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ORIGINAL ARTICLE

Formulation and Characterization of Embelin Nanoemulsion-Loaded Nanogel: Enhanced Solubility and Antifungal Enzyme Inhibition

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ABSTRACT
Embelin, a lipophilic benzoquinone from Embelia ribes, exhibits antifungal potential but is limited by poor aqueous solubility and bioavailability. This study developed and optimized Embelin-loaded nanoemulsions (NEs) via a Simple Lattice design and converted the optimized NE into a nanogel (F11). Solubility screening identified olive oil, Tween-80, and PEG-400 as optimal excipients. Fourteen formulations were prepared and characterized for droplet size, zeta potential, pH, viscosity, and drug content. Particle sizes ranged 225–269 nm with optimized batch F11 exhibiting a mean hydrodynamic diameter of 225 nm and zeta potential −19.4 mV. Drug content in formulations varied between 81.9% and 98.4% (F11 = 98.36%). In vitro release from dialyzed NE and nanogel in PBS pH 7.4 showed sustained release; F11 nanoemulsion achieved 98.91 ± 2.25% cumulative release by 480 min, while F11 nanogel reached 95.74 ± 2.96% at 12 h. Stability at 40°C/75% RH for 3 months indicated negligible changes in pH, viscosity, and drug content. Antifungal virulence enzyme inhibition assays (phospholipase and protease) revealed enhanced activity for formulated products: pure Embelin inhibited phospholipase 39.89 ± 1.36% and protease 32.47 ± 1.29%; NE F11 inhibited phospholipase 66.46 ± 1.93% and protease 59.23 ± 1.68%; nanogel inhibited phospholipase 78.72 ± 1.84% and protease 72.81 ± 1.49%. These values are comparable to ketoconazole (phospholipase 79.41 ± 1.18%, protease 74.92 ± 1.04%). The results indicate that nanoformulation markedly improves Embelin's functional inhibition of fungal virulence enzymes and supports the potential of Embelin nanogels as topical antifungal therapeutics.
Keywords: Embelin; Nanoemulsion; Nanogel; Antifungal enzymes; Phospholipase inhibition; Protease inhibition; Solubility enhancement; Green formulation

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INTRODUCTION

Natural products continue to be a major source of novel antimicrobial agents, with plant-derived phytochemicals showing promise against fungal pathogens that are increasingly resistant to conventional therapies [1,2]. Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone), a major constituent of *Embelia ribes*, has been reported to possess diverse bioactivities including anticancer, anti-inflammatory, antioxidant, and antimicrobial effects [3,4]. However, its clinical translation is hindered by low aqueous solubility and rapid metabolic clearance, which diminish in vivo potency despite favorable in vitro activity. Formulation science offers strategies to overcome these physicochemical barriers; lipid-based carriers such as nanoemulsions (NEs) and derived nanogels provide improved solubilization, enhanced permeation, controlled release, and the potential for targeted topical application [5–7].

Nanoemulsions are thermodynamically unstable but kinetically stable dispersions of oil droplets in water stabilized by surfactants and co-surfactants. Their small droplet size (typically <300 nm) increases interfacial area, facilitating solubilization of lipophilic drugs and improving absorption across biological barriers. Conversion of an optimized NE into a nanogel by incorporating a biocompatible gelling agent (e.g., Carbopol) combines the advantages of nanoscale drug carriers with the rheological properties necessary for topical application — superior spreadability, residence time, and controlled release. Moreover, adopting greener formulation approaches — selecting safe oils (olive oil), generally recognized

as safe (GRAS) surfactants (Tween-80), and moderate co-solvents (PEG-400) — aligns development with sustainability and regulatory acceptability [8–11].
Fungal virulence is mediated by extracellular hydrolytic enzymes such as phospholipases and proteases, which facilitate host tissue invasion and immune evasion [12,13]. Enzyme inhibition assays provide mechanistic insight into antifungal potential beyond simple growth inhibition; suppression of these enzymes may attenuate pathogenicity, rendering treatments more effective [14–16]. In this investigation, Embelin was formulated into multiple NE prototypes using a factorial design and characterized for particle size, zeta potential, pH, viscosity, drug content, and in vitro release. The optimized NE (F11) was converted into a nanogel and evaluated in antifungal enzyme inhibition assays alongside pure Embelin and ketoconazole as a standard. The aim was to quantitatively evaluate whether nanoformulation enhances Embelin's functional inhibition of fungal virulence factors and to characterize the physicochemical attributes supporting such activity. Findings could support further preclinical development of Embelin nanogels as topical antifungal agents with improved efficacy and formulation-driven advantages.

MATERIAL AND METHODS

Embelin was purchased from Total Herb Solutions, Mumbai, India. Pectin was obtained from Hi-Media Lab, Mumbai. Olive oil, almond oil, and eucalyptus oil was purchased from Moychem Pvt. Ltd., Methanol, Ethanol, Chloroform, Acetone, and Potassium dihydrogen phosphate (KH_2PO_4), Sodium chloride, PEG-400 and Tween80 were obtained from Loba Chem. Pvt. Ltd. Mumbai. All material were used of analytical grade. Double distilled water was used as aqueous solvent.

Physical Characterization and Identification

Physical examination

In the pre-formulation study the physical inspection of Embelin is done firstly for its appearance, colour and taste for identification [17,18].

UV-Visible spectroscopy in Methanol

An accurately weighed 100 mg of Embelin pure drug was dissolved in 100 ml of Methanol using a 100 ml volumetric flask. The solution was then sonicated for 10 min and the final volume was adjusted up to the mark with the same solvent, to give the final concentration of 1000µg/ml. Out of this stock, 10ml was pipetted and diluted up to 100ml (100µg/ml) by Methanol and examined between 200-400 nm. The maximum absorbance was determined using UV-Vis Spectrophotometer to confirm the λmax of the drugs [19–21].

Calibration curve in Methanol

Five concentrations of standard solution (2, 4, 6, 8, 10 µg/ml) were prepared by pipetting out 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 10 ml of standard stock solution and transferring into a series of 10 ml volumetric flask. The volume was then adjusted up to the mark with Methanol. The absorbance of each solution was recorded at 395 nm using Methanol as a blank. A calibration graph was prepared by plotting absorbance vs respective concentration.

FTIR Analysis

FTIR analysis was done on FTIR spectrometer (Ver. 7.03 Shimadzu, Japan) with KBr disc. In the FTIR infrared spectroscopy the spectrum was recorded in the wavelength region of 4000-400 cm^{-1}. 10 mg of drug was mixed with KBr and triturated then it was placed in holder and pressed to form a pellet. Then it was placed under IR beam and a spectrum was obtained on computer [22–25].

Differential Scanning Calorimetry

Thermogram for Embelin was obtained using DSC (Mettler DSC 1 star system, Mettler-Toledo, Switzerland). The drug was sealed in perforated aluminum pan and heated at constant rate of 10°C/min over the temperature ranges of 30-350°C at 20ml/min nitrogen purging (26–28).

Solubility Determination

When the solubility of a drug is less than 10 mg/mL it affects the bioavailability due to the absorption problem since prediction of solubility is a useful parameter. The apparent solubility of Embelin was determined in methanol, DMSO, Buffer pH 1.2, 6.8 and 7.4 at 37±0.5°C. Excess of drug was added to 10 mL of solvent in glass vials with rubber closers. Then the vials were kept on an orbital shaking incubator maintained at 37±0.5°C for 24 h. After shaking, the vials were kept in an incubator at 37±0.5°C for equilibrium for 12 h. The solution was then filtered through 0.45µm millipore filter and the filtrate was assayed by UV spectrophotometer at λmax 395nm and the solubility was calculated by respective calibration curve [29–31].

Drug-Excipients Compatibility Study

FTIR Analysis

FTIR analysis was done on FTIR spectrometer (Ver. 7.03 Shimadzu, Japan) with KBr disc. In the FTIR infrared spectroscopy the spectrum was recorded in the wavelength region of 4000-400 cm-1. 10 mg of drug and excipients was mixed with KBr and triturated then it was placed in holder and pressed to form a pellet. Then it was placed under IR beam and a spectrum was obtained on computer [22–25].

Differential Scanning Calorimetry

Thermogram for Embelin and other excipients was obtained using DSC (Mettler DSC 1 star system, Mettler-Toledo, Switzerland). The drug was sealed in perforated aluminum pan and heated at constant rate of 10°C/min over the temperature ranges of 30-350°C at 20ml/min nitrogen purging [26–28].

Screening of Oil, Surfactant and Co-surfactant (Solubility Study)

The solubility study of Embelin in various oils, surfactants, and co-surfactants was performed to identify the most suitable components for developing a stable and efficient lipid-based drug delivery system. Given Embelin's lipophilic nature and limited aqueous solubility, it is essential to select excipients that can provide maximum solubilization, thereby enhancing drug loading, stability, and bioavailability. The oils (almond oil, eucalyptus oil, olive oil), surfactants (Tween 80, Span 20, Tween 20), and co-surfactants (PEG-400, ethanol, propylene glycol) were selected based on their emulsifying properties, safety profiles, and potential to solubilize lipophilic drugs. Excess Embelin was equilibrated with each vehicle, and after 24 hours, the concentration of drug solubilized in the supernatant was quantified using UV-Visible spectrophotometry at 395 nm. The excipients showing the highest solubility for Embelin were considered optimal, as they would allow the formulation of a more effective delivery system with improved drug dispersion and absorption. Thus, this solubility screening forms the rational basis for the selection of components in the formulation development of Embelin [29–31].

Preparation of Embelin loaded nanoemulsion

On the basis of their visual observation like transparency and zeta potential, 14 formulations were selected out as per factorial design (Table 1) for preparing Embelin loaded nanoemulsion. The required amount of Embelin was dissolved in the calculated quantity of oil phase for the said volume of nanoemulsion. The calculated quantity of Smix (surfactant and co-surfactant) were added and mixed thoroughly in beaker using magnetic stirrer at room temperature. Then double distilled water was added drop wise drop till a clear and transparent liquid was obtained. The prepared nanoemulsion was stored in tightly closed suitable container at ambient temperature [32–34].

Factorial Design

Construction of and Formula optimization using SLD

A Simple Latex Design (SLD) was used. All variables explained in Table 1.

Table 1: Independent Variables for QbD (SLD)

Independent Variables Coded Values	Low (-)	High (+)
X1: Concentrations of water (%)	62	64
X2: Olive oil (%)	2	4
X3: Smix (%)	32	34

TW80:TP (Smix) were blended in various proportions, and a mixture of OO with Smix(s) was created, resulting in OO:Smix (TW80:TP) ratios as outlined in Table 2 and 3. The optimization of N-NEs was carried out using Design-Expert (Stat-Ease Inc., USA), where the variables included concentrations of water (X1), OO (X2), and Smix (X3), and the corresponding response measured was particle size (Y1) and Zeta Potential (Y2). The statistical relationships between independent variables and 3D Response surface plot were also generated. The formulation layout for the factorial design batches is shown in Table 2 and 3.

Table 2: Levels of variables for optimization

Batch Code	X1: Water (%)	X2: Olive oil (%)	X3: Smix (%)
F1	0.5	0	0.5
F2	0.5	0.5	0
F3	0.333333	0.333333	0.333333
F4	1	0	0
F5	0.666667	0.166667	0.166667
F6	1	0	0
F7	0.166667	0.666667	0.166667
F8	0	1	0
F9	0.5	0.5	0
F10	0	0	1
F11	0	0	1
F12	0	0.5	0.5
F13	0.166667	0.166667	0.666667
F14	0	1	0

Table 3: Simple Latex Design (SLD)

Batch Code	X1: Water (%)	X2: Olive oil (%)	X3: S_{mix} (%)	Particle Size (nm)	Zeta Potential (mV)
F1	62	2	34	261	-16
F2	64	2	32	262	-12
F3	66	2	32	269	-26
F4	63	2	33	247	-25
F5	62.21	2.64	32.16	254	-22
F6	63	2	32	248	-20
F7	62.36	1.32	32.64	250	-19
F8	62	2	34	246	-21
F9	63.56	0.64	32.16	260	-14
F10	64	2	32	238	-24
F11	**62.21**	**0.64**	**33.56**	**225**	**-19**
F12	62	2	33	259	-11
F13	62	4	32	255	-23
F14	62	4	32	243	-20

Characterization of Nanoemulsion

Physical characterization

The prepared nanoemulsion formulations were visually inspected for their colour, transparency, homogeneity and consistency.

Droplet Size and Size Distribution

Droplet size was determined by photon correlation spectroscopy (PCS) that analyses the fluctuations in light scattering due to Brownian motion of the droplets using a Zetasizer (1000 HS, Malvern Instruments, Italy). 0.1ml nanoemulsion was dispersed in 50ml of water in a volumetric flask, mixed thoroughly with vigorous shaking and light scattering was monitored at 25°C a 90° angle.

Zeta potential analysis

Zeta potential of a droplet is the overall charge that the particle acquires in a particular medium. Knowledge of the zeta potential of nanoemulsion helps to assess the stability of the formulation during storage [35].

Measurement of pH

The pH values of the nanoemulsion were measured at 25ºC using digital pH meter. 10% w/w dispersion (1gm of nanoemulsion was dispersed in 10 ml of distilled water. At first the reading of pH- meter was adjust with a known pH solution (pH 4 and pH 7). Then the prepared formulations were subjected for pH analysis.

Surface Morphological Study

Surface morphology of the nanoemulsion was performed by using SEM/TEM. A nanoemulsion was placed on Formvars coated copper grids and allowed to equilibrate. Excess liquid was removed with a filter paper and dried at room temperature for about half an hour. The dried grid containing the nanoemulsion was visualized using SEM/TEM.

***In-vitro* Release Study of Embelin Loaded Nano-emulsion Formulations**

The *in-vitro* permeation studies were carried out using Franz diffusion cell, which is a reliable method for prediction of drug transport across the skin. These studies were conducted employing dialysis

membrane. The receptor compartment of the diffusion cell was filled with 25 ml of phosphate buffer pH 7.4. The whole assembly was fixed on a magnetic stirrer and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads at 100 rpm and the temperature was maintained at 37±0.50°C throughout the experiments [36,37].

Accelerated Stress Stability Study

Stability studies were done as per ICH guidelines for 3 months. The optimized nano-emulsion batch was kept in an amber color glass bottle then placed in an accelerated stability chamber at 40°C±5°C temperature and 70%±5% RH. After three-month, nano-emulsion was tested for pH, particle size, and PDI.

Preparation of Gel from Nanoemulsion

An appropriate amount of carbopol 940 (3% solution) was added in above prepared NEs and kept for stirring for about half an hour. This led to the formation of nanogel.

Characterization of Nanogel

Measurement of pH

The pH values of the Nanogel were measured at 25ºC using digital pH meter. 10% w/w dispersion (1gm of Nanoparticles was dispersed in 10 ml of distilled water. At first the reading of pH- meter was adjust with a known pH solution (pH 4 and pH 7). Then the prepared formulations were subjected for pH analysis.

Measurement of Viscosity

The viscosities of Nanogel were measured at 25ºC using Brookfield viscometer (Brookfield DV-E Viscometer) using spindle no 6 at 10, 20.30 and 60 rpm.

Drug Content Determination

The drug content in nanogel was determined by UV-Spectrophotometer. 1.0 g of formulation was accurately weighed, dissolved in 100 ml of methanol: phosphate buffer (2:8). It was filtered and diluted if required. Absorbance was determined using UV spectrophotometer at 395 nm.

Spreadability

1g Nanogel preparation was placed above ground slide and second glass slide having same dimension as that of the fixed ground slide. The second glass slide is provided with the hook. A weight of 100 g was placed on the top of the two slides for 5 min to expel air and to provide a uniform film of the gel between the two slides. Measured quantity of weight (35g) was placed in the pan attached to the pulley with the help of hook. Time in seconds taken by two slides to slip off from gel and placed in between the slides under the direction of certain load. Lesser the time taken for separation of two slides, better the spreadability. It is calculated by using the formula [38,39].

$$S = m \text{ X } t/l \text{ x } 100$$

Where S is spreadability, m is weight placed on upper slide, l is length of upper slide, and t is the time taken.

***In-vitro* Release Study of Nanoemulsion Loaded Nanogel Formulations**

The *in-vitro* permeation studies were carried out using Franz diffusion cell, which is a reliable method for prediction of drug transport across the skin. These studies were conducted employing dialysis membrane. The receptor compartment of the diffusion cell was filled with 25 ml of phosphate buffer pH 7.4. The whole assembly was fixed on a magnetic stirrer and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads at 100 rpm and the temperature was maintained at 37±0.50°C throughout the experiments. 50mg of nanoemulsion loaded in nanogel.

Accelerated Stability Study

Stability studies were done as per ICH guidelines for 3 months. The optimized nanogel was kept in an amber color glass bottle then placed in an accelerated stability chamber at 40°C±5°C temperature and 70%±5% RH. After three-month, gel was tested for pH, Viscosity, and drug content.

***In vitro* Antifungal Enzyme Assay**

The antifungal enzyme inhibitory activity of Embelin formulations was evaluated using in vitro assays targeting extracellular phospholipases and proteases, which are key virulence factors of pathogenic fungi. A 0.5 mL aliquot of each sample—control (untreated), standard (ketoconazole, 20 µg/mL), pure Embelin, optimized Embelin nanoemulsion (F13), and Embelin nanogel—was incubated with equal volumes of substrate solution in phosphate buffer (pH 7.0). For the phospholipase assay, egg yolk phosphatidylcholine (2 mg/mL) was used as the substrate, while for the protease assay, bovine serum albumin (2 mg/mL) served as the substrate. The mixtures were incubated at 37 °C for 30 minutes. Enzyme activity was quantified by measuring the release of free amino groups (protease assay) or free fatty acids (phospholipase assay) spectrophotometrically. Enzyme inhibition (%) was calculated relative to the control using the formula:

$$\%\ Inhibition = \frac{A(control) - A(sample)}{A(control)} \times 100$$

where $A_{control}$ is the absorbance of the enzyme reaction without inhibitor, and A_{sample} is the absorbance in the presence of test formulations. All experiments were performed in triplicate, and mean values were recorded.

RESULT AND DISCUSSION

UV-Visible spectroscopy in Methanol

The wavelength of maximum absorption (λmax) of Embelin was found to be 395nm methanol solvent as shown in **Figure 1**.

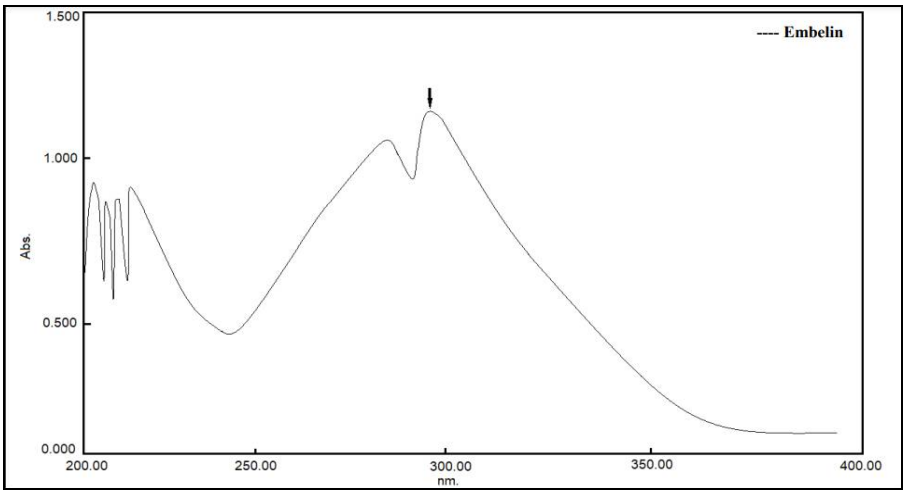


Figure 1: UV spectrum of Embelin in Methanol

Solubility Determination

The solubility of Embelin was assessed in different solvents like methanol, DMSO and different buffers at 37±0.5°C. The solubility was shown in Table 4.

Table 4: Solubility Study in different solvents

Sr. No.	Solvent	Observed Solubility (µg/ml)
1	Methanol	50.35±0.25
2	DMSO	60.15±0.41
3	pH buffer 1.2	09.26±0.22
4	pH buffer 6.8	54.45±0.16
5	pH buffer 7.4	66.36±0.21

Drug-Excipients Compatibility Study

FTIR Analysis

In Figure 2, the FTIR spectra of Embelin is given, which showed an intense absorption peak at 3304 cm^{-1}, which was because of the OH group of an embelin. Absorption at 3089, 2953, 2918 and 2848 $cm^{-1}$was because of the absorption peak of C–H stretching. Peak at 1641 cm^{-1} was observed because of C=O, whereas two C–O stretching at 1324 and 1191 cm^{-1} were because of alcohol group, respectively. The FTIR spectra of Embelin and physical mixture of all excipients (Pectin, Tween-80, PEG-400 and Carbopol 980) is given and which showed an intense absorption peak at 3304 cm^{-1}, which was because of the OH group of an embelin. Absorption at 3089, 2953, 2918 and 2848 $cm^{-1}$was because of the absorption peak of C–H stretching. Peak at 1641 cm^{-1} was observed because of C=O, whereas two C–O stretching at 1324 and 1191 cm^{-1} were because of alcohol group, respectively. There was no major deviation in peaks of the FTIR spectrum for the physical mixture and pure Embelin drug, which confirmed the absence of molecular interaction between the drug and the formulation components. All the peaks in the FTIR spectrum of the pure drug and the physical mixture were present as per the functional groups present in the structure of the drug. This indicates the drug is compatible with other excipients.

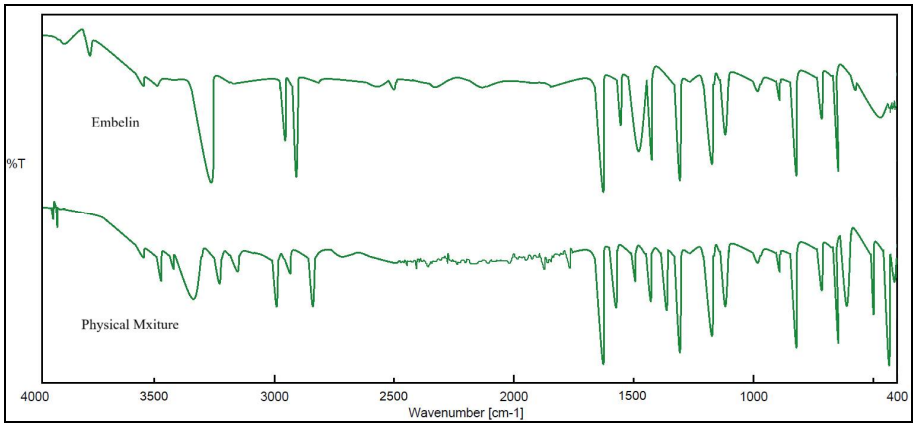


Figure 2: FTIR spectra of Embelin and Physical Mixture (Pectin + PEG-400 + Tween 80 + Carbopol 980)

Differential Scanning Calorimetry (DSC)

DSC analysis of Embelin, conducted at a scanning rate of 10°C/min, showed a prominent melting endothermic peak at 126.46 ºC, as illustrated in **Figure 3**. This peak signifies the phase transition from a semi-solid to a liquid, reflecting the heat flow linked to this transformation. Additionally, as the temperature continues to rise, certain drug components may decompose, releasing energy. This decomposition is observed on the DSC curve as an endothermic peak, indicating the heat absorbed during this thermal event. The DSC study serves as a valuable tool for evaluating the thermal behavior and potential interactions between Embelin and selected excipients. The distinct endothermic peak at 126.46 °C corresponds to the melting point of pure Embelin, indicating its crystalline nature. Pectin, Tween 80, and Carbopol 980 exhibited their respective melting points at 118.39 °C, 64.38 °C, and 147.76 °C. In the DSC thermograms of the 1:1 physical mixture of Embelin with each excipient, no significant shifts, disappearance of peaks, or formation of new thermal events were observed. This preservation of characteristic melting transitions suggests that no chemical or physical incompatibility occurred between Embelin and the excipients. Therefore, the DSC analysis confirms that Embelin is thermally and physically compatible with Pectin, Tween 80, and Carbopol 980, supporting their suitability for use in formulation development (**Figure 3**).

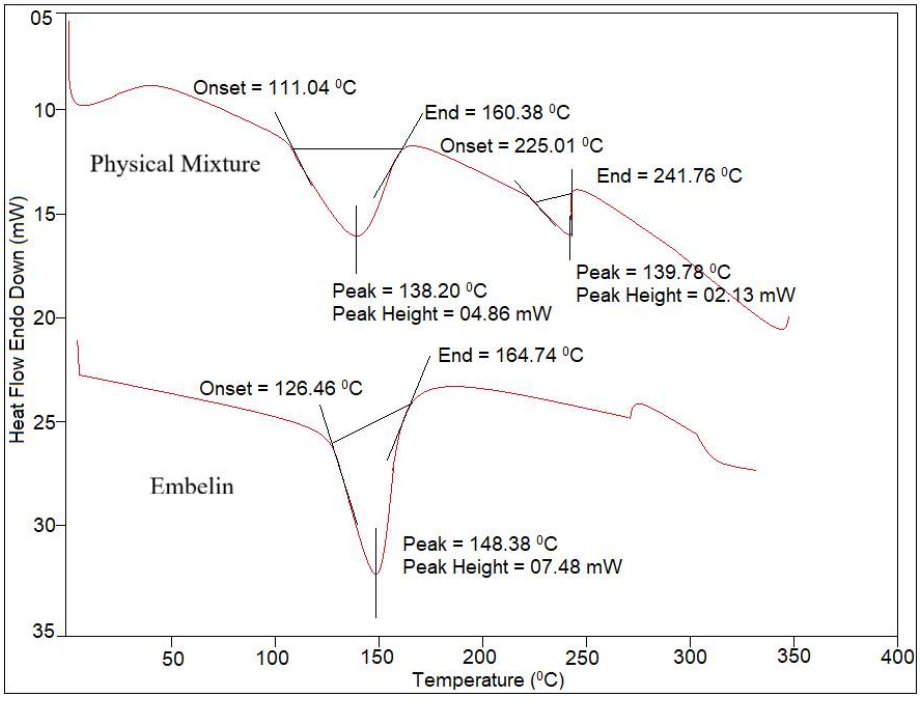


Figure 3: DSC thermogram of Embelin and Physical Mixture (Pectin + PEG-400 + Tween 80 + Carbopol 980)

Screening of Oil, Surfactant and Co-surfactant (Solubility Study)

Screening of oils, surfactants and co-surfactants are based on their solubility profile for Embelin as shown in Table 5. The Olive oil was selected oil, Tween 80 as surfactant and PEG-400 as co- surfactant.

Table 5: Solubility of Embelin

Name of Excipients	Solubility (mg/ml)
Olive Oil	48.53±0.6
Eucalyptus Oil,	26.65±0.5
Almond Oil,	29.45±0.9
Tween 80	37.39±0.2
PEG-400	36.25±0.5

Formulation and selection of Nanoemulsion

On the basis of their visual observation like transparency and pH, 14 formulations were selected out as per factorial design (Table 1) for preparing Embelin loaded nanoemulsion.

Formulation Design

The two factors with lower, middle and upper design points in coded and un-coded values are shown in Table 1. The ranges of responses Y1 and Y2 were 225-269 d.nm and -11 to -26mV respectively. All the responses observed for nine formulations prepared were fitted to main effect model, which was found as the best fitted model for Y1 and Y2, using Design Expert® software. The values of R^2 SD and % CV are given in (Table 6), along with the regression equation generated for each response. The results of ANNOVA in (Table 6), for the dependent variables demonstrate that the model was significant for all the response variables. It was observed that independent variables X1, X2 and X3 had a positive effect on the zeta potential and an desired particle size of nano-formulation i.e. nano-emulsion was achieved.

Model Assessment

After putting the data in Design Expert® software for, Fit summary applied to data in that Main Effect Model had been suggested by the software for all the responses. The statistical evaluation was performed by using ANNOVA. Results are shown in (Table 6). The coefficients with more than one factor term in the regression equation represent interaction terms. It also shows that the relationship between factors and responses is not always linear. When more than one factor are changes simultaneously and used at different levels in a formulation, a factor can produce different degrees of responses.

Table 6: Results of Analysis of Variance for Measured Response

Parameters	Values	
	Particle Size	Zeta Potential
Model	Quadratic Model (Significant)	Special Quartic model (Significant)
Model p-value	0.0044	0.0341
Standard Deviation	5.71	2.35
F-value	8.67	5.83%
CV	2.27%	12.10%
R^2	0.8441	0.9031
Adequate Precision	8.5238	8.0573
Regression Equation	Y1 = 246.96 X1 + 243.63 X2 + 232.07 X3 + 53.57 X1 X2 + 78.97 X1 X3 +74.97 X2 X3	Y1 = -22.51X1 -20.51X2 -21.51X3 + 33.95 X1 X2 + 23.84 X1 X3 + 39.84 X2 X3 -372.97$X1^2X2X3$-300.97$X1X2^2X3$-587.99$X1X2X3^2$

Response Surface Plot Analysis

The size of particles in nanoemulsion plays a crucial role as it determines both the rate and extent of drug release, as well as drug absorption. A smaller particle size provides a larger surface area for drug absorption, thereby enhancing bioavailability. The polydispersity index calculation considers factors such as the mean particle size, refractive index of the solvent, measurement angle, and distribution variance. A low polydispersity index indicates high homogeneity in the particle population, while high values suggest a broader size distribution or multiple populations. Analyzing the 3D response surface plot (Figure 4) and considering that nanoparticles are structures with nanoscale dimensions, the formulation batch with the smallest particle size is favored and chosen as the optimized batch. Specifically, Design Batch F11, with a Smix concentration of approximately 33.56% and olive oil concentration of 0.64%, exhibits the optimized particle size of 225 nm.

Zeta potential holds a crucial role in determining the stability of nanoemulsions, making it a vital parameter in both their formulation and characterization. In the context of nanoemulsions, zeta potential denotes the electric charge present on the surface of the dispersed droplets in the system. A high absolute value of zeta potential signifies robust electrostatic repulsion between adjacent droplets, preventing their coalescence and aggregation. This electrostatic repulsion is essential for sustaining the long-term stability of nanoemulsions by impeding the formation of larger droplets or phase separation.

Maintaining an optimal zeta potential, typically falling within the range of ±30 mV ensures that repulsive forces prevail over attractive forces, such as van der Waals and hydrophobic interactions. This, in turn, prevents the destabilization of the nanoemulsion over time. Through the control and optimization of zeta potential, formulators can enhance the physical stability and shelf life of nanoemulsions, rendering them suitable for diverse applications in pharmaceuticals, food, and the cosmetic industries.

Understanding and manipulating zeta potential in nanoemulsions are essential for achieving stable and functional formulations with improved dispersion, bioavailability, and overall performance. Analysis of the 3D response surface plot (Figure 4) and the regression coefficient values of factors led to the conclusion that the zeta potential of the nanoemulsion increases with an elevation in the concentration of olive oil. Notably, there were no observed interactions or nonlinearity. The results further indicated that olive oil exerted a more significant effect on zeta potential.

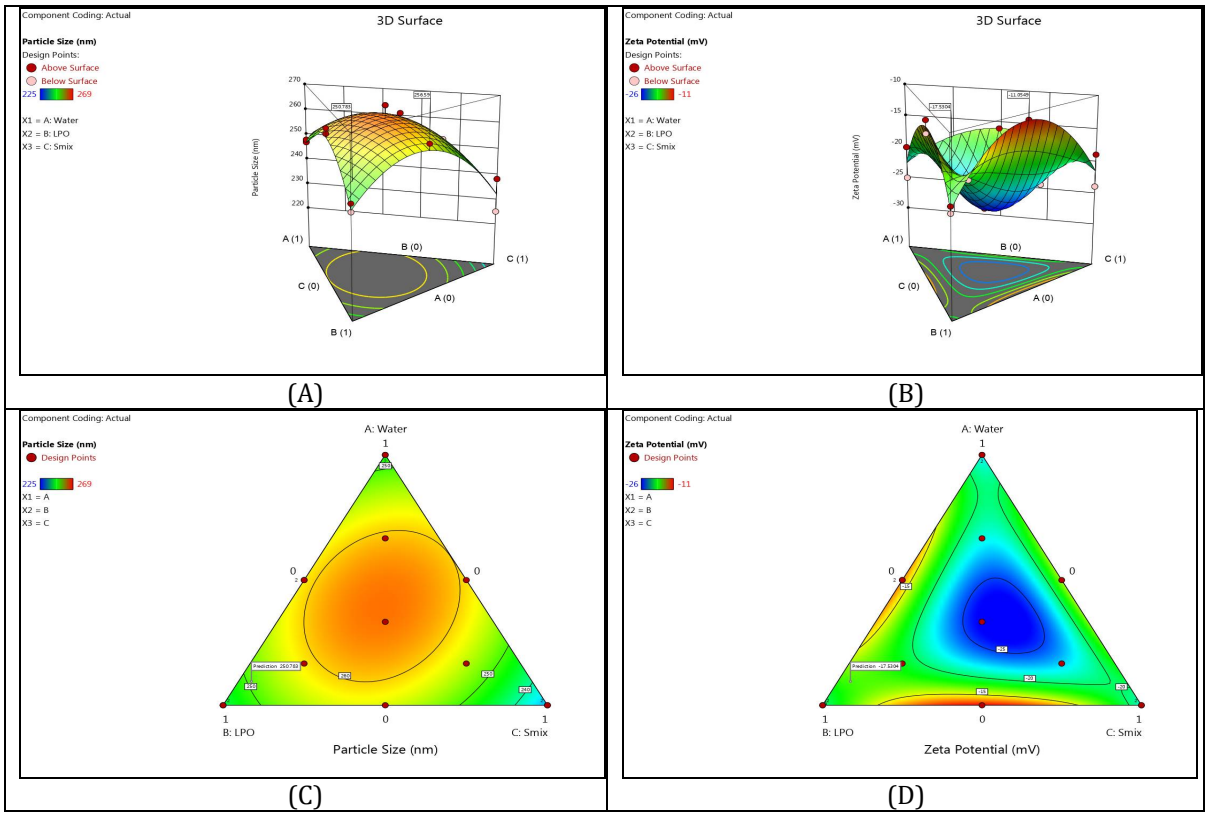


Figure 4: Response surface plots for X1, X2 and X3 on (A) Mean Particle Size (Y1); (B) ZP (Y2); and contour plots for (C) Mean Particle Size (Y1); (D) ZP (Y2)

Figures 4 depict Contour plots within the framework of Quality by Design (QbD). These graphical representations illustrate the correlation between critical quality attributes (CQAs) and critical process parameters (CPPs). These plots serve as valuable tools for visualizing the design space and comprehending how alterations in process parameters may influence product quality. In both cases, the flat contours in the plots suggest that the critical quality attribute is not significantly affected by variations in the associated process parameters within that particular region. This observation points to a robust design space, indicating that fluctuations in those parameters are unlikely to have a substantial impact on product quality. Both responses demonstrate optimized outcomes within the defined design space.

Characterization of Nanoemulsion

Physical characterization

All formulations are clear, transparent, and homogenous and no grittiness and no clogs were found and suitable consistency.

Droplet Size and Size Distribution

The particle size of the PNs is a fundamental factor because it decides the rate and extent of drug release as well as drug absorption. The smaller particle size offers a larger interfacial surface area for drug absorption and improves the bioavailability. The calculation of polydispersity index takes into account the particle mean size, the refractive index of the solvent, the measurement angle and the variance of the distribution. Low polydispersity index value might be associated with a high homogeneity in the particle population, whereas high polydispersity index values suggest a broad size distribution or even several populations. The optimized formulation batch (F11) showed mean particle size 250 nm with PDI 0.209.

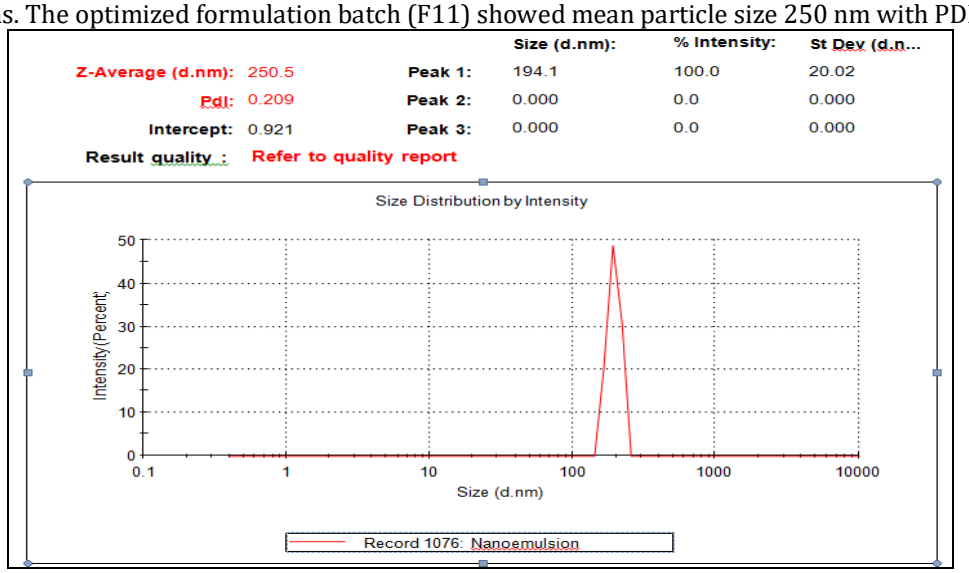


Figure 5: Particle size of F11 formulation

Zeta potential analysis

The zeta potential values of drug loaded nanoemulsion that was found to be -19.4 mV. The high value of zeta potential confirms the stability of nanoemulsion.

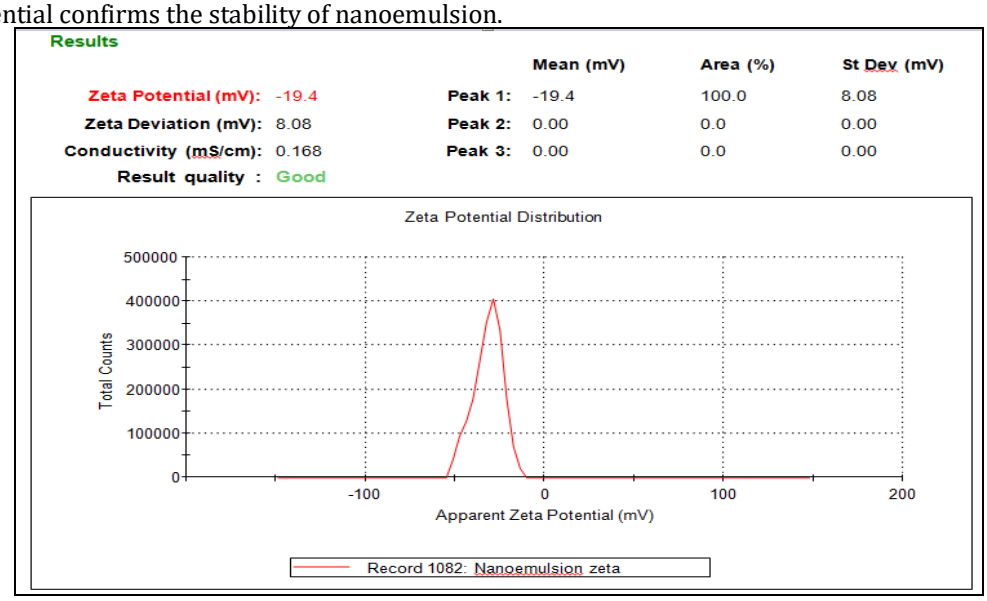
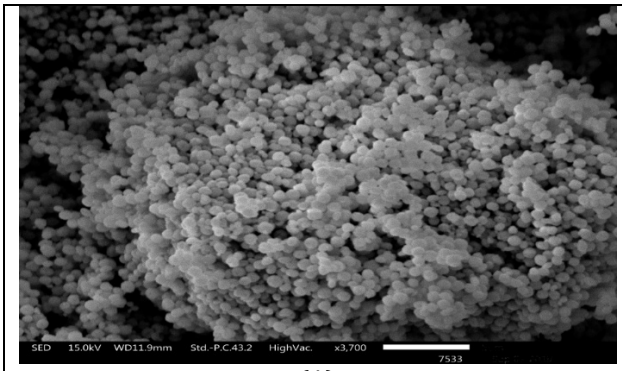


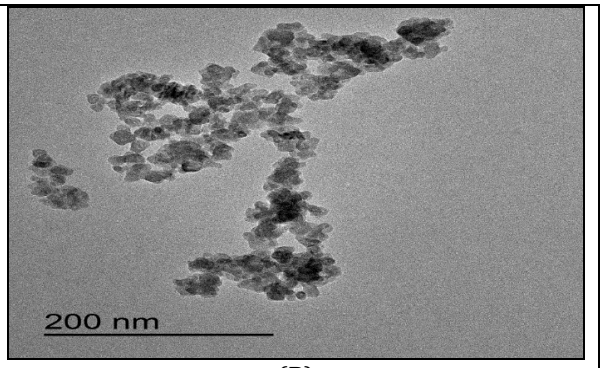
Figure 6: Zeta Potential of F11 formulation

Surface Morphological Study

Surface morphology of the nanoemulsion was evaluated using SEM and TEM from which it can be seen that the droplets have smooth surfaces. Droplets show spherical shape with size 200 nm (**Figure 21**).



(A)



(B)

Figure 7: (A) SEM and (B) TEM image of F11 formulation

Characterization of Nanoemulsion

Measurement of pH

The pH value for the selected NE (F1-14) formulation was found to be 5.25-5.97 as shown in Table 7. The pH of the NE was found to be within the range of pH of skin (5-7) and would not cause any irritation to the skin. Thus, prepared NE formulations are suitable for skin application.

Table 7: Characterization of Nanoemulsion

Formulation code	pH value of NE	Viscosity (cps)	Drug Content (%)
F1	5.25±0.03	1731±2.33	84.19±0.27%
F2	5.33±0.07	1530±2.28	85.35±0.29%
F3	5.45±0.04	1635±2.21	81.92±0.30%
F4	5.65±0.01	1721±2.29	85.38±0.31%
F5	5.36±0.03	1732±2.31	83.91±0.32%
F6	5.39±0.05	1525±2.35	84.28±0.26%
F7	5.89±0.02	1885±2.36	92.64±0.28%
F8	5.67±0.04	1751±2.39	93.47±0.32%
F9	5.77±0.07	1665±2.33	91.21±0.31%
F10	5.17±0.01	1545±2.27	94.38±0.30%
F11	5.45±0.05	1976±2.26	98.36±0.28%
F12	5.43±0.03	1709±2.21	89.38±0.29%
F13	5.97±0.02	1417±2.28	87.65±0.35%
F14	5.35±0.03	1878±2.31	92.35±0.39%

Measurement of Viscosity

Brookfield viscometer was used to measure the viscosity of nanoemulsion at different spindle speeds. Viscosity reveals the rheological properties of all nanoemulsion formulation. All formulation shows shear thinning effect as the shear stress increased the viscosity was decreased. Formulation F11 was found more viscous than other formulations.

Drug Content of Nanoemulsion

Drug content in the nanoemulsion is supposed to be decreased in some extent because of pectin which occupies some volume as it swells in formulations so it was determined by UV spectrometer at 395 nm for the same. The range of percentage drug content of nanoemulsion was 81.92% to 98.36% as shown in Table 7. The percentage drug content of formulations was within a permissible range.

***In-vitro* Release Study**

Embelin loaded emulsion of all batches was studied *in-vitro* for drug release using Franz diffusion cell. For batches F1-F14, the maximal drug release (F11) was found to be about 98.91±2.25% as shown in Table 8. The *in-vitro* release of produced emulsion in phosphate buffer saline (PBS) (PH 7.4) at 37ºC was studied. Nanoemulsion was dialyzed for 60 min. The quantity of drug released was measured by using a UV-visible spectrophotometer to measure absorbance. A burst drug release was observed in the beginning, which may be due to the smaller particle size that attributed to the large surface area of the emulsion, apart from it diffusion of the drug from the outer shell of the emulsion may be responsible for initial burst release.

Table 8: *In-vitro* release profile of Nano-emulsion

Time (Min.)	Pure Drug Solution	Nano-emulsion (F11)
0	0	0
60	14.56±1.11	15.42±1.11
120	53.41±1.41	27.40±2.29
180	73.62±1.22	41.26±1.58
240	97.25±1.32	57.25±2.39
300	-	75.20±2.45
360	-	89.42±2.84
420	-	95.20±2.69
480	-	98.91±2.25

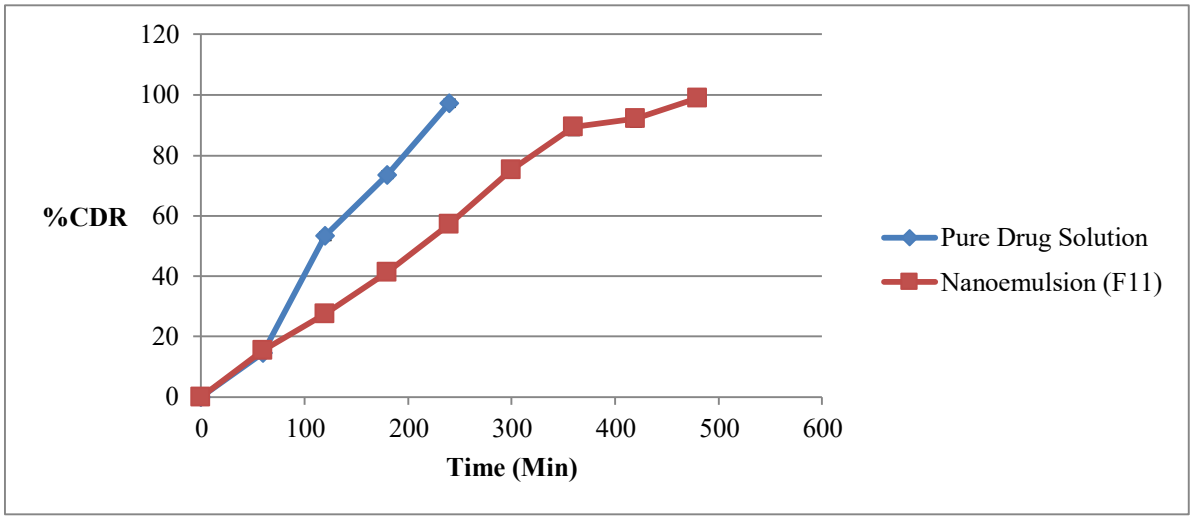


Figure 8: *In-vitro* drug release study of F11

Accelerated Stability Study

The optimized emulsion was subjected to stability studies and the results are given in Table 9. Based on these results it is revealed that, Embelin loaded nanoemulsion (Formulation batch F11) was found to be stable formulation at the given temperature and humidity condition.

Table 9: Stability study of parameters of the optimized formulation (F11)

Parameters	Initial Month	1st Month	2nd Month	3rd Month
pH	5.45 ± 0.05	5.49 ± 0.04	5.73 ± 0.02	5.35 ± 0.03
Viscosity (cps)	1976 ± 2.26	1961 ± 2.28	1981 ± 2.33	1973 ± 2.30
Drug content (%)	98.36 ± 0.28	97.31 ± 0.35	98.33 ± 0.31	98.28 ± 0.39

Characterization of Nanoemulsion Gel

Measurement of pH

The pH value for the selected NE and its gel (F11) formulation was found to be 5.85±0.09 as shown in Table 10. The pH of the NE was found to be within the range of pH of skin (5-7) and would not cause any irritation to the skin. Thus, prepared NE formulations are suitable for skin application and the formulated nanogels are also suitable for skin application.

Measurement of Viscosity

Brookfield viscometer was used to measure the viscosity of Nanogel (NE gel) at different spindle speeds. Viscosity reveals the rheological properties of all NE formulation. All formulation shows shear thinning effect as the shear stress increased the viscosity was decreased. Formulation F11 was found more viscous than other formulations as shown in Table 10.

Drug Content of Nanogel

Drug content in the Nanogel (which is made by adding gelling agent in NE) is supposed to be decreased in some extent because of gelling agent which occupies some volume as it swells in formulations so it was determined by UV spectrometer at 395 nm for the same. The range of percentage drug content of Nanogel was 92.47% as shown in Table 10. The percentage drug content of formulations was within a permissible range.

Spreadability

Spreadability determined as % increase in area of gel upon pressing with certain weight. F11 formulations have shown good Spreadability as shown in Table 10.

Table 10: Characterization of Nanoemulsion Gel

Formulation code	Drug Content (%)	Spreadability (%)	pH	Viscosity (cps)
F11	92.47±0.19%	94.49±1.09%	5.85±0.09	1912±1.17

***In-vitro* Release Study**

The *in-vitro* drug release from nanoemulsion loaded gel of F11 batch was investigated using a Franz diffusion cell. For batches F11, the maximum drug release was 95.74±2.96%, as indicated in Table 11. The in-vitro release of the formulated gel in phosphate buffer saline (PBS) at pH 7.4 and 37ºC was examined, with nanogels undergoing a 60-minute dialysis. The quantity of released drug was assessed using a UV-visible spectrophotometer to measure absorbance. Initial burst drug release was observed, likely attributed to the smaller particle size, leading to a larger surface area of the gel. Additionally, the diffusion of the drug from the outer shell of the gel may contribute to the observed initial burst release.

Table 11: *In-vitro* release profile of Nanogel

Sr. No.	Time (Hours)	Nanogel (F11)
1	0	0
2	2	16.41±1.36
3	4	33.23±2.41
4	6	58.58±1.69
5	8	80.28±2.32
6	10	87.29±2.56
7	12	95.74±2.96

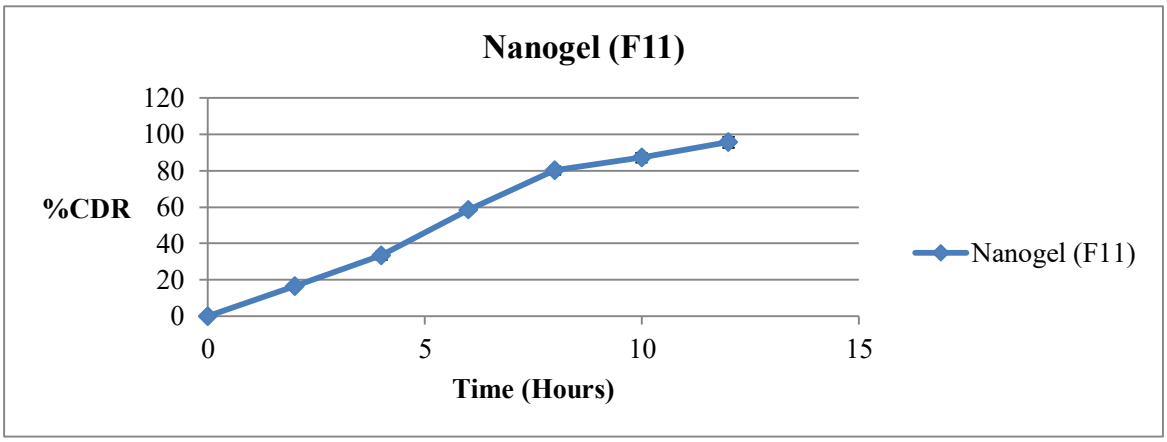


Figure 9: *In-vitro* drug release study of F11 formulation

Accelerated Stability Study

The optimized gels were subjected to stability studies and the results are given in Table 12. Based on these results it is revealed that, Embelin nanoemulsion loaded nanogels (Formulation batch F11) were found to be stable formulation at the given temperature and humidity condition.

Table 12: Stability study of parameters of the optimized formulation (F11)

Parameters	Initial Month	1st Month	2nd Month	3rd Month
pH	5.85 ± 0.19	5.79 ± 0.14	5.89 ± 0.12	5.81 ± 0.13
Viscosity (cps)	1912 ± 1.17	1972 ± 1.20	1974 ± 1.39	1969 ± 1.14
Drug content (%)	92.47 ± 0.19	92.35 ± 0.25	92.39 ± 0.13	92.40 ± 0.26

***In vitro* Antifungal Assay**

The control enzyme reactions exhibited maximum activity in the absence of inhibitors. The pure Embelin produced moderate inhibition of phospholipase (39.89 ± 1.36**%**) and protease (32.47 ± 1.29**%**) activity. The optimized nanoemulsion (F11) demonstrated significantly higher inhibition, with values of 66.46 ±

1.93% for phospholipase and 59.23 ± 1.68% for protease. The nanogel formulation further enhanced activity, inhibiting 78.72 ± 1.84% of phospholipase and 72.81 ± 1.49% of protease activity. These results were comparable to the standard ketoconazole, which achieved 79.41 ± 1.18% inhibition of phospholipase and 74.92 ± 1.04% inhibition of protease. The enzyme inhibition assays clearly demonstrated that Embelin formulations were capable of suppressing fungal virulence enzymes, which play a pivotal role in tissue invasion and pathogenicity. While the pure Embelin showed only modest inhibition, incorporation into nanoemulsion significantly improved enzyme suppression, likely due to enhanced solubility and better interaction of Embelin with enzyme active sites. The nanogel formulation provided the highest inhibition, which may be attributed to its sustained release and greater bioavailability on the enzyme–substrate interface. Importantly, the nanogel's inhibition values were statistically comparable to those of ketoconazole, suggesting that Embelin, when delivered via nanocarrier systems, could serve as a promising natural alternative in antifungal therapy.

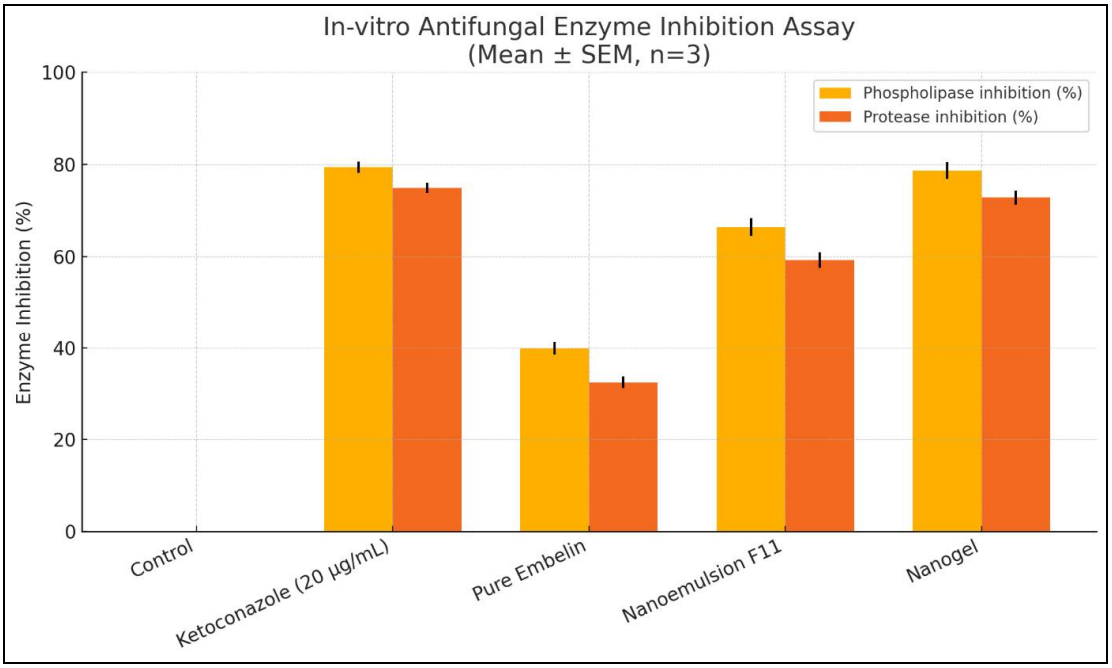


Figure 10. *In-vitro* inhibition of fungal virulence enzymes by Embelin formulations. Grouped bar chart showing mean ± SEM (n = 3) percent inhibition of phospholipase and protease for Control, Ketoconazole (20 µg/mL), Pure Embelin, Nanoemulsion F11, and Nanogel.

CONCLUSION

This study demonstrates that nanoformulation substantially improves the functional antifungal activity of Embelin, as measured by inhibition of two key virulence enzymes — phospholipase and protease. The optimized nanoemulsion (F11) and its nanogel derivative displayed markedly greater enzyme inhibition than pure Embelin and approached the efficacy of ketoconazole, the clinical standard. Physicochemical characterization showed that formulation F11 offers favorable droplet size (~225 nm), acceptable zeta potential (−19 mV), high drug content (~98%), and robust stability under accelerated conditions. Conversion to nanogel provided sustained release and superior enzyme inhibition, suggesting improved local bioavailability at the enzyme–substrate interface. Together, these findings indicate that green-formulated Embelin nanoemulsions and nanogels are promising topical antifungal delivery systems warranting further investigation, including MIC/MBIC testing, time-kill studies, ex vivo skin permeation models, and ultimately in vivo efficacy and safety assessments. The combined approach — rational excipient selection, factorial optimization, and enzyme-based functional assays — offers a translational pathway for phytochemical therapeutics that maximizes biological performance through formulation engineering.

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