rate of 10 °C/min under dry nitrogen purge at a rate of 25 mL/min and a temperature range of 25 to 300 °C to record the change in enthalpy.[7,28]

3.2.4.4. MTT Cytotoxicity Assay. To assess the cytotoxic effect of the tested materials (EZ, optimized NLC formula, and blank NLC), Vero cells were purchased from Nawah Scientific Inc., Egypt, and have been used as normal cell lines during this investigation. The 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was utilized to investigate the cytotoxic activity of the samples according to ref 46 after slight modifications. To illustrate, the cells were seeded in 96-well plates (100 μL/well with 3×10^5 cells/mL density) followed by incubation for 1 day at 5% CO_2 at 37 °C. Then, the cells were treated with serial concentrations (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 μg/mL) of all test samples in triplicate. After 72 h, the supernatant was thrown out, sterile phosphate-buffered saline (PBS) was used to wash cell monolayers 3 times, and MTT solution (20 μL of 5 mg/mL stock solution) was added to each well and then incubated for 4 h at 37 °C followed by medium aspiration. The formed formazan crystals inside each well were dissolved in 200 μL of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 mL of HCL in 50 mL isopropanol). Finally, a multiwell plate reader (BMGLABTECH FLUO star Omega, Germany) was used to determine the formazan solutions' absorbance at 570 nm.

To calculate cell viability percent, the following equation was applied

$$\text{viability\%} = \frac{\text{mean OD treated}}{\text{mean OD control}} \times 100$$

where OD is optical density.

The IC_{50} is the concentration of tested material required to inhibit 50% of cell growth, and the value was calculated by an online tool.[47]

3.2.5. Assessment of the Antihyperlipidemic Effect in Rats. 3.2.5.1. Animals. Adult Sprague−Dawley male rats weighing between 140 ± 10 g (age: 8−10 weeks) were obtained from Nile Co. for the Pharmaceutical and Chemical industries (Cairo, Egypt). They were housed under standard laboratory conditions of 28 °C and 55% humidity, alternating 12 h light and dark cycles, and free access to standard diet pellets and water. To ensure animal comfort, rats were housed in cages of appropriate size (a maximum of three animals per cage). The experimental protocol was carried out in accordance with ethical guidelines and was approved by the Research Ethics Committee of the Faculty of Pharmacy, Cairo University (PI 1719).

3.2.5.2. Hyperlipidemia Induction. Hyperlipidemia was attained through feeding of the hypercholesterolemic inductors orally in rats (10%/kg body weight from a mixture of cholesterol and cholic acid at a ratio of 3:1) in addition to feeding a high-fat diet for 10 consecutive weeks.[9]

The lipid profile was assessed weekly; at the end of the sixth week, induction of hyperlipidemia was confirmed by the elevation of serum lipid levels, and the hyperlipidemic rats were included in the study. After 6 weeks, the treatment was started and continued for 4 weeks.[19]

EXPERIMENTAL DESIGN

Twenty-four adult male Sprague−Dawley rats (140 ± 10 g: 8−10 weeks) were weighed and randomly assigned into four groups ($n = 6$) as follows:

1. Group I (negative control): represented healthy normal rats who received a normal diet.
2. Group II (positive control): rats received the hyperlipidemic regimen for 10 weeks.
3. Group III (HFD + EZ-NLC): rats received the hyperlipidemic regimen for 10 weeks and EZ-NLC (0.9 mg/kg p.o) for 4 weeks.
4. Group IV (HFD + EZ): rats received the hyperlipidemic regimen for 10 weeks and EZ- suspension (0.9 mg/kg p.o) for 4 weeks.

By completion of the experimental protocol, blood samples were withdrawn from the retro-orbital plexus following overnight fasting. Then, they were subjected to centrifugation at 3000 rpm for 15 min to obtain serum samples, which were tested for (TC), (TG), (HDL-c), and (LDL-c).

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