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Development, Characterization and in Vivo Hepatoprotective Evaluation of Ellagic Acid-Loaded Nanocapsules: A Novel Nanoformulation Approach

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ABSTRACT: Ellagic acid (EA) is a naturally occurring polyphenolic compound widely investigated for its antioxidant, anti-inflammatory, and hepatoprotective properties. However, its therapeutic application may be limited by poor aqueous solubility, low bioavailability, and restricted dissolution, which can reduce its effective delivery to biological systems. The present study was undertaken to develop and characterize ellagic acid-loaded nanocapsules (EAN) as a potential nanoformulation for improving the delivery and hepatoprotective activity of EA. Initially, comprehensive preformulation studies of EA were performed to evaluate its physicochemical characteristics and compatibility with selected formulation components. EAN were subsequently prepared using a suitable nanocapsule formulation technique and evaluated for physicochemical and pharmaceutical characteristics, including particle size, polydispersity index, zeta potential, morphology, drug content, entrapment efficiency, and in vitro drug-release behavior. The optimized formulation was further investigated for its hepatoprotective potential in an appropriate experimental animal model of liver injury and compared with free EA. Biochemical parameters associated with hepatic function, oxidative stress, and tissue architecture were assessed to determine the protective effect of the formulation. The developed EAN formulation demonstrated nanoscale characteristics and effective incorporation of EA within the nanocapsule system. Comparative biological evaluation was performed to determine whether nanoencapsulation could improve the hepatoprotective response of EA. Histopathological examination was additionally conducted to assess hepatic tissue protection. Overall, the findings support the potential of ellagic acid-loaded nanocapsules as a promising nano-based delivery system for hepatoprotective applications, subject to further optimization and comprehensive pharmacological and safety evaluation.

1. INTRODUCTION

The liver is one of the most important organs of the human body and plays a central role in metabolism, detoxification, biotransformation, storage, and maintenance of physiological homeostasis. Continuous exposure to drugs, environmental contaminants, alcohol, dietary factors, and other xenobiotics can generate excessive oxidative stress and inflammatory responses, ultimately leading to hepatocellular injury. Oxidative stress is considered an important contributor to the progression of several forms of liver damage because excessive production of reactive oxygen species (ROS) can result in lipid peroxidation, protein oxidation, mitochondrial dysfunction, and cellular membrane damage. Therefore, the development of effective hepatoprotective strategies, particularly those involving bioactive compounds with antioxidant and anti-inflammatory properties, remains an important area of pharmaceutical and biomedical research.

Natural products and plant-derived polyphenolic compounds have attracted considerable attention as potential therapeutic agents for the management of oxidative-stress-associated disorders. Ellagic acid (EA) is a naturally occurring polyphenolic compound belonging to the family of hydrolysable tannin-derived phytochemicals. It is present in several fruits, vegetables, and medicinal plants and has been investigated for diverse pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, cardioprotective, and hepatoprotective effects.

The biological activity of EA is closely associated with its ability to interact with reactive oxygen species and modulate cellular antioxidant and inflammatory pathways. Experimental studies have suggested that EA may protect hepatic cells against chemically induced oxidative injury by reducing oxidative stress, lipid peroxidation, and inflammatory responses while supporting endogenous antioxidant defense mechanisms. These properties make EA an attractive candidate for investigation as a hepatoprotective phytoconstituent.

Despite its promising pharmacological profile, the pharmaceutical development of EA is associated with important physicochemical and biopharmaceutical challenges. Poor aqueous solubility and limited dissolution can adversely affect its absorption and systemic availability. Consequently, administration of free EA may not always provide optimal exposure at the site of pharmacological action. This creates a need for suitable drug-delivery approaches capable of improving the physicochemical and biopharmaceutical performance of EA.

Nanotechnology-based drug-delivery systems have emerged as an important approach for addressing limitations associated with poorly soluble bioactive compounds. Reduction of formulation components to the nanoscale can provide advantages such as increased surface area, improved dispersion, enhanced drug incorporation, modified drug-release characteristics, and potential improvement in bioavailability. Among various nanocarrier systems, nanocapsules consist of a core surrounded by a polymeric or lipid-based shell and can provide a suitable environment for incorporating active pharmaceutical or bioactive compounds. Depending on their composition and formulation characteristics, nanocapsules may protect the encapsulated compound from environmental degradation and facilitate controlled or modified drug release. Nanoencapsulation of natural compounds has therefore received increasing interest as a strategy for improving their therapeutic performance. In the case of EA, incorporation into a nanocarrier may overcome some of the limitations associated with its physicochemical characteristics and may provide improved delivery of the active compound. Importantly, formulation development should not be restricted to achieving nanoscale particle size; the resulting system must also demonstrate appropriate physicochemical characteristics, drug-loading capacity, stability, release behavior, and biological performance. Preformulation characterization represents an essential stage in the development of a nanopharmaceutical formulation. Evaluation of parameters such as solubility, melting behavior, partition characteristics, spectroscopic properties, crystallinity, and drug–excipient compatibility can provide important information for selecting appropriate formulation components and processing conditions. Such studies can subsequently guide the rational development and optimization of the nanocapsule system. In addition to physicochemical characterization, *in vivo* biological evaluation is essential for establishing the pharmacological relevance of a newly developed nanoformulation. Comparison of free EA with EA-loaded nanocapsules can provide useful information regarding whether nanoencapsulation influences the hepatoprotective response. Assessment of serum biochemical markers of hepatic injury, antioxidant status, and histopathological changes can provide complementary evidence of hepatic protection. Although the hepatoprotective properties of EA have been investigated, the development of an optimized ellagic acid-loaded nanocapsule (EAN) system combined with comprehensive physicochemical characterization and comparative *in vivo* hepatoprotective evaluation provides an integrated formulation–pharmacological approach. The present investigation was therefore designed to develop EA-loaded nanocapsules, characterize their physicochemical and pharmaceutical properties, and evaluate their hepatoprotective potential in an experimental model of liver injury. The therapeutic response of EAN was compared with that of free EA to investigate the potential contribution of nanoencapsulation to the biological performance of EA.

2. MATERIAL AND MATERIAL

2.1 Chemicals and Instruments required

1.1 Preliminary Trials for Preparation of Ellagic acid-loaded nanocapsules

Ellagic acid-loaded nanocapsules were prepared using the interfacial deposition technique as described by Patrick Fessi (1989). Initially, 13 formulations (NC1–NC13) were developed to evaluate variations in particle size

and zeta potential and to optimize the formulation using a three-level experimental design. The formulation and processing variables investigated included the concentration of polymer (PLGA), surfactant concentration, and magnetic stirring time. The proposed composition of the ellagic acid-loaded nanocapsules is presented in Table 1. Briefly, the organic phase containing PLGA (150–200 mg), soy lecithin (150 mg), isopropyl myristate (100 mg), methanol (5 mL), acetone (22 mL), and ellagic acid (20 mg) was mixed at room temperature. The aqueous phase consisted of phosphate buffer (pH 7.4) containing 120–150 mg of a mixture of Pluronic F68 and Pluronic F127 (1:1). The organic phase was slowly injected into the aqueous phase under magnetic stirring using a 10 mL syringe fitted with a needle of 0.3175 mm internal diameter. The resulting nanoemulsion was maintained under magnetic stirring for 30–45 minutes at room temperature. Subsequently, the organic solvents were removed by evaporation under reduced pressure (600 mmHg) at 40°C using a rotary evaporator until a final volume of 10 mL was obtained

Table 1 Composition of *ellagic acid* loaded nanocapsules

Formulation	Factor 1	Factor 2	Factor 3	Solid lipid	Liquid lipid	Drug
	A:Polymer	B:Surfactant Pluronic F68 and Pluronic F127 (1:1)	C:Stirring Time	Soy lecithin	Isopropyl myristate	Ellagic acid
	mg	mg	mins	mg	mg	mg
NC 1	150	150	37.5	150	100	20
NC 2	150	120	37.5	150	100	20
NC 3	175	120	30	150	100	20
NC 4	200	135	30	150	100	20
NC 5	175	150	30	150	100	20
NC 6	175	150	45	150	100	20
NC 7	150	135	30	150	100	20
NC 8	175	120	45	150	100	20
NC 9	200	120	37.5	150	100	20
NC 10	200	150	37.5	150	100	20
NC 11	175	135	37.5	150	100	20
NC 12	150	135	45	150	100	20
NC 13	200	135	45	150	100	20

1.2 Experimental Factorial Design

A three-factor, three-level Box–Behnken design (BBD) was employed to optimize the formulation of ellagic acid-loaded

nanocapsules. The independent variables were polymer concentration (A), surfactant concentration (B), and magnetic stirring time (C), each evaluated at three levels. A total of 13 experimental runs were generated by the design. The responses studied were particle size and zeta potential.

Table 2 Independent variables

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Polymer	Mg	Numerical	150.00	200.00	-1 ↔ 150.00	+1 ↔ 200.00	175.00	20.41
B	Surfactant	Mg	Numerical	120.00	150.00	-1 ↔ 120.00	+1 ↔ 150.00	135.00	12.25
C	Stirring Time	Mins	Numerical	30.00	45.00	-1 ↔ 30.00	+1 ↔ 45.00	37.50	6.12

Table 3 Dependent variables

Response	Name	Units
R1	Particle size	nm
R2	Zeta potential	mV

1.3 Evaluation of Formulation

1.3.1 Particle Size Measurement

The particle size of the prepared nanocapsule formulation was measured using Dynamic Light Scattering (DLS) with a particle size analyzer. A small volume of the formulation was appropriately diluted with distilled water to achieve optimal scattering conditions. The diluted sample was transferred into a clean, dust-free cuvette and analyzed at room temperature. The instrument provided values for the average particle size and polydispersity index (PDI), which indicate the uniformity of particle distribution. These parameters are essential for evaluating the stability of the formulation and its suitability for oral or transdermal drug delivery applications (Sangolkar *et al.*, 2012).

1.3.2 Zeta Potential

The zeta potential of the nanocapsules formulation was measured using a Horiba Zetasizer to evaluate the surface charge and predict colloidal stability. A small amount of the formulation was appropriately diluted with distilled water to optimize conductivity and reduce the likelihood of particle aggregation during measurement. The diluted sample was placed in a specialized zeta potential cell and analyzed at room temperature. The instrument determined the electrophoretic mobility of the nanocapsules and calculated the corresponding zeta potential, providing insight into the electrostatic interactions between particles. High absolute values of zeta potential, whether positive or negative, indicate strong repulsive forces that help prevent aggregation, thereby enhancing the physical stability and shelf life of the nanocapsules formulation.

1.3.3 Determination of Entrapment Efficiency

The entrapped drug concentration was determined by lysis of the nanocapsules using absolute alcohol followed by sonication. An accurately weighed amount of drug-loaded nanocapsules (10mg) was dissolved in 10 mL of absolute alcohol and tightly covered to prevent evaporation. The solution was sonicated for 15 min to obtain a clear solution. An aliquot of 1 mL of this solution was then diluted with 9 mL of absolute alcohol and sonicated for an additional 15 min. The concentration of ellagic acid in absolute alcohol was determined spectrophotometrically (Shimadzu UV-1700) at 366 nm after appropriate dilution (Nasr et al., 2008).

Unloaded nanocapsules showed insignificant absorbance at the same wavelength. Each sample was analyzed in triplicate.

The entrapment efficiency was calculated using the following equation:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Entrapped drug}}{\text{Total drug}}$$

Scanning electron microscopy (SEM)

An optimized formulation was selected and analyzed for superficial morphology and shape using a SEM (ZEISS). For surface morphology, microphotographs were obtained at a greater magnification (200X).

1.3.4 *In vitro* release study by dialysis membrane method using Franz Diffusion cell

The *in vitro* drug release of nanocapsules was conducted by dialysis membrane method using Franz Diffusion cell assembly (25 ml capacity and 2.0 cm diameter). The dialysis membrane with mol. wt. cut-off between 12 and 14 kDa was selected as diffusion membrane, and phosphate buffer (PBS) pH 7.4 was used as dissolution media. Prior to the experiment, the dialysis membrane was cut into pieces of desirable size and treated with the dissolution medium by soaking in PBS, pH 7.4 for 48 h. The receptor compartment of the assembly was filled with PBS, pH 7.4 and the open end was mounted with pre-treated dialysis membrane. The nanocapsules formulation was fill in the donor compartment. The sample was preincubated at 37 °C for

20 min before use. The assembly was maintained at 37 °C temperature throughout the study. Periodic sampling was done from the receptor compartment and replaced with equal amount of fresh medium.

Aliquots withdrawn were assayed at each time interval for the drug released at λ max of 366 nm using a UV-visible spectrophotometer by keeping phosphate buffer pH 7.4 blank and the amount of released drug was estimated by the standard curve (Agrawal et al., 2021).

• *In vitro* drug release kinetics

The drug release kinetics of nanocapsules were determined by plotting the following kinetic models, using the data collected from *in vitro* release studies (zero order, first order, and Higuchi equations). The mechanism of drug release was determined by using the Korsmeyer-Peppas equation (Paarakh et al., 2018).

1. Zero-order kinetics: The zero-order rate (Eq. 1) describes the systems where the drug release rate is independent of its concentration.

$$C = K_0 t \quad (1)$$

Where K_0 is the zero-order rate constant expressed in units of concentration/time and t is the time.

A plot of the amount of drug released versus time will be linear for zero-order kinetics. The dosage forms following this profile, release the same amount of drug by unit time and it is the ideal method of drug release in order to achieve a prolonged pharmacological action.

2. First-order kinetics: First-order kinetics was first applied for drug dissolution studies. The first-order equation (Eq 2 & 3) describes the release of a drug from a system where the release rate is concentration-dependent.

$$\text{Log } C_t = \text{Log } C_0 + K_1 t / 2.303 \quad (2) \quad \text{Log } C_0 - \text{Log } C_t = K_t / 2.303 \quad (3)$$

Where C_t is the amount of drug released in time t , C_0 is the initial concentration of the drug, k_1 is the first order constant, and t is the time. Here the graphical representation of the log cumulative of % drug remaining versus time will be linear with a negative slope. The dosage form follows this profile in the amount of drug released by unit time diminishes.

3. Higuchi's model: Higuchi equation (Eq 4) describes drug release as a diffusion process based on Fick's law, square root time dependent. For diffusion-controlled processes a plot of Q versus square root of time is linear.

$$Q = K_H t^{1/2} \quad (4)$$

here

Q is the amount of drug released in time t per unit area, K_H is the Higuchi dissolution constant and t is the time in hours.

4. Korsmeyer- peppas equations: Korsmeyer- Peppas equation (Eq 5) to analyze both Fickian and non-Fickian release of drugs.

$$M_t / M_\infty = K t^n \quad (5)$$

The logarithm form of equation (Eq 6) could be written as: $\text{Log } (M_t / M_\infty) = \text{Log } k + n \text{ Log } t \quad (6)$

Where M_t/M_∞ is the fraction of drug released at time t , n is the diffusion exponent indicative of the mechanism of transport of the drug, and K is the kinetic constant (having units of t) incorporating structural and geometric characteristics of the delivery system

4. RESULTS

4.1 Optimization process by Design of experiment approach

Table 4 Build Information

File Version	12.0.1.0		
Study Type	Response Surface	Subtype	Randomized
Design Type	Box-Behnken	Runs	13
Design Model	Quadratic	Blocks	No Blocks
Build Time (ms)	38.00		

Table 5 Formulation trials as per Box–Behnken design

Formulation	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	A:Polymer	B:Surfactant	C:Stirring Time	Particle size	Zeta potential
	mg	mg	Mins	nm	mV
NC 1	150	150	37.5	418.24	-30.5
NC 2	150	120	37.5	210.3	-25.1
NC 3	175	120	30	335.4	-33.5
NC 4	200	135	30	254.52	-26.2
NC 5	175	150	30	356.7	-31.7

NC 6	175	150	45	213.74	-17.4
NC 7	150	135	30	480.6	-39.6
NC 8	175	120	45	287.1	-25.4
NC 9	200	120	37.5	459	-31.4
NC 10	200	150	37.5	212.7	-19.8
NC 11	175	135	37.5	342.9	-31.4
NC 12	150	135	45	152.7	-14.7
NC 13	200	135	45	379.3	-28.2

Table 6 Independent variables

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev.
A	Polymer	mg	Numerical	150.00	200.00	-1 ↔ 150.00	+1 ↔ 200.00	175.00	20.41
B	Surfactant	mg	Numerical	120.00	150.00	-1 ↔ 120.00	+1 ↔ 150.00	135.00	12.25
C	Stirring Time	mins	Numerical	30.00	45.00	-1 ↔ 30.00	+1 ↔ 45.00	37.50	6.12

Table 7 Dependent variables (Responses)

Response	Name	Units	Observations	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Transform	Model
R1	Particle size	nm	13	Polynomial	152.7	480.6	315.63	103.42	3.15	None	2FI
R2	Zeta potential	mV	13	Polynomial	-39.6	-14.7	-27.30	6.93	2.69	None	2FI

4.2 Fit Summary

Table 8 Response 1: Particle Size

Source	Sequential p-value	Adjusted R ²	Predicted R ²	
Linear	0.6445	-0.1183	-0.8453	
2FI	< 0.0001	0.9597	0.9131	Suggested
Quadratic	0.0550	0.9916		
Cubic				Aliased

4.3 ANOVA for 2FI model

Response 1: Particle size

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	1.258E+05	20959.31	48.68	< 0.0001	significant
A-Polymer	238.49	238.49	0.5539	0.4848	
B-Surfactant	1021.97	1021.97	2.37	0.1743	
C-Stirring Time	19441.95	19441.95	45.16	0.0005	
AB	51583.49	51583.49	119.81	< 0.0001	
AC	51229.80	51229.80	118.99	< 0.0001	
BC	2240.13	2240.13	5.20	0.0627	
Residual	2583.30	430.55			
Cor Total	1.283E+05				

Factor coding is **Coded**.

Sum of squares is **Type III - Partial**

The **Model F-value** of 48.68 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case C, AB, AC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

4.4 Fit Statistics

Table 9 Regression coefficient (R²) value

Std. Dev.	20.75	R ²	0.9799
Mean	315.63	Adjusted R²	0.9597
C.V. %	6.57	Predicted R²	0.9131
		Adeq Precision	21.3406

The **Predicted R²** of 0.9131 is in reasonable agreement with the **Adjusted R²** of 0.9597; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 21.341 indicates an adequate

signal. This model can be used to navigate the design space.

Table 10 Coefficients in Terms of Coded Factors

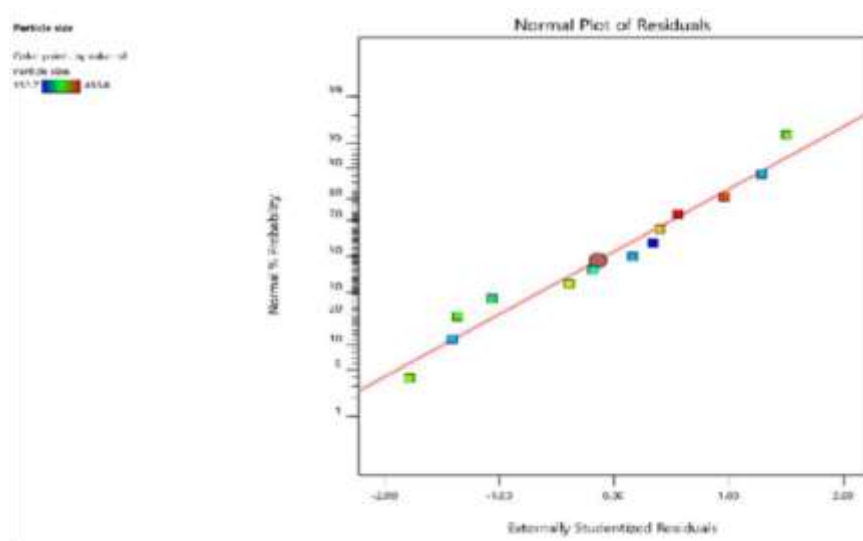
Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High	VIF
Intercept	315.63	5.75	301.55	329.71	
A-Polymer	5.46	7.34	-12.49	23.41	1.0000
B-Surfactant	-11.30	7.34	-29.25	6.65	1.0000
C-Stirring Time	-49.30	7.34	-67.25	-31.35	1.0000
AB	-113.56	10.37	-138.95	-88.17	1.0000
AC	113.17	10.37	87.78	138.56	1.0000
BC	-23.66	10.37	-49.05	1.72	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-collinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

4.5 Final Equation in Terms of Coded Factors

Particle size (R1) = +315.63 Intercept +4.46AX1 -11.30BX2 -49.30CX3 -113.56AX1BX2
+113.17AX1CX3 -23.66BX2CX3

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.



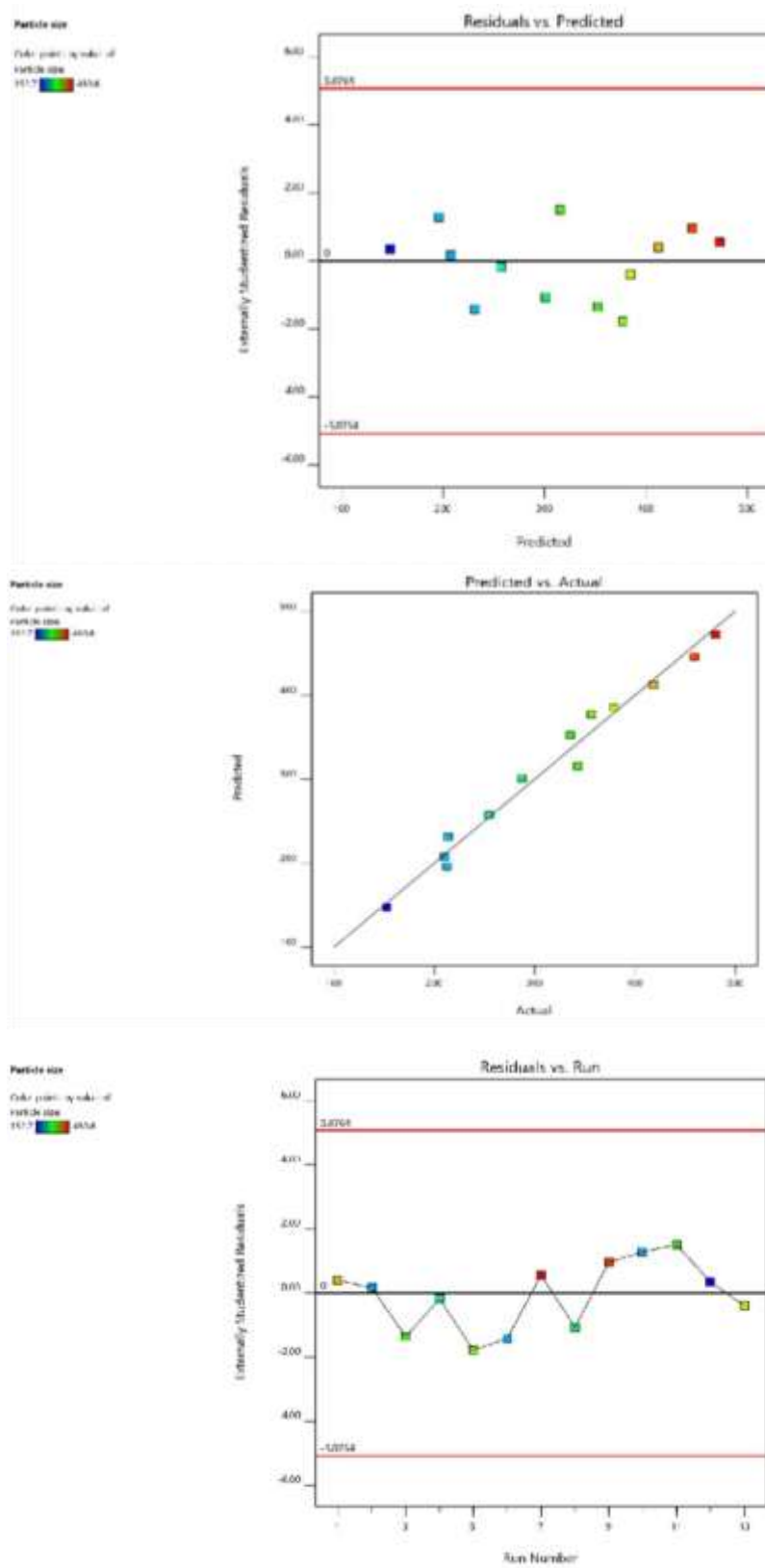
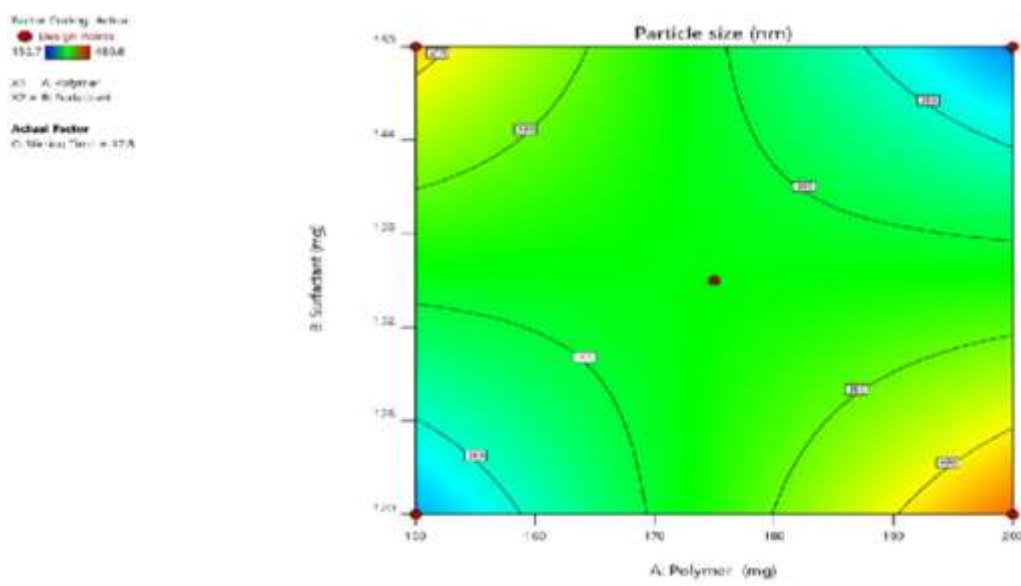


Figure 1 Two-dimensional response surface plots (normal plot vs residual, residual vs predicted and residual vs. run of Particle size) revealing relative effects of independent variables on dependent variable particle size of drug loaded formulation

4.6 Actual value versus predicted value of Particle size Table 11 Report

Formulation	Actual Value	Predicted Value
NC 1	418.24	412.43
NC 2	210.30	207.91
NC 3	335.40	352.57
NC 4	254.52	257.22
NC 5	356.70	377.29
NC 6	213.74	231.37
NC 7	480.60	472.64
NC 8	287.10	301.30
NC 9	459.00	445.95
NC 10	212.70	196.23
NC 11	342.90	315.63
NC 12	152.70	147.70
NC 13	379.30	384.96

Figure 2 Two-dimensional (2D) contour plots for the effect of polymer and surfactant concentration on particle size



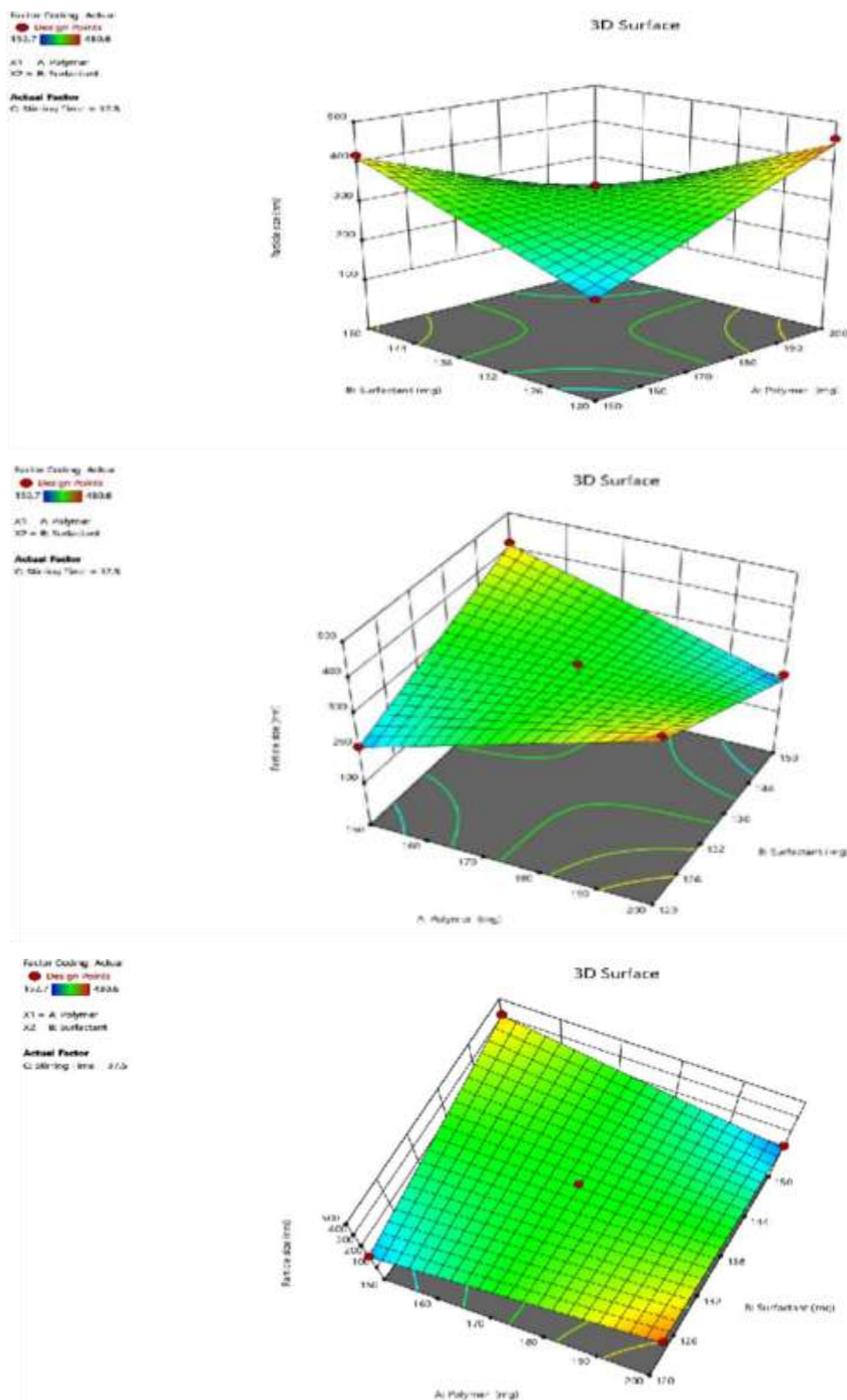


Figure 3 Three-dimensional (3D) Response surface plot showing combined effect of polymer (PLGA) and surfactant on particle size of formulation

4.7 Fit Summary

Table 12 Response 2: Zeta potential

Source	Sequential p-value	Adjusted R ²	Predicted R ²	
Linear	0.0845	0.3394	-0.0636	
2FI	0.0011	0.9207	0.9072	Suggested
Quadratic	0.1253	0.9710		
Cubic				Aliased

4.8 ANOVA for 2FI model

Table 13 Response 2: Zeta potential

Source	Sum of Squares	Mean Square	F-value	p-value	
Model	553.59	92.26	24.22	0.0006	significant
A-Polymer	2.31	2.31	0.6068	0.4656	
B-Surfactant	32.00	32.00	8.40	0.0274	
C-Stirring Time	256.51	256.51	67.34	0.0002	
AB	72.25	72.25	18.97	0.0048	
AC	180.90	180.90	47.49	0.0005	
BC	9.61	9.61	2.52	0.1633	
Residual	22.85	3.81			
Cor Total	576.44				

Factor coding is **Coded**.

Sum of squares is **Type III - Partial**

The **Model F-value** of 24.22 implies the model is significant. There is only a 0.06% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case B, C, AB, AC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Table 14 Fit Statistics

Std. Dev.	1.95	R ²	0.9604
Mean	-27.30	Adjusted R²	0.9207
C.V. %	7.15	Predicted R²	0.9072
		Adeq Precision	17.2990

The **Predicted R²** of 0.9072 is in reasonable agreement with the **Adjusted R²** of 0.9207; i.e. the difference is less than 0.2.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 17.299 indicates an adequate signal. This model can be used to navigate the design space.

Table 15 Coefficients in Terms of Coded Factors

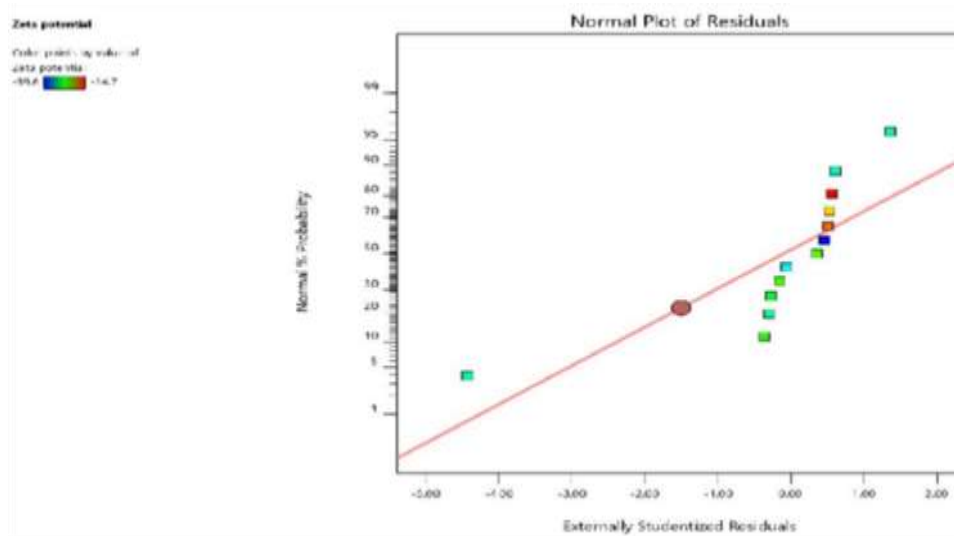
Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High	VIF
Intercept	-27.30	0.5413	-28.62	-25.98	
A-Polymer	0.5375	0.6900	-1.15	2.23	1.0000
B-Surfactant	2.00	0.6900	0.3115	3.69	1.0000
C-Stirring Time	5.66	0.6900	3.97	7.35	1.0000
AB	4.25	0.9759	1.86	6.64	1.0000
AC	-6.72	0.9759	-9.11	-4.34	1.0000
BC	1.55	0.9759	-0.8378	3.94	1.0000

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-collinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

4.9 Final Equation in Terms of Coded Factors

Particle size (R1) = -27.30 Intercept +0.5375AX1 +2.00BX2 +5.66CX3 +4.25AX1BX2 -6.73AX1CX3 +1.55BX2CX3

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.



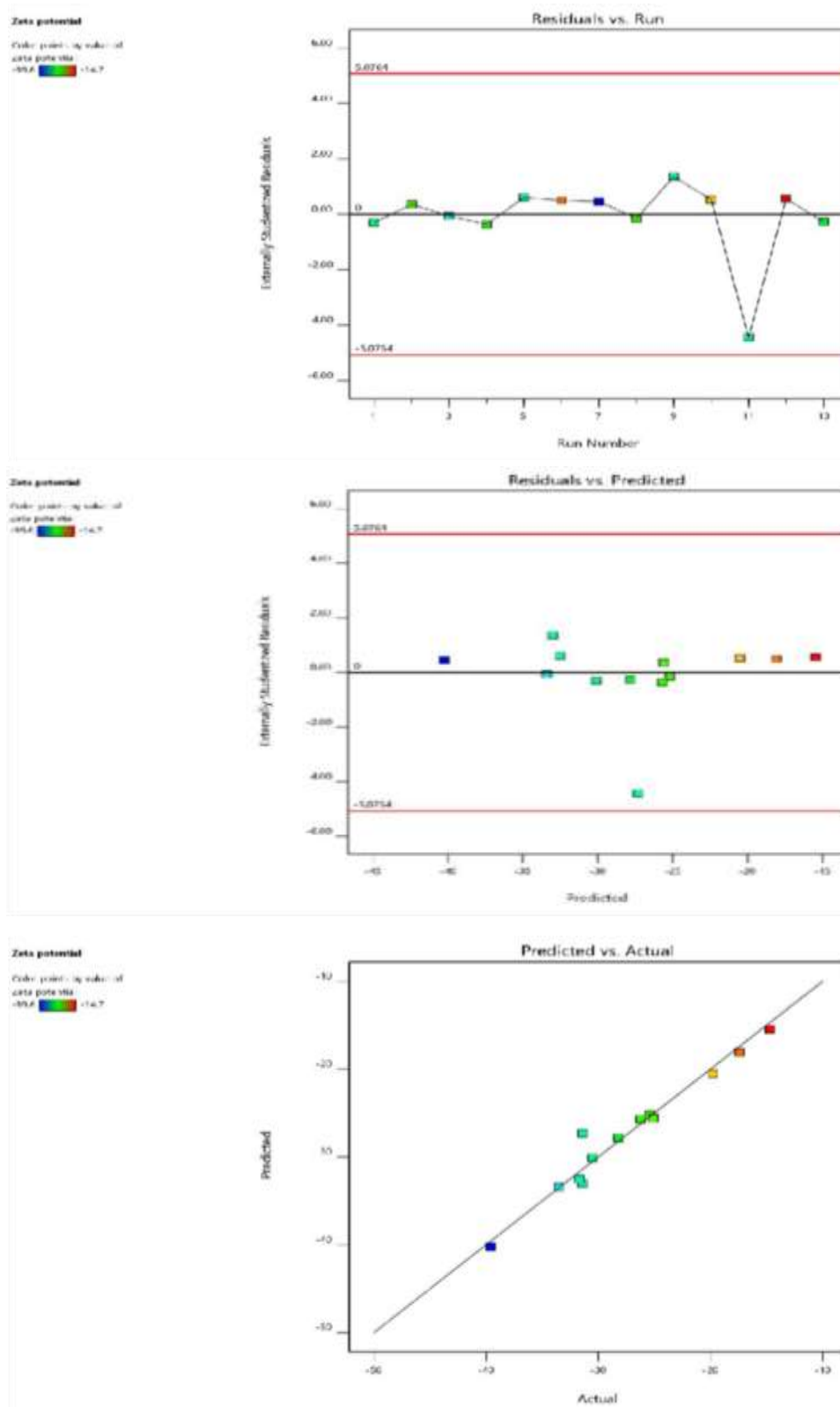


Figure 4 Two-dimensional response surface plots (normal plot vs residual, residual vs predicted and residual vs. run of zeta potential) revealing relative effects of independent variables on dependent variable zeta potential of drug loaded formulation

4.10 Actual value versus predicted value of Zeta potential

Table 16 Report

Formulation	Actual Value	Predicted Value
NC 1	-30.50	-30.09
NC 2	-25.10	-25.59
NC 3	-33.50	-33.41
NC 4	-26.20	-25.70
NC 5	-31.70	-32.51
NC 6	-17.40	-18.09
NC 7	-39.60	-40.23
NC 8	-25.40	-25.19
NC 9	-31.40	-33.01
NC 10	-19.80	-20.51
NC 11	-31.40	-27.30
NC 12	-14.70	-15.45
NC 13	-28.20	-27.83

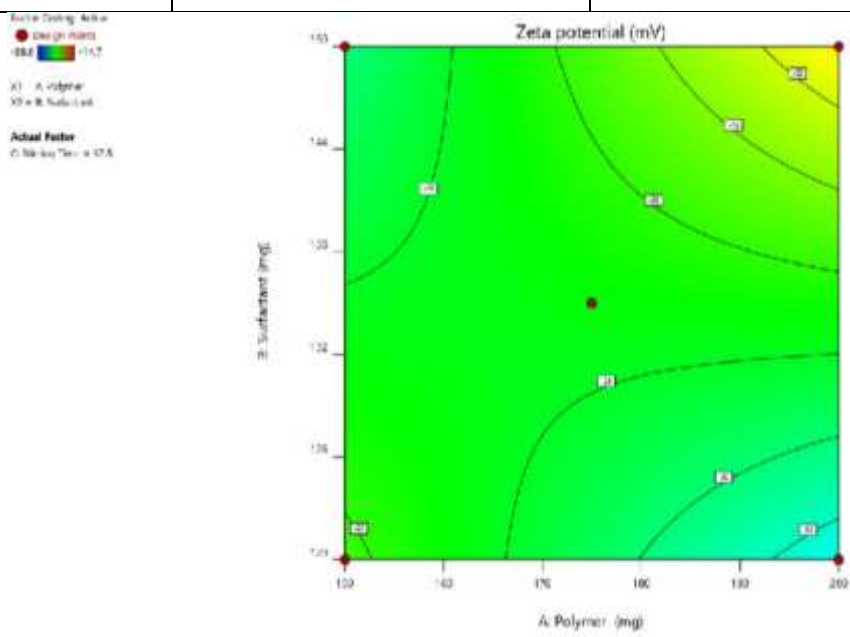
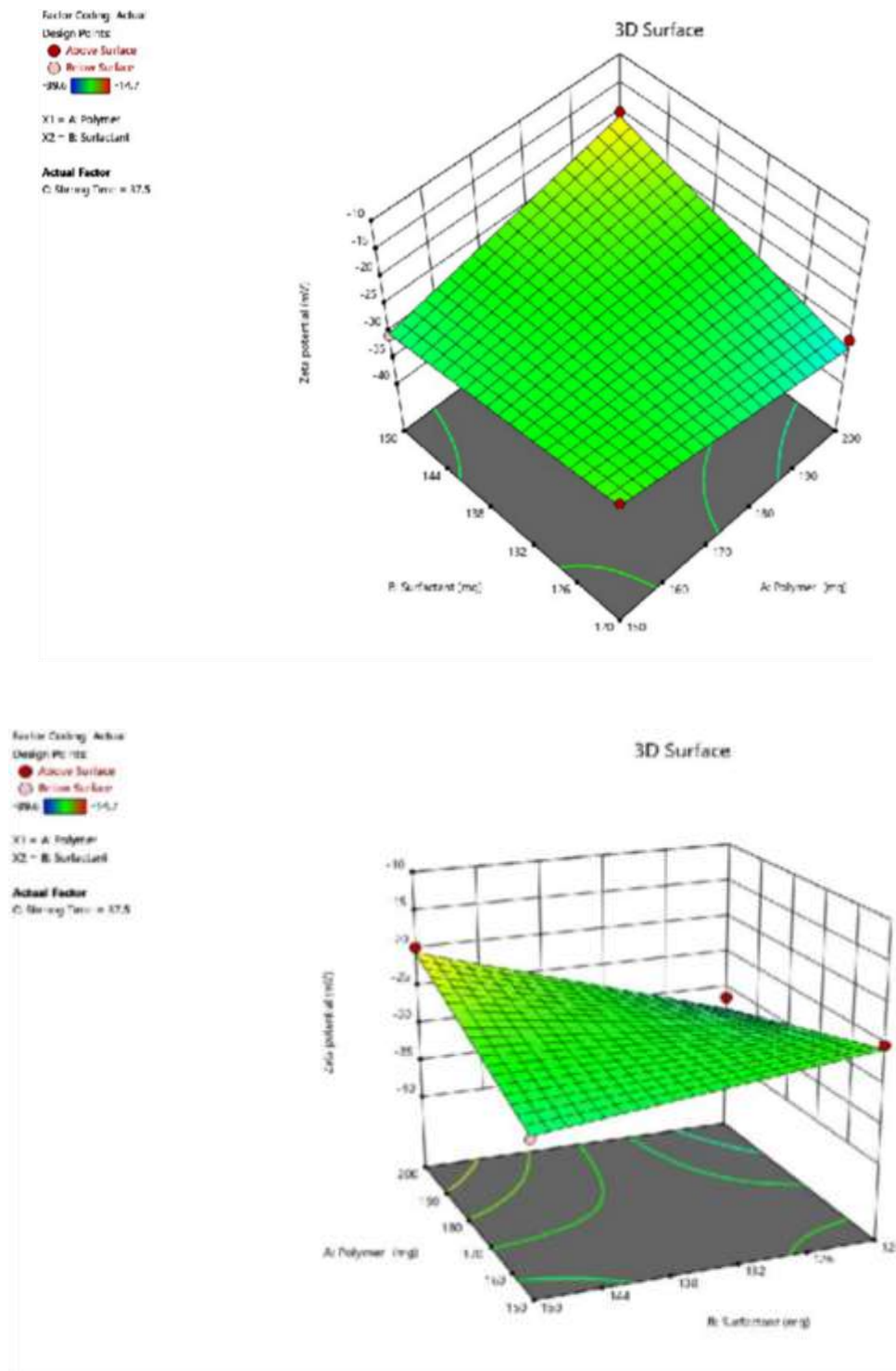


Figure 5 Two-dimensional (2D) contour plots for the effect of polymer (PLGA) and surfactant concentration on zeta potential



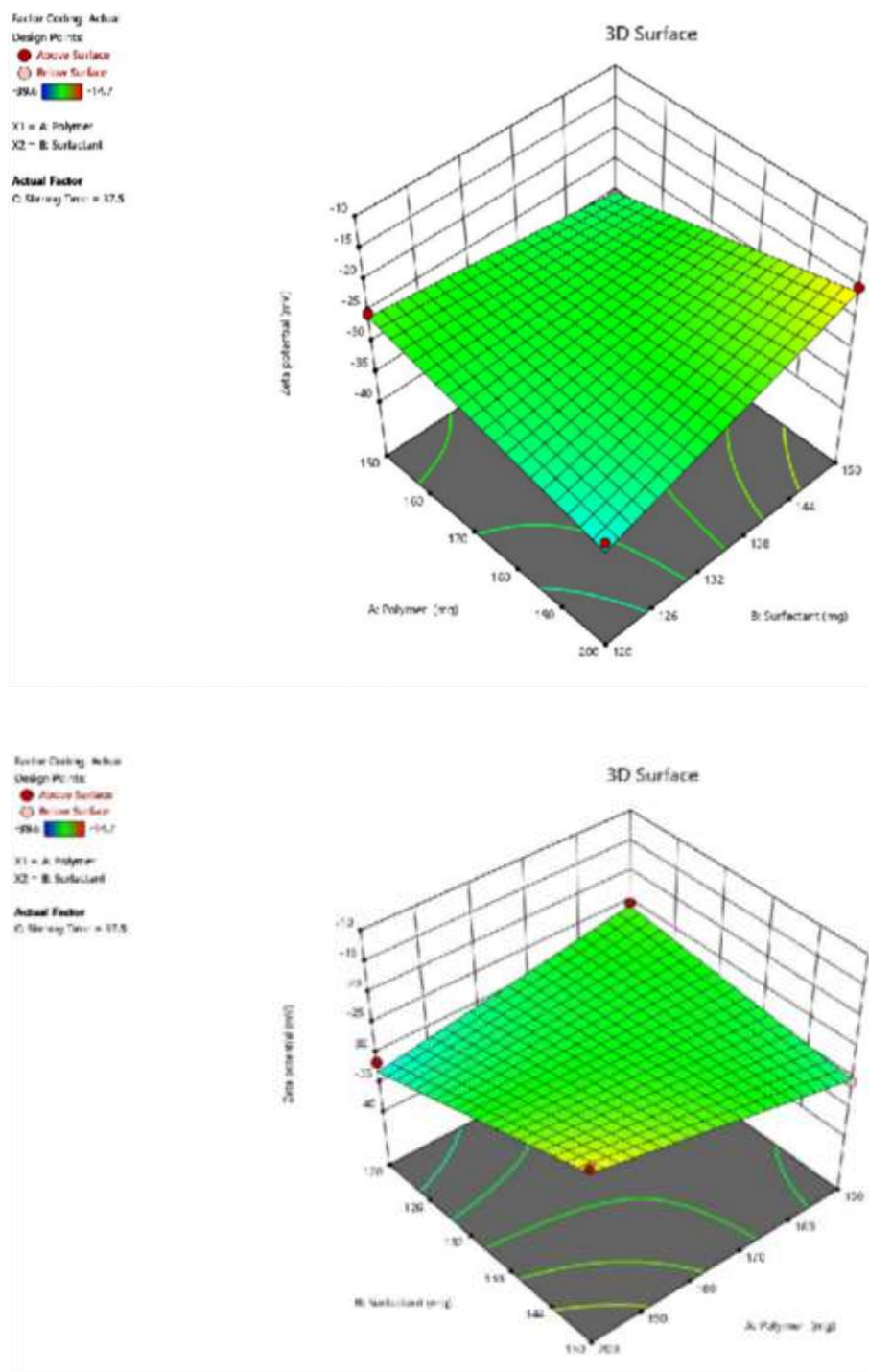
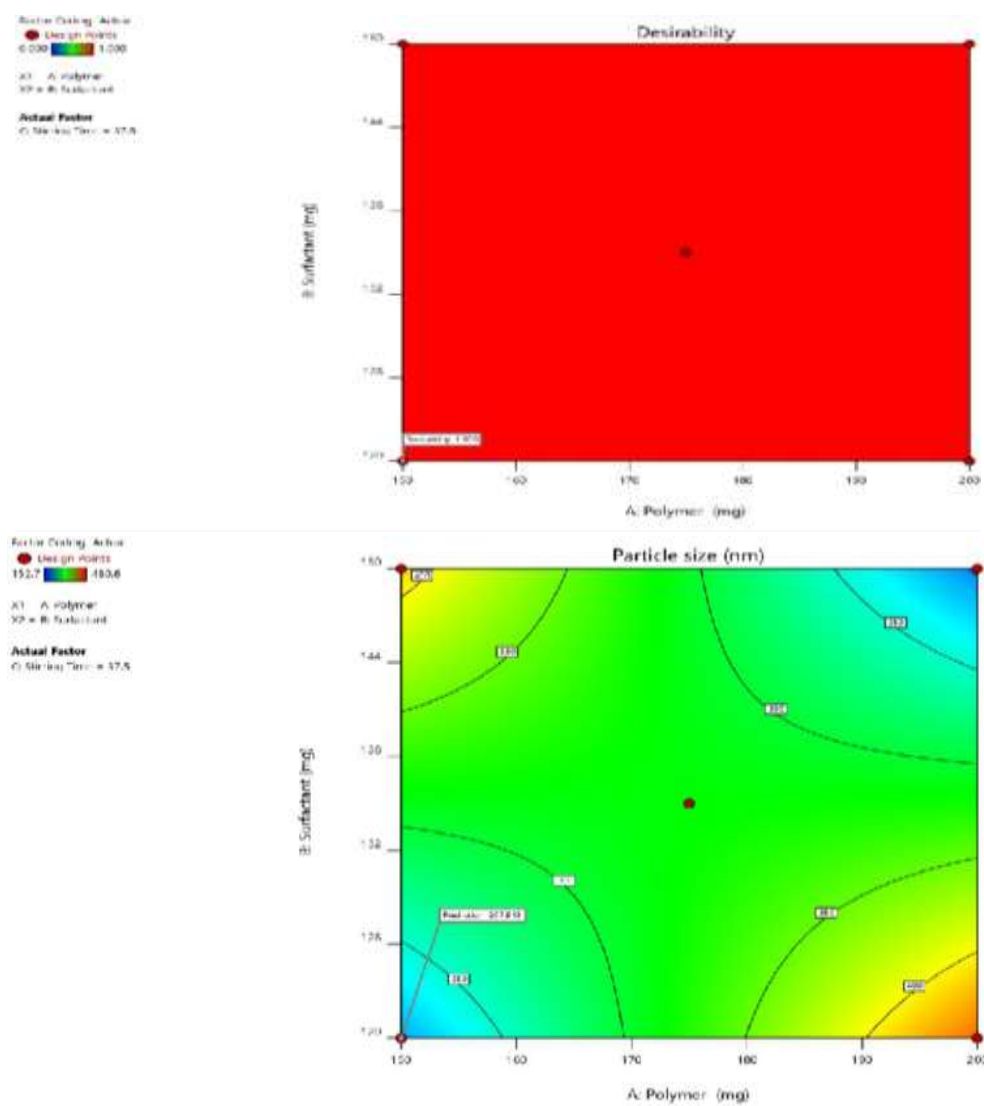


Figure 6 Three-dimensional (3D) Response surface plot showing combined effect of polymer and surfactant on zeta potential of drug loaded formulation

Table 17 Limit of Variables

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:Polymer	is in range	150	200	1	1	3
B:Surfactant	is in range	120	150	1	1	3
C:Stirring Time	is in range	30	45	1	1	3
Particle size	none	152.7	480.6	1	1	3
Zeta potential	none	-39.6	-14.7	1	1	3



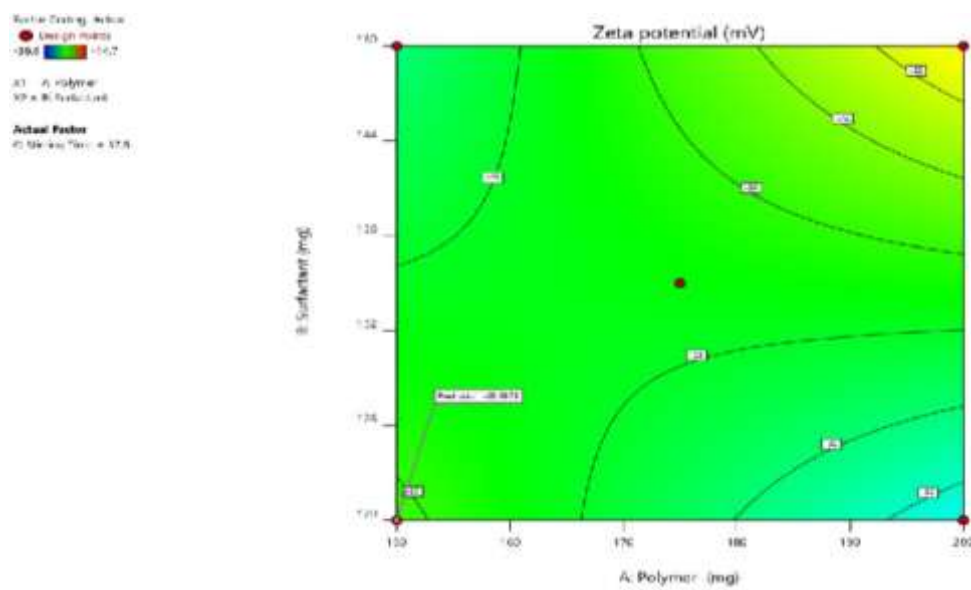


Figure 7 Response surface plot showing prediction data for optimization

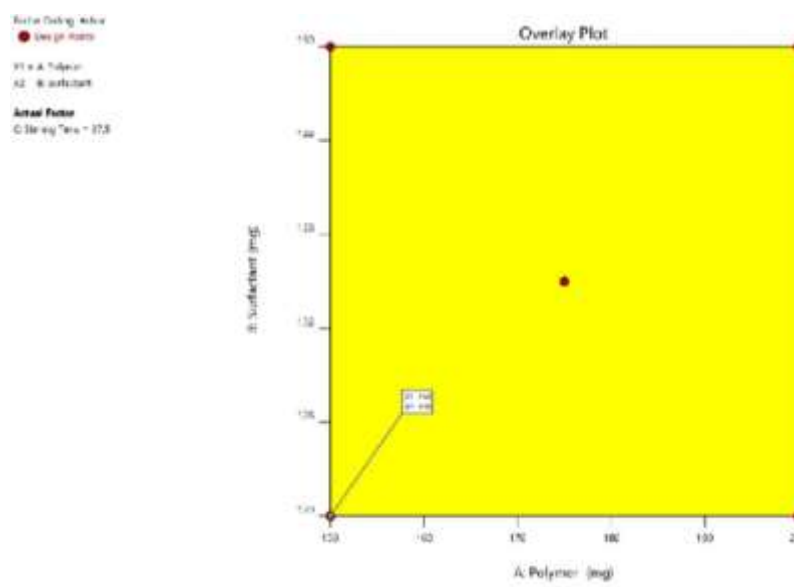


Figure 8 Overlay plots of optimization formulation

Table 18 Solutions

Number	Polymer (PLGA)	Surfactant	Stirring Time	Particle size	Zeta potential	Desirability	
1	200.000	150.000	37.500	196.228	-20.512	1.000	
2	150.000	120.000	37.500	207.913	-25.587	1.000	Selected
3	175.000	150.000	30.000	377.291	-32.513	1.000	
4	150.000	150.000	37.500	412.428	-30.087	1.000	

Table 19 Final Composition of Optimized formulations

S. No	Formulation code	Polymer (PLGA) mg	Surfactant mg	Stirring Time mins	Soy lecithin mg	Isopropyl myristate mg	Ellagic acid mg
1.	NC	150	120	37.5	150	100	20

4.11 Evaluation of nanocapsules formulation

4.11.1 Particle size analysis

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	191.9 nm	58.1 nm	181.5 nm
2	---	---nm	---nm	---nm
3	---	---nm	---nm	---nm
Total	1.00	191.9 nm	58.1 nm	181.5 nm

Cumulant Operations

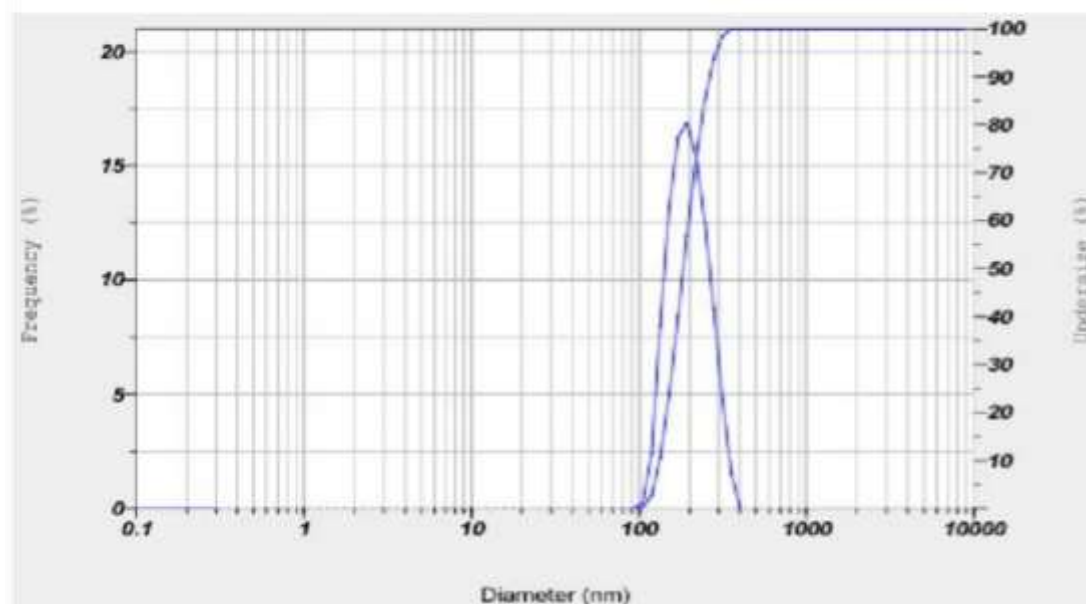
Z-Average : 170.1 nm

PI : 0.583

Molecular weight measurement

Molecular weight : ---

Mark-Houwink-Sakurada parameters : ---



Particle size of optimized nanocapsules formulation

Table 20 Particle size of nanocapsules formulation

S. No	Formulation	Particle size (predicted value)	Particle size (Actual value)
1.	NC	207.913nm	170.1nm

4.11.2 Zeta potential analysis

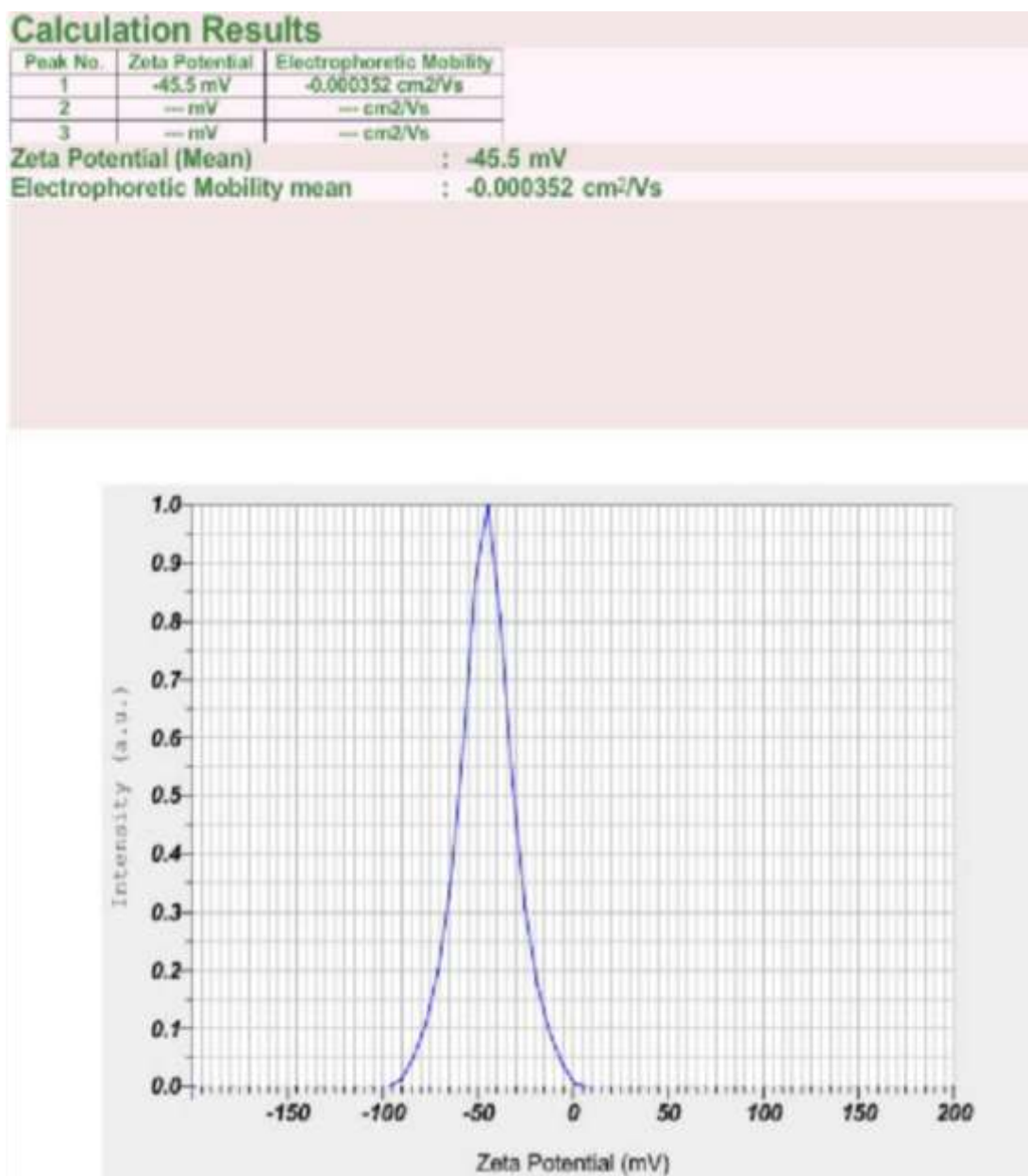


Figure 9 Zeta potential of nanocapsules formulation

Table 21 Zeta potential of nanocapsules formulation

S. No	Formulation	Zeta potential (Predicted value)	Zeta potential (Actual value)
1.	NC	-25.587mV	-45.5 mV

4.11.3 Entrapment efficiency

Drug entrapment efficiency is a measure of the drug-loading capacity of a system. The entrapment efficiency of betulinic acid loaded nanocapsule was found to be 85.43% for the optimized formulation.

4.11.4 SEM analysis

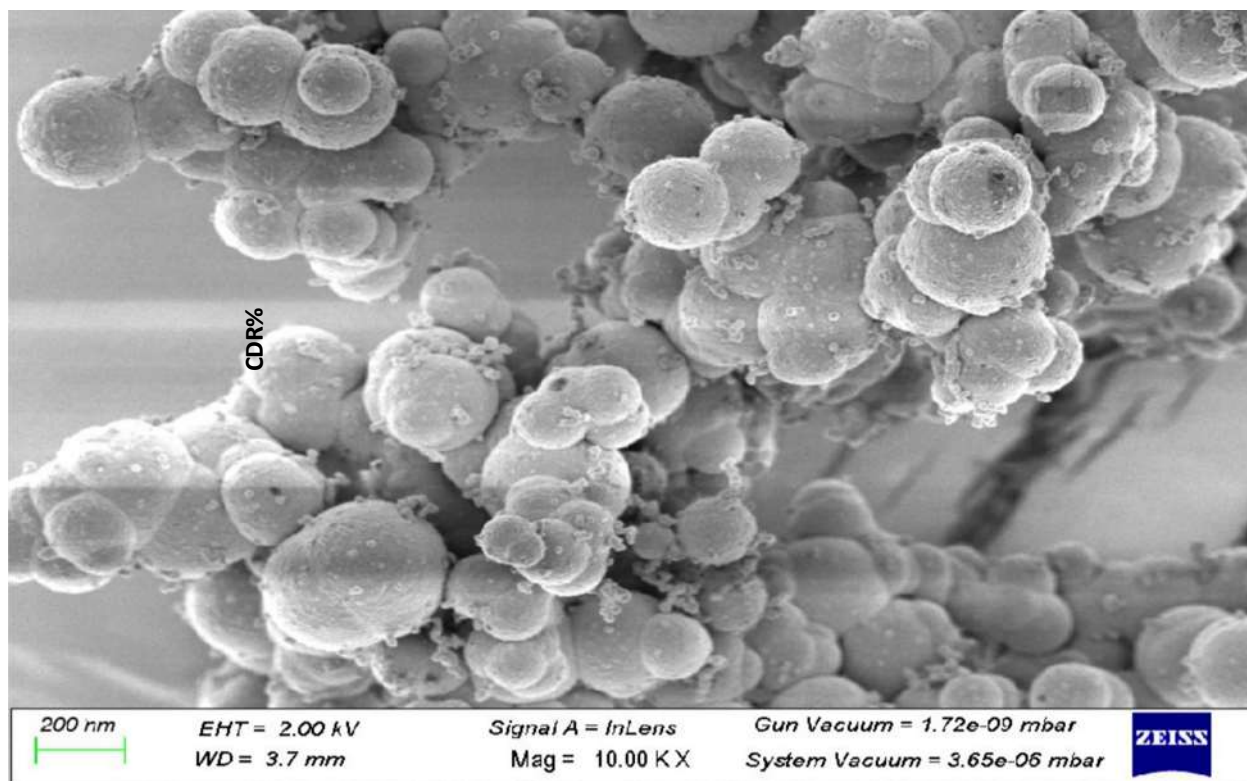
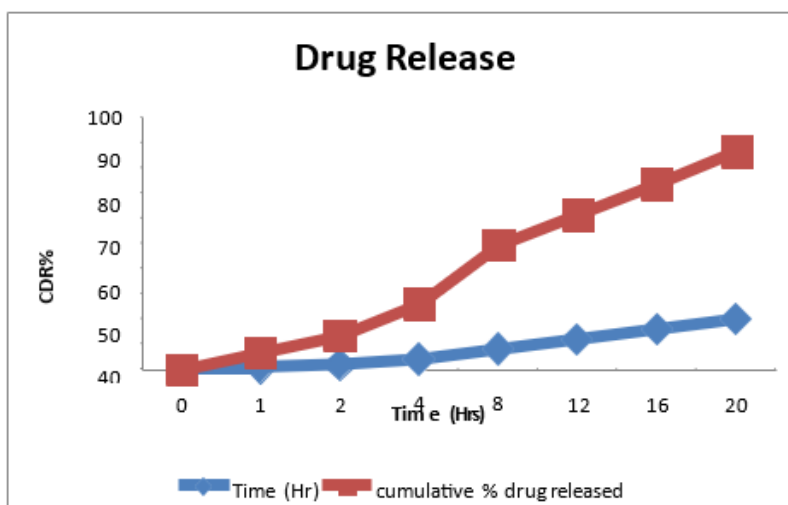


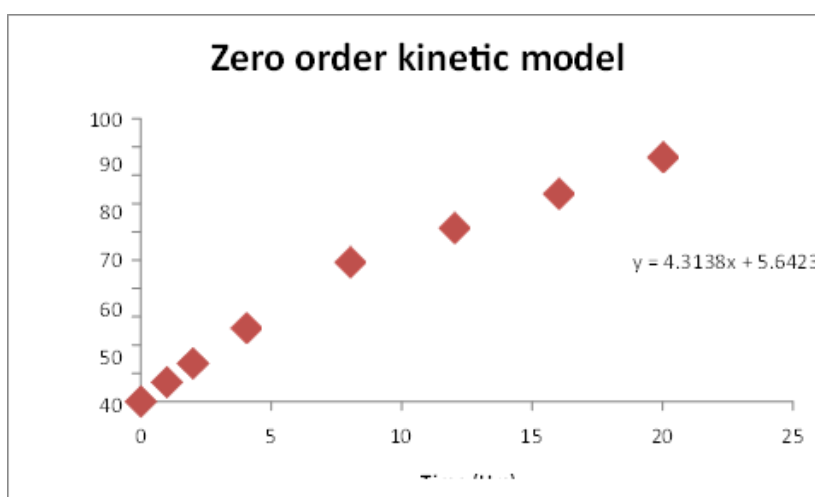
Figure 10 SEM image of nanocapsule formulation

Table 21 In vitro drug release study of ellagic acid loaded nanocapsules

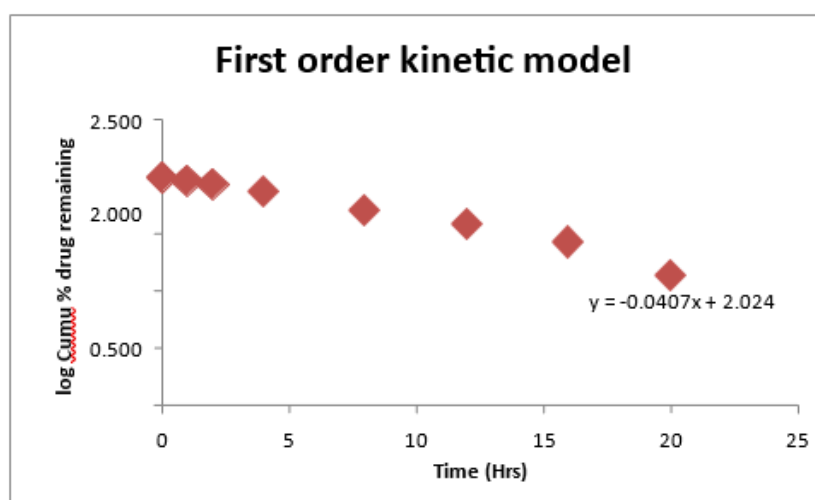
Time (hrs)	Cumulative % drug released	% drug remaining/ drug present	Square root time	Log cumulative % drug remaining	Log time	Log cumulative % drug released	% drug released
0	0	100	0.000	2.000	0.000	0.000	100
1	6.83	93.17	1.000	1.969	0.000	0.834	6.83
2	13.47	86.53	1.414	1.937	0.301	1.129	6.64
4	26.02	73.98	2.000	1.869	0.602	1.415	12.55
8	49.28	50.72	2.828	1.705	0.903	1.693	23.26
12	61.38	38.62	3.464	1.587	1.079	1.788	12.1
16	73.51	26.49	4.000	1.423	1.204	1.866	12.13
20	86.42	13.58	4.472	1.133	1.301	1.937	12.91



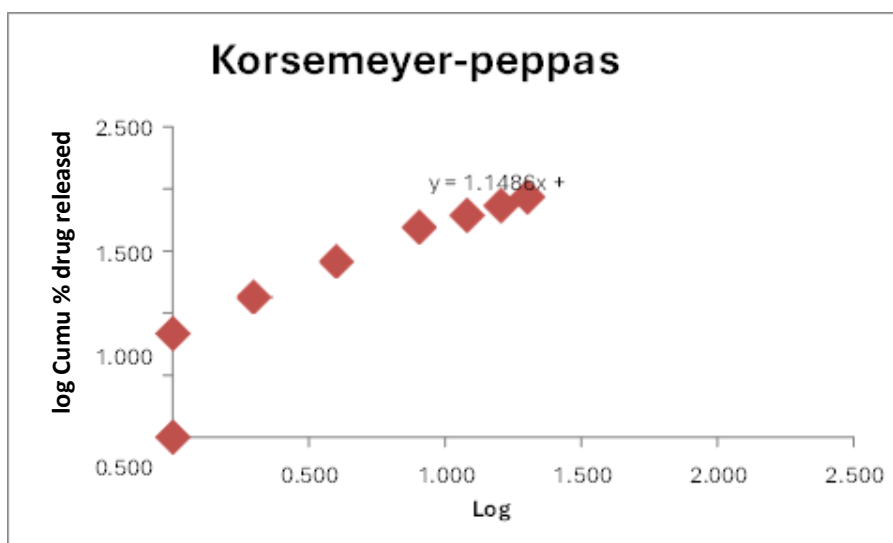
Graph 1 In-vitro drug release for nanocapsules prepared by optimization



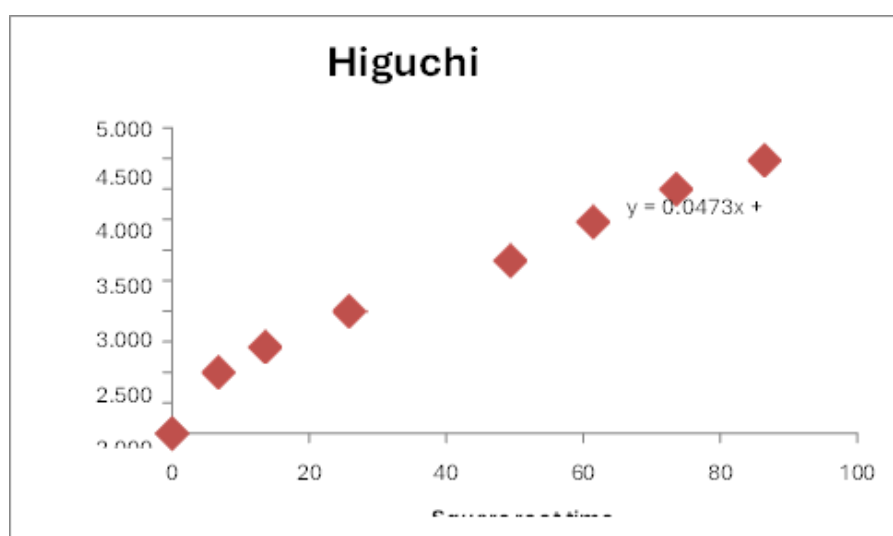
Graph 2 Zero order plot



Graph 3 First order plot



Graph 4 Korsmeyer-peppas plot



Graph 5 Higuchi plot

Table 22 Correlation Value (R^2 value)

Formulation	Kinetic Model	Regression value
NC	Zero order	0.975
	First order	0.981
	Korsmeyer-peppas	0.838
	Higuchi	0.973

The drug release data of the nanocapsule formulation (NC) were fitted to different kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer–Peppas models, to understand the release mechanism. The regression coefficient (R^2) values indicated that the first-order kinetic model ($R^2 = 0.981$) showed the highest correlation compared to the zero-order model (R^2

= 0.975), Higuchi model ($R^2 = 0.973$), and Korsmeyer–Peppas model ($R^2 = 0.838$). Overall, the results indicate that the drug release from the nanocapsules follows predominantly first-order kinetics with diffusion-controlled characteristics.

Stability study of Nanocapsule

Stability studies of the developed formulation were carried out in accordance with International Council for Harmonisation (ICH) guidelines to determine the physical stability and estimate the shelf life of the product. The studies were designed to evaluate the effect of environmental factors such as temperature and humidity on the formulation over a defined period.

The prepared formulations were packed in suitable, well-closed glass containers and stored under controlled conditions. The stability studies were performed by keeping the formulations at $40^\circ\text{C} \pm 2^\circ\text{C}/75\% \text{ RH} \pm 5\% \text{ RH}$ for the period of three month. The formulations were stored for a period of three months, and samples were withdrawn at predetermined time intervals (0, 1, 2, and 3 months) for evaluation.

The following parameters such as color, appearance, phase separation, particle size, zeta potential, and entrapment efficiency were observed.

3.3 Evaluation of NLC formulation

At each time point, the formulations were analyzed for the following physicochemical parameters:

1. **Color:** Visually inspected for any change in color.
2. **Appearance:** Examined for clarity, uniformity, and any visible changes.

3.3.1 Particle Size Measurement

The particle size of the prepared nanocapsule formulation was measured using Dynamic Light Scattering (DLS) with a particle size analyzer. A small volume of the formulation was appropriately diluted with distilled water to achieve optimal scattering conditions. The diluted sample was transferred into a clean, dust-free cuvette and analyzed at room temperature. The instrument provided values for the average particle size and polydispersity index (PDI), which indicate the uniformity of particle distribution. These parameters are essential for evaluating the stability of the formulation and its suitability for oral or transdermal drug delivery applications (Sangolkar *et al.*, 2012).

3.3.2 Zeta Potential

The zeta potential of the nanocapsules formulation was measured using a Horiba Zetasizer to evaluate the surface charge and predict colloidal stability. A small amount of the formulation was appropriately diluted with distilled water to optimize conductivity and reduce the likelihood of particle aggregation during measurement. The diluted sample was placed in a specialized zeta potential cell and analyzed at room temperature. The instrument determined the electrophoretic mobility of the nanocapsules and calculated the corresponding zeta potential, providing insight into the electrostatic interactions between particles. High absolute values of zeta potential, whether positive or negative, indicate strong repulsive forces that help prevent aggregation, thereby enhancing the physical stability and shelf life of the nanocapsules formulation

3.3.3 Determination of Entrapment Efficiency

The entrapped drug concentration was determined by lysis of the nanocapsules using absolute alcohol followed by sonication. An accurately weighed amount of drug-loaded nanocapsules (10mg) was dissolved in 10 mL of absolute alcohol and tightly covered to prevent evaporation.

The solution was sonicated for 15 min to obtain a clear solution. An aliquot of 1 mL of this solution was then diluted with 9 mL of absolute alcohol and sonicated for an additional 15 min. The concentration of ellagic acid in absolute alcohol was determined spectrophotometrically (Shimadzu UV-1700) at 366 nm after appropriate dilution (Nasr *et al.*, 2008).

Unloaded nanocapsules showed insignificant absorbance at the same wavelength. Each sample was analyzed in triplicate.

The entrapment efficiency was calculated using the following equation:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Entrapped drug}}{\text{Total drug}} \times 100$$

4.1 Stability study of formulation

Table 1: Evaluation of Nanocapsules stored at $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $75\% \text{ RH} \pm 5\% \text{ RH}$ after 3 months

Properties	Initial	After 1 month	After 2 month	After 3 month
Physical Characteristics				
Color	White	White	White	White
Consistency	Semi liquid	Semi liquid	Semi liquid	Semi liquid
Phase separation	No separation	No separation	No separation	No separation
Chemical Properties				
Particle size (nm)	170.1	178.97	183.2	205.5
Zeta potential (mV)	-45.5	-41.2	-38.5	-37.9
Entrapment efficiency (%)	85.43	85.21	83.57	82.49

4.12 Gross Morphological Examination of Experimental Mice and Isolated Liver Tissues

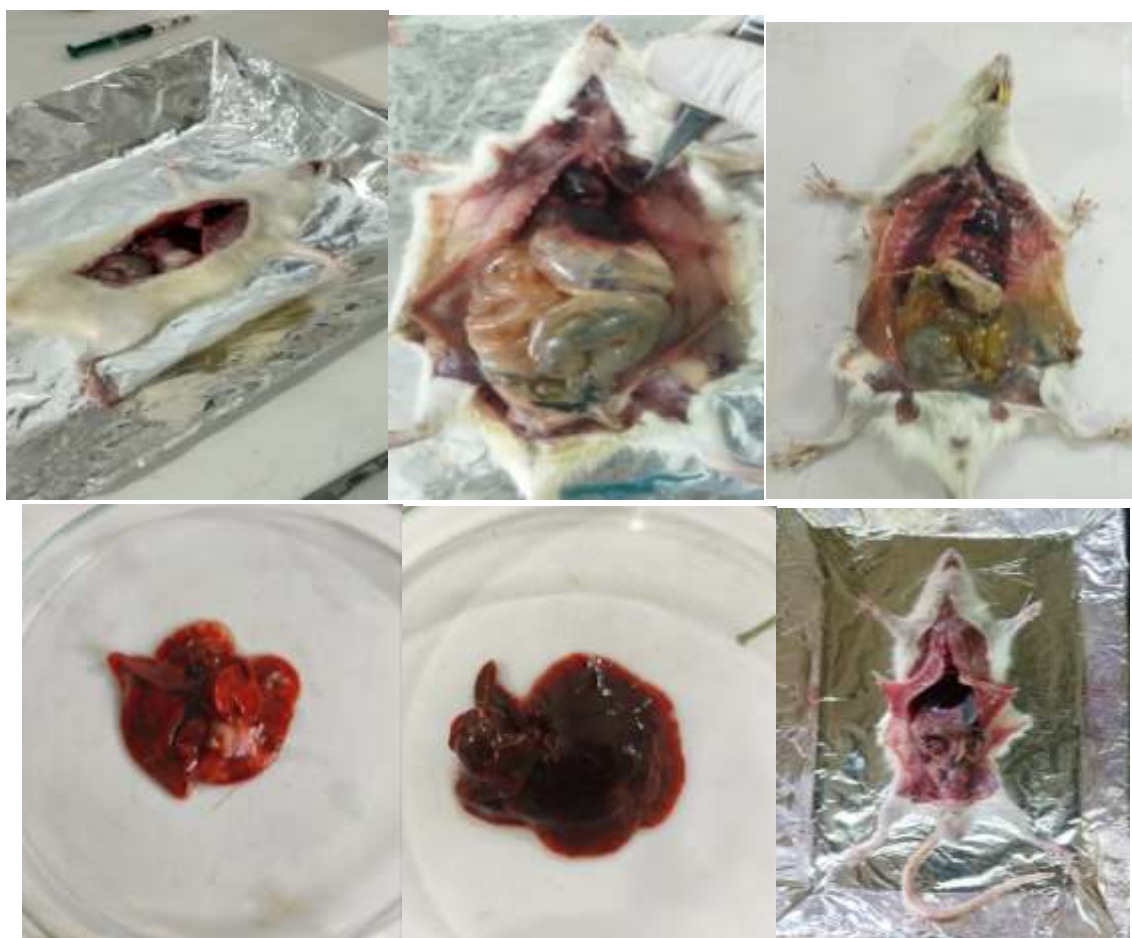


Figure 11. Gross morphological observations during experimental animal examination: (A–C) dissected experimental mice and (D–F) isolated liver tissues.

5. DISCUSSION

The present study was designed to develop an ellagic acid-loaded nanocapsule system using PLGA as the polymeric component and to optimize the formulation through a three-factor, three-level Box–Behnken design. The formulation variables were polymer concentration, surfactant concentration, and stirring time, while particle size and zeta potential were selected as the principal responses. The use of a design-of-experiments approach enabled assessment of both individual factors and their interactions rather than relying only on one-factor-at-a-time experimentation. The optimization results demonstrated that the selected formulation (NC2) contained 150 mg PLGA, 120 mg surfactant and 37.5 min stirring time. The model predicted a particle size of 207.913 nm and a zeta potential of -25.587 mV, whereas the experimentally obtained values were 170.1 nm and -45.5 mV, respectively. The particle-size response model showed a high R^2 of 0.9799, adjusted R^2 of 0.9597 and predicted R^2 of 0.9131, with adequate precision of 21.34. Similarly, the zeta-potential model showed R^2 of 0.9604, adjusted R^2 of 0.9207 and predicted R^2 of 0.9072, with adequate precision of 17.299. These statistics support the usefulness of the fitted models for navigating the investigated design space. The particle size of 170.1 nm places the optimized formulation within the nanoscale range targeted in the present work. Nanocapsules are core–shell carrier systems in which a liquid or oily core is surrounded by a polymeric membrane, and interfacial deposition is an established method for their preparation. The negative zeta potential of -45.5 mV also suggests a substantial electrostatic surface charge, which is consistent with the physical stability rationale described in the manuscript. However, zeta potential alone should not be considered a complete prediction of long-term stability because colloidal stability is also influenced by steric effects, medium composition, ionic strength, temperature and other formulation variables. The optimized formulation showed an entrapment efficiency of 85.43%. This indicates that a substantial proportion of the added ellagic acid was incorporated into the nanocapsule system. The result is particularly relevant for ellagic acid because its poor aqueous solubility and limited oral bioavailability have been repeatedly identified as formulation challenges. Nanoencapsulation can provide a carrier environment that may improve dispersion and protect the incorporated compound while allowing modified release. Nevertheless, the present study establishes formulation performance rather than clinical or therapeutic superiority, and comparative pharmacokinetic studies would be needed to demonstrate improved systemic exposure. The *in vitro* release study showed a gradual release of ellagic acid from the optimized nanocapsules, reaching 86.42% cumulative release at 20 h. Mathematical fitting showed R^2 values of 0.975 for zero-order, 0.981 for first-order, 0.973 for Higuchi and 0.838 for Korsmeyer–Peppas models. On the basis of the highest R^2 , the manuscript identifies first-order kinetics as the best-fitting model, with diffusion-controlled characteristics also indicated by the relatively high Higuchi correlation. Because model selection based solely on R^2 has limitations, additional mechanistic analysis and replicate release studies would strengthen the interpretation. The stability study conducted at 40 ± 2 °C/ $75 \pm 5\%$ RH for three months showed no visible phase separation and no change in color or consistency. Particle size increased from 170.1 nm initially to 205.5 nm after three months, while zeta potential changed from -45.5 to -37.9 mV and entrapment efficiency decreased from 85.43% to 82.49%. These changes suggest some physicochemical evolution during accelerated storage, although the formulation retained its general physical appearance and nanoscale characteristics during the study period. Longer-term studies under both accelerated and recommended storage conditions would be required for a robust shelf-life assessment. The gross animal images included in the manuscript provide visual documentation of experimental animal dissection and isolated liver tissues. Such photographs can support documentation of the experimental procedure and gross appearance, but they should not be interpreted as quantitative evidence of hepatoprotection. The uploaded manuscript does not provide group-wise serum biochemical values, oxidative-stress markers, histopathological scoring or a defined animal-group table corresponding to these images. Therefore, a definitive comparison of free ellagic acid versus ellagic acid-loaded nanocapsules on hepatic injury cannot be established from the images alone. If those biochemical and histopathological results are available separately, they should be incorporated into the Results and Discussion to substantiate the *in vivo* hepatoprotective claim. Overall, the formulation findings are consistent with the rationale for using a nanocapsule platform to improve the pharmaceutical performance of ellagic acid. Previous literature has reported antioxidant and hepatoprotective effects of ellagic acid in experimental liver-injury models, while reviews have emphasized poor solubility and bioavailability as important barriers to its therapeutic translation. The current study adds a formulation-development component by combining interfacial nanocapsule preparation, statistical optimization, physicochemical characterization, release analysis and accelerated stability testing. Further work should include complete *in vivo* pharmacodynamic data, pharmacokinetic comparison with free ellagic acid, detailed histopathology, dose optimization, toxicity evaluation and longer-term stability studies.

6. CONCLUSION

The present investigation successfully developed an ellagic acid-loaded nanocapsule formulation using an interfacial deposition approach and optimized the formulation using a Box–Behnken experimental design. The selected formulation, NC2, contained 150 mg PLGA, 120 mg surfactant and 37.5 min stirring time. It showed an experimentally determined particle size of 170.1 nm, zeta potential of -45.5 mV and entrapment efficiency of 85.43%. The formulation exhibited sustained release behavior, with 86.42% cumulative ellagic acid release at 20 h, and the first-order model provided the highest correlation among the models evaluated ($R^2 = 0.981$). Accelerated stability testing for three months showed no visible phase separation, while particle size, zeta potential and entrapment efficiency showed moderate changes. These findings demonstrate that the developed nanocapsule system has promising pharmaceutical characteristics for delivery of ellagic acid. The animal images document the experimental liver examination, but quantitative hepatoprotective efficacy cannot be concluded from photographs alone. Complete biochemical, oxidative-stress, histopathological, pharmacokinetic and safety data should therefore be included before making a definitive therapeutic claim. Further in vivo and long-term stability studies are warranted to establish the translational potential of the optimized formulation.

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