RESEARCH PAPER

Formulation Development and in Vitro - In Vivo Evaluation of Luliconazole Nanoemulsions for Targeted Antifungal Delivery Against Candida Albicans Using Design of Experiments.

Pravin Narayan Kirdat* and Meenakshi B Patel

Department of Pharmaceutics, School of Pharmacy, Parul University, Waghodia, Vadodara, Gujarat, 391760, India
**Corresponding author: pravinkirdat31@gmail.com*

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ABSTRACT

Background: Drugs such as Luliconazole, which have low aqueous solubility and undergo extensive first-pass metabolism, typically exhibit reduced bioavailability. Nanoemulsion-based delivery systems are utilised to enhance absorption and efficacy in the treatment of fungal infections.

Objective: This study aimed to develop and optimize nanoemulsion formulations of Luliconazole to improve their solubility, stability, and bioavailability.

Methods: Preliminary characterization of Luliconazole included solubility analysis in various solvents, melting point determination, particle size, zeta potential, FTIR spectroscopy, DSC, and XRD. Pseudo-ternary phase diagrams were utilized to classify suitable Smix ratios for nanoemulsion formation. Formulations were prepared using a homogenization technique and optimized via a full factorial design of experiments. Key variables included globule size, zeta potential, homogenization speed, and time. The final formulation further assessed for its in vitro and in vivo performance by using animal model.

Results: The optimized nanoemulsion formulation of Luliconazole (batch 8) showed a globule size of 342.8 nm, a zeta potential of –27.9 mV, a polydispersity index (PDI) of 0.568, drug loading efficiency of 99.34%, and light transmittance of 99.57%. Stability testing under accelerated conditions demonstrated that the formulation remained stable for up to 90 days. Optimized Nanoemulsion formulation shows comparable antifungal activity with marketed formulation.

Conclusion: Nanoemulsion formulations of Luliconazole demonstrated enhanced solubility, stability, and physicochemical characteristics, indicating their potential as effective delivery systems for antifungal therapy.

Keywords: *Nanoemulsion, fungal infection, Luliconazole, DoE, Particle size, zeta potential, Antifungal activity, In vitro - In vivo study.*
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1. INTRODUCTION

Fungal infections constitute a major global health issue, especially among individuals with compromised immune systems. The therapeutic approach to these infections frequently involves antifungal agents such as Luliconazole, which possess broad-spectrum efficacy against pathogenic fungi. Despite their effectiveness, these agents are categorized as Biopharmaceutical Classification System (BCS) Class II compound, denoting low aqueous solubility and good permeability. Consequently, their oral bioavailability and therapeutic outcomes are often inadequate when delivered via conventional dosage forms [1,2].

Luliconazole, an imidazole antifungal, is almost insoluble in water, which limits its effectiveness for systemic or topical use [4]. Nanoemulsion (NE) drug delivery systems have been developed to overcome this, using oil, surfactants, co-surfactants, and water with droplet sizes of 20–500 nanometres [5]. NEs enhance the solubility of hydrophobic drugs, improve permeability, offer protection from degradation, and increase retention at infection sites. [6,7]

Recent studies indicate that nanoemulsions can significantly improve the absorption and antifungal efficacy of drugs with poor water solubility. In addition, nanoemulsions formulated with oils such as castor oil and

**Author for Correspondence: pravinkirdat31@gmail.com*

surfactants like Polysorbate 60 and 80 have demonstrated compatibility with lipophilic compounds and enhanced emulsification efficiency [8,9].

In light of these benefits, the present investigation was undertaken to develop and optimize nanoemulsion-based formulations of Luliconazole utilizing a Design of Experiment (DoE) methodology. Initial procedures encompassed solubility assessments, particle characterization, and structural analyses to verify drug identity and inform formulation strategies. A pseudo-ternary phase diagram facilitated the determination of the optimal Smix ratio, while a central composite design (CCD) was implemented to systematically assess the influence of homogenization speed and duration on key quality parameters. The finalized formulations were subsequently characterized for stability, drug content, particle size, zeta potential, and in vitro performance.

This study examines the formulation of nanoemulsions containing Luliconazole [16-19], using castor oil, Polysorbate 60, and Polysorbate 80 as polymers. The homogenization technique was used for preparation, and the resulting nanoemulsions were evaluated to determine their drug release characteristics for the management of fungal diseases.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Luliconazole were provided as gift samples by Aadhaar Life Sciences Pvt. Ltd. (India). Acetonitrile (HPLC grade) was procured from Qualigens Fine Chemicals (Mumbai, India), and formic acid was obtained from Thomas Baker Chemicals Pvt. Ltd. (Mumbai, India). Surfactants, including Polysorbate 60 and Polysorbate 80, were purchased from Croda Inc. (India), while castor oil was supplied by AOS Products Pvt. Ltd. (India).

All weighing procedures were conducted using NABL-calibrated analytical balances to ensure measurement accuracy. Sample preparation was carried out using Class A borosilicate glassware, and all measurements were performed under standardized laboratory conditions unless otherwise specified.

2.2. Fungal species and medium

The Nutrient broth culture Candida Albicans obtained from IABRD, Muller Hilton Agar obtained from HiMedia laboratories Pvt.Ltd. India.

2.3. Methodology

Preliminary analysis of Drugs

The initial characterization of Luliconazole was undertaken to verify its identity and assess the physicochemical attributes necessary for formulation development. The study encompassed solubility assessment in diverse solvents, melting point determination, Fourier-transform infrared (FTIR) spectroscopy, particle size evaluation, zeta potential analysis, differential scanning calorimetry (DSC), and X-ray diffraction (XRD). These tests were performed to confirm the purity, crystallinity, and thermal profile of the active pharmaceutical ingredient (API).

Formulation of Prototype Nanoemulsion of Luliconazole

Screening of nanoemulsion components

For nanoemulsion development, Luliconazole solubility was screened using the shake flask method by mixing oils, surfactants, and cosurfactants in sealed vials. Samples were stirred at 37 °C for 48 hours, then centrifuged at 10,000 rpm for 10 minutes after standing for 72 hours. The supernatant concentration was measured at 235 nm with a UV–Vis spectrophotometer. Compounds with high solubility were chosen to create the phase diagram.

Formulation of Nanoemulsion with selected Smix

Luliconazole Nanoemulsion prepared using castor oil, Smix (2:1) and water by using high shear homogenization method. The formulation table is given below:

Table 2: Formulation table of Luliconazole Nanoemulsion

Batches	Luliconazole	Castor oil	Smix (2:1) (ml)		Water
	(mg)	(ml)	Polysorbate 80 (ml)	Polysorbate 60 (ml)	(ml)
Luliconazole Nano emulation	35.59	5.71	15.24	7.62	71.43

The prepared solution was homogenized at 7000 rpm for 90 minutes. This prepared Nanoemulsion was evaluated for Globule size and zeta potential of the drug.

Evaluation of Nanoemulsion of Luliconazole

The prepared Nanoemulsion was evaluated for Globule size, Zeta Potential, PDI, %Transmittance and Drug content.

Design of experiment

A three-level, two-factor, randomized full factorial design was used to optimize the developmental trail, leading to a 9-run experimental plan created with Design expert software. The experimental design determines which factors have a statistically significant impact on the responses. It models main effects, two factor interactions and quadratic terms. The results from these experiments were analysed using Design of Experiment software and the terms with p-values >0.1 were removed from the model.

Experimental Design Summary

Experimental Study Range and Quality Attributes					
Type of Experiment	Randomized full factorial designed experiment with Three-level, two factors.				
Parameters Investigated					**Quality Attributes Measured**
Parameter	**Units**	**Low**	**Mean**	**High**	Globule size and Zeta potential
Homogenization speed	RPM	3000	5000	7000	
Homogenization time	Min	30	60	90	

Table below shows the experimental design with run order.

Run order	Factor 1 A: Speed (RPM)	Factor 2 B: Time (min)	Response 1 Globule size (nm)	Response 2 Zeta potential (mV)
1	7,000	90	342.8	-25.1
2	5,000	30	424.8	-29.6
3	5,000	90	395.4	-28.9
4	3,000	30	490.4	-30.5
5	7,000	60	365.1	-26.1
6	3,000	60	465.2	-30.2
7	5,000	60	412.7	-29.1
8	3,000	90	439.5	-29.9
9	7,000	30	390.9	-28.7

In-Vitro evaluation of Nanoemulsion of Luliconazole for Antifungal activity

The antifungal study of Luliconazole nanoemulsion was conducted against human fungal pathogen Candida Albicans by the principal of Kirby-Bauer diffusion method (well diffusion method). Sterile MHA (Muller Hinton Agar) poured into the plate at depth of approx.4 mm and kept for solidification for 30 min. Inoculum was prepared by 5-6 colonies of candida albicans inoculated in nutrient broth and incubated at 37C for 2-5 hrs. A sterile cotton swab dipped in to the diluted inoculum and swab on MHA media Petri plates for further inoculation with agar. Prepared well at 7mm diameter in plate with the help of syringe The antifungal activity of Luliconazole nanoemulsion was tested against Candida albicans. Test samples included the nanoemulsion at 0.04% concentration and a marketed 1% Luliconazole cream as reference. Results were observed after incubation at 37°C for 24-48 hours.

In-Vivo evaluation of Nanoemulsion of Luliconazole

The in vivo study of Luliconazole nanoemulsion was conducted by using the Nine healthy species of Wistar Rats (weights 180- 250 g) divided in to 3 groups of study. Group I consists of control whereas the group II with newly prepared Luliconazole nanoemulsion and group III with alternative formulation of Amphotericin B nano emulsion to compare the outcomes. The *in-vivo* study was conducted in accordance with Animal Ethical Guidelines for investigations in laboratory with Committee for the Purpose of Control and Supervision of Experiments on Animals with Registration number CPCSEA/IAEC/BiRD/Sangli/2025-26/02/13

The samples were injected intramuscularly from the thigh muscle and volume is around the 0.5 mL per injection. The blood samples were collected Retro-orbital plexus under mild anaesthesia at the interval of 0 (pre-dose), 0.5, 1, 2, 4, 6, 8, 12, 24 hr. collected samples centrifuged at 3000 rpm for 10 minutes and separated plasma stored at -20°C until taken for analysis. The samples were further analysed by using the HPLC method validated for formulation testing and results were analysed by using the PKSolver software to understand the Pharmacodynamic properties.

3. RESULTS AND DISCUSSION

Preliminary analysis of Drugs

a) Description

Luliconazole is off-white to pale yellow crystalline powder.

b) Solubility

Luliconazole is practically insoluble in water. It is freely soluble in DMF and acetone and soluble in Acetonitrile and methanol and sparingly soluble in ethanol.

c) Melting Point

The melting of Luliconazole was found to be approximately 152°C.

d) FTIR analysis

The FT-IR spectrum of Luliconazole is given below:

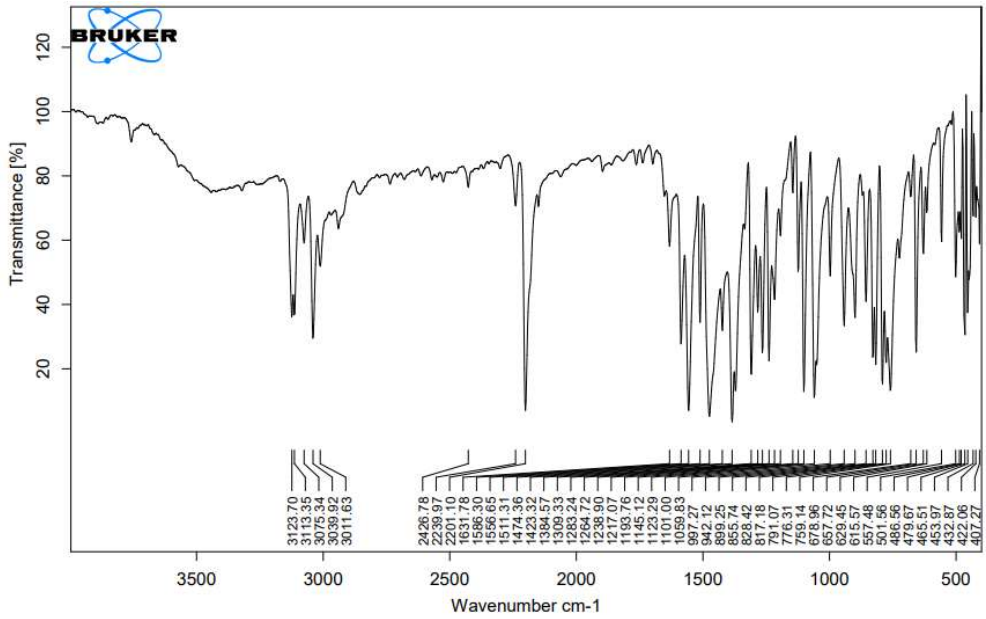


Figure 1: IR spectrum of Luliconazole

Luliconazole shows characteristic transmittance as below listed wavenumber:

Key peaks in the IR spectrum of Luliconazole include the stretching vibrations of following:

3011.63 cm^{-1}	C - H stretch
2426.78 cm^{-1}	C ≡ N stretch
1511.31 cm^{-1}	C=C-C Aromatic Ring Stretch
1474.36 cm^{-1}	C ≡ C aromatic Stretch
855.74 cm^{-1}	Para C-H distribution

The spectrum was compared with the standard spectrum of Luliconazole and it was found to comply.

e) Particle Size:

The measured particle size of Luliconazole was approximately 254.4 nm.

f) Zeta potential:

Pure Luliconazole exhibited a zeta potential of about -25.1 mV, suggesting a similar level of electrostatic repulsion and dispersion stability.

g) DSC analysis of drugs:

The DSC Curve of Luliconazole is given in Figure 3

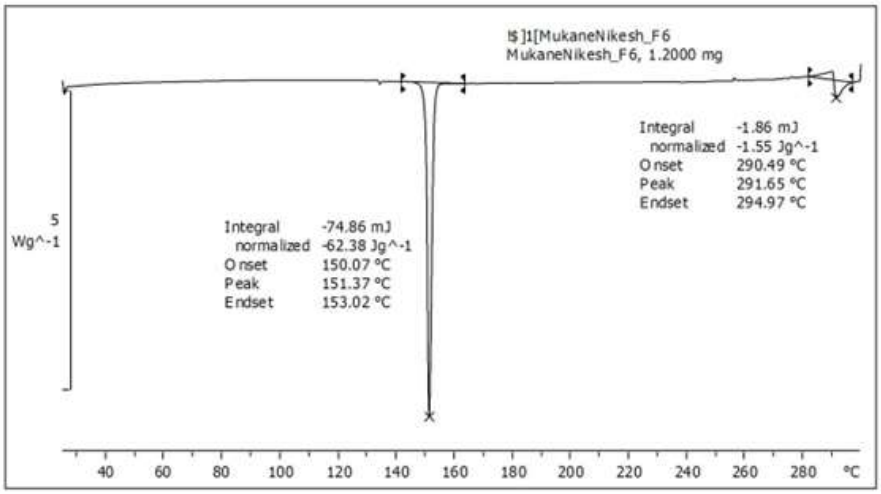


Figure 3: Thermogram of Luliconazole

The results significantly match with the reference in literature for DSC Luliconazole thermo analysis.

g) XRD of Drugs

The XRD Graph of Luliconazole is given in Figure 4.

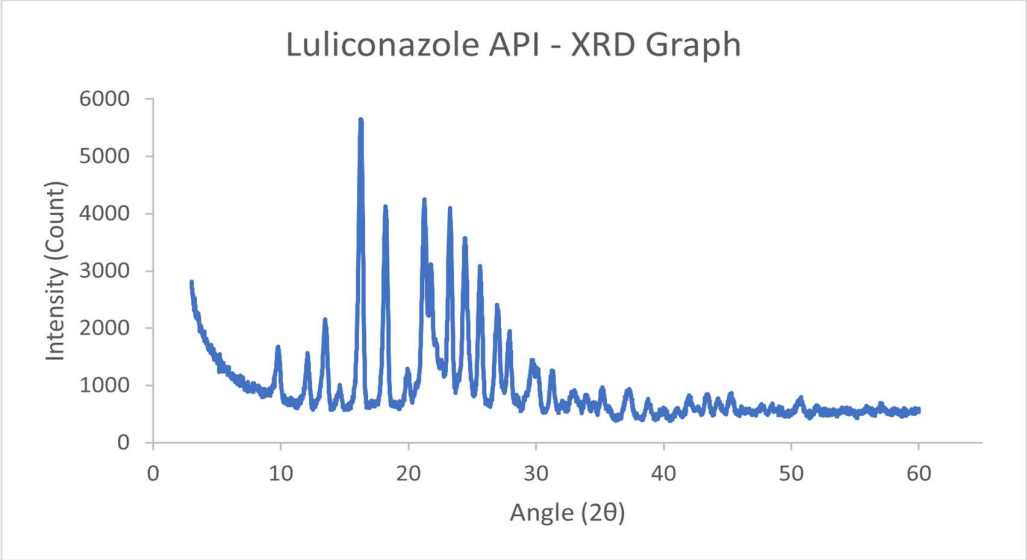


Figure 4: XRD Graph of Luliconazole

The Luliconazole API was found to be crystalline in nature as it show significant diffraction during its time course of analysis.

Preformulation Studies

Solubility of Luliconazole in different oils

The solubility of Luliconazole was estimated in different oils. The maximum solubility of Luliconazole was found to be in Transcutol P with the concentration of 120 mg/mL. Based on the results of solubility in oils, Castor Oil was selected for the formulation of Nanoemulsion. The graphical representation of solubility in different oils is given in Figure 5.

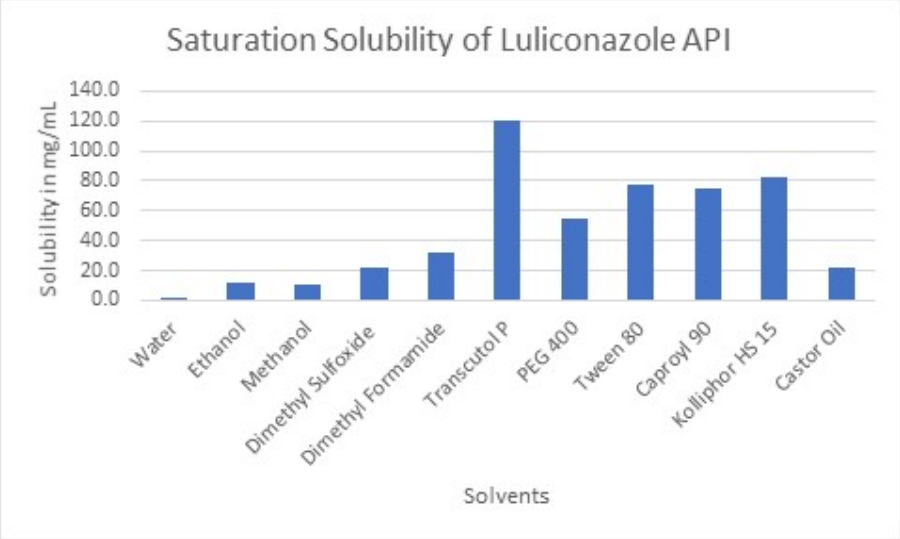


Figure 5: Solubility of Luliconazole API in Oils

Solubility of Luliconazole in different surfactants

The solubility of Luliconazole was estimated in different surfactants

From the solubility data, it was observed that Amphotericin B had the maximum solubility in polysorbate 80 and 60 with 1023.97 and 909.54 µg/ml, respectively; so, these were selected as surfactant and co-surfactant for preparation of Smix.

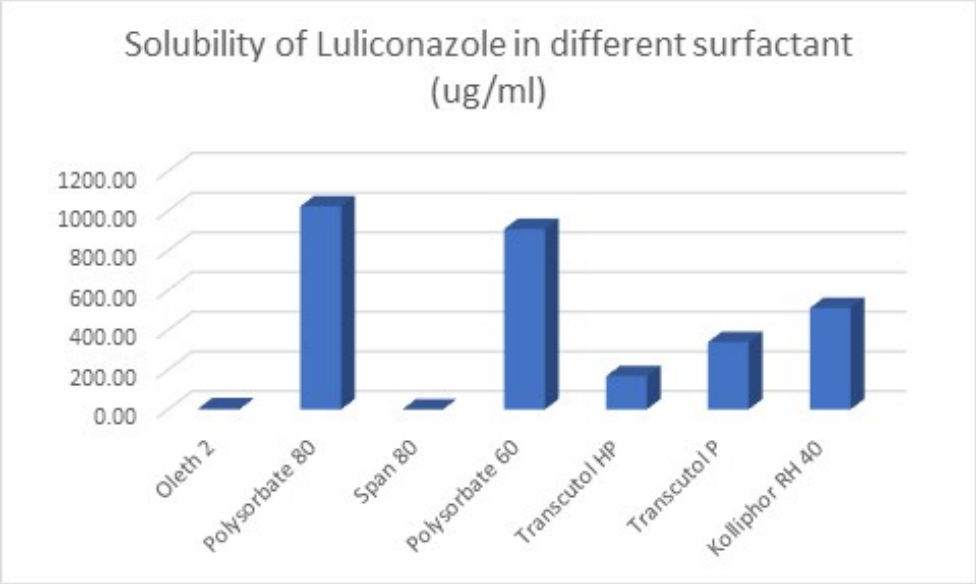


Figure 6: Solubility of Amphotericin B in surfactants

Pseudo ternary phase diagram

The pseudo-ternary phase diagram was developed utilizing the water titration technique.

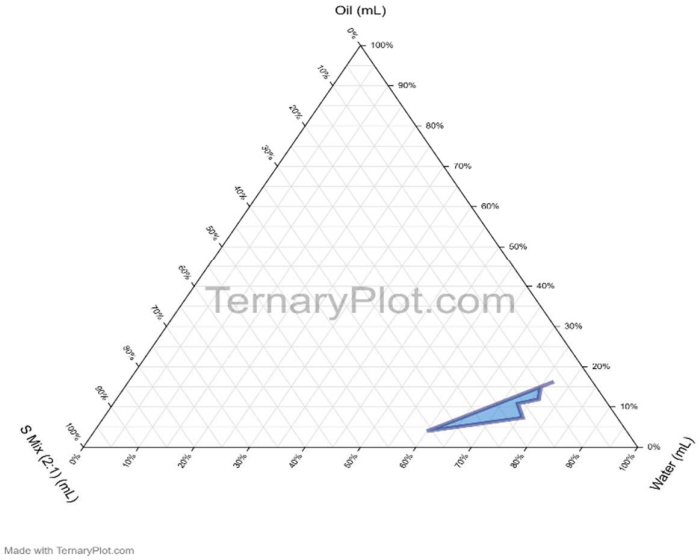


Figure 7: Pseudo ternary phase diagram using Smix 2:1

The Blue region in the pseudo ternary phase diagram represents the nanoemulsion region. The region in phase diagrams was smaller due to lower solubility of castor oil in the water and Smix. The Smix with ratio 2:1 was found to have maximum nanoemulsion region hence was found suitable for formulation of Nanoemulsion.

Formulation of Prototype Nanoemulsion

The prepared Nanoemulsion was checked for phase separation visually.

Table 4: Results of Nanoemulsion formulation with Smix

Batches	Observation
NE-1	No Phase separation

NE-2	No Phase separation
NE-3	Phase Separation
NE-4	Phase Separation
NE-5	Phase Separation
NE-6	Phase Separation
NE-7	Phase Separation

Optimization of Luliconazole Nanoemulsion formulation

Table 5: Actual design for Luliconazole Nanoemulsion formulations

	Factor 1	Factor 2	Response 1	Response 2
Run	**A: Homogenization Speed**	**B: Homogenization Time**	**Globule Size**	**Zeta Potential**
	RPM	**mins**	**nm**	**mV**
1	3000	30	490.4	-30.5
3	5000	30	424.8	-29.6
4	7000	30	390.9	-28.7
5	5000	60	412.7	-29.1
6	3000	60	465.2	-30.2
7	7000	60	365.1	-26.1
2	5000	90	395.4	-28.9
8	7000	90	342.8	-25.1
9	3000	90	439.5	-29.9

ANOVA spectrums for Luliconazole from DoE software

Response 1: Globule size

Homogenization speed and time both are found to be the statistically significant factor for the Globule size with a decrease in globule size as the speed and time increases.

For the model has no transformation of the responses was applied. **P-values** less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms.

Response 1: Globule size

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	17424.18	4	4356.04	121.89	0.0002	significant
A-Speed	14676.15	2	7338.07	205.33	< 0.0001	
B-time	2748.03	2	1374.01	38.45	0.0024	
Residual	142.95	4	35.74			
Cor Total	17567.13	8				

The ANOVA findings (**Response 1- Globule size)** demonstrate that the model is statistically significant ($p < 0.0001$). All examined factors, indicating that the selected factors explain a significant portion of the variation in the response. This suggests that the Homogenization speed is influenced by changes in homogenization time, which is essential for ensuring the model provides adequate predictive capability for optimization purposes.

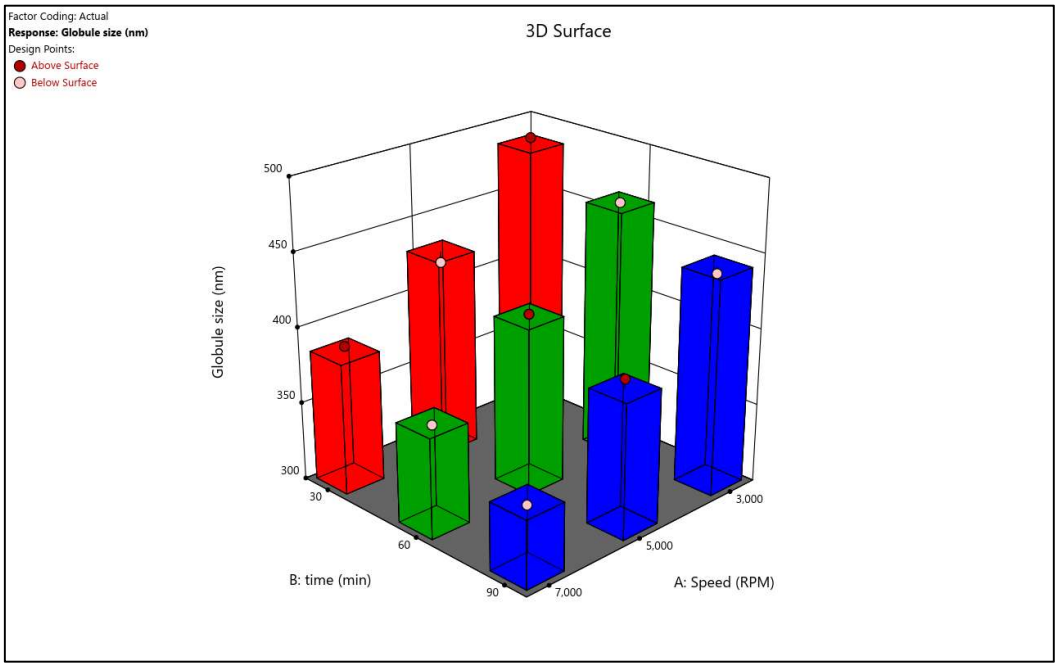


Figure 10: 3D Surface Plot- Globule size

Response 2: Zeta Potential

A Homogenization speed is found to be the statistically significant factor for the **Zeta Potential** with an increase in **Zeta Potential** as the Homogenization speed increases.

For the model Inverse transformation of the responses was applied. **P-values** less than 0.0500 indicate model terms are significant. In this case A is significant model terms.

ANOVA for selected factorial model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.0000	2	0.0000	7.38	0.0241	significant
A-Speed	0.0000	2	0.0000	7.38	0.0241	
Residual	0.0000	6	2.281E-06			
Cor Total	0.0000	8				

The ANOVA findings for Response 2 (**Zeta Potential**) with reduced model is statistically significant ($p < 0.0001$), confirming that the selected factors explain a significant portion of the variability in the response. Based on p-values (< 0.0500), the significant model terms is A (Homogenization speed), while B (Homogenization time) is not significant (removed from model).

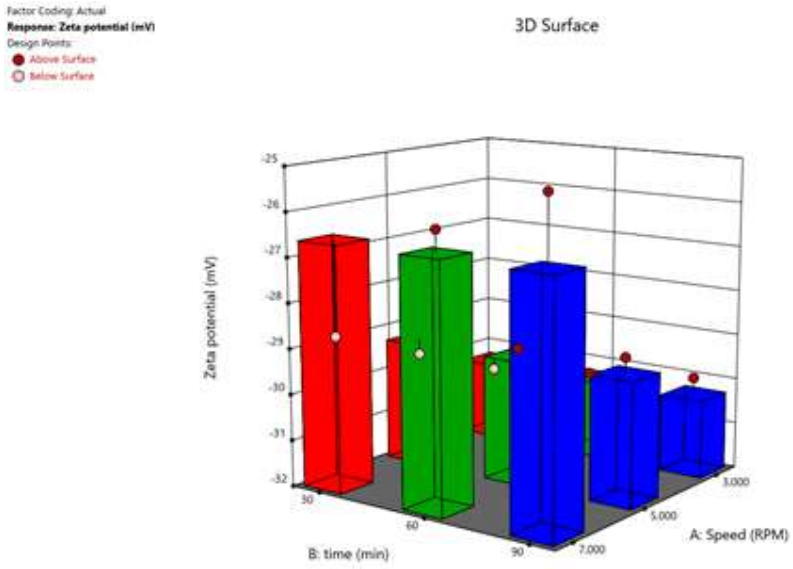


Figure 11: 3D Surface Plot- Zeta Potential

Evaluation of Luliconazole Nanoemulsion

Globule Size

The Globule size analysis was performed all the Luliconazole Nanoemulsion. Batch 8 was found to have the least particle size of 342.8 nm. The results of particle size are given in Table 6.

Zeta potential

Zeta potential measurements were conducted to evaluate the stability of the nanoemulsion formulations. The pure drug exhibited a zeta potential of –25.1 mV, while all formulations showed comparable negative zeta potential values, indicating satisfactory stability. Notably, increased homogenization time and speed led to a further enhancement in the negative zeta potential, contributing to improved electrostatic stabilization of the system. The highest zeta potential observed was –27.9 mV for batch 8. Detailed zeta potential values for all batches are presented in Table 6.

Polydispersity Index

The PDI analysis was performed all the Luliconazole Nanoemulsion. Batch 8 was found to have a high PDI of 0.578. The results of PDI are given in Table 6.

Drug Content

Drug content was evaluated to confirm the integrity of the active pharmaceutical ingredient (API) throughout the formulation process and to ensure consistent therapeutic efficacy. All formulations exhibited drug content within the range of 97–100%, indicating no significant drug loss or degradation during processing. These results also reflect the accuracy of the API incorporation method. Detailed drug content data are presented in Table 6.

% Transmittance

The percentage transmittance (%T) of the selected batch 8 formulations was measured at 650 nm using distilled water as the blank. The %T values for nanoemulsions were found to be close to 100%, indicating that the formulations were clear and transparent. The highest transmittance recorded was 99.57 ± 0.28% for batch 8. These results confirm the optical clarity and homogeneity of the nanoemulsions. Detailed %T values are presented in Table 6.

Table 6: Results of Evaluation of Luliconazole Nanoemulsion

Batch	Globule Size (nm)	Zeta potential (mV)	PDI	Drug Content (%)	Transmittance (%)
Batch 1	490.4	-30.5	0.342	98.31±0.34	97.92±0.45
Batch 2	424.8	-29.6	0.389	99.23 ±0.17	99.45±0.18
Batch 3	390.9	-28.7	0.354	98.27±0.12	97.26±0.14
Batch 4	412.7	-29.1	0.389	98.19±0.23	97.12±0.34
Batch 5	465.2	-30.2	0.411	97.32±0.46	97.25±0.33
Batch 6	365.1	-26.1	0.378	98.24±0.18	98.31±0.19
Batch 7	395.4	-28.9	0.471	97.62±0.42	98.13±0.36
Batch 8	342.8	-27.9	0.568	99.34±0.19	99.57±0.28
Batch 9	439.5	-29.9	0.478	98.24±0.54	99.29±0.16

Stability Studies

Stability testing was conducted for a period of three months under accelerated conditions (40 ± 2°C and 75 ± 5% relative humidity) in accordance with ICH guidelines. The optimized formulation, batch 8, was subjected to stability assessment to evaluate its performance at elevated temperature, providing insight into its potential long-term stability under ambient storage conditions. The comparative results of these evaluations are summarized in Table 7.

Table 7: Results of Stability study of batch 8

Time interval	Globule Size (nm)	Zeta potential (mV)	PDI	Drug Content (%)	Transmittance (%)
0 Day	342.8	-27.9	0.489	99.78±0.25	99.28±0.33
1 month	346.8	-27.5	0.512	99.37±0.13	99.09±0.18
2 month	349.3	-26.8	0.531	99.03±0.22	98.86±0.27
3 month	354.6	-26.1	0.548	98.73±0.35	98.41±0.42

The formulation was found to be stable over the 90 days interval at at 40ºC ± 2ºC and 75% ± 5% relative humidity.

Antifungal Study

The zone of Inhibition after 48 Hours of incubation was measured and results are summarized as below in table 8

Table 8: Results of Zone of inhibition study (n=3)

Sr. No	Sample name	Identification mark	Zone of inhibition (mm)
1	Negative control	Blank	0 mm
2	Luliconazole nano emulsion at 0.04% concentration	Formulation	18.2 mm ± 0.2 mm
3	Marketed sample- Luliconazole cream 1 % concentration (Std.)	Standard	20.1 mm ± 0.3 mm

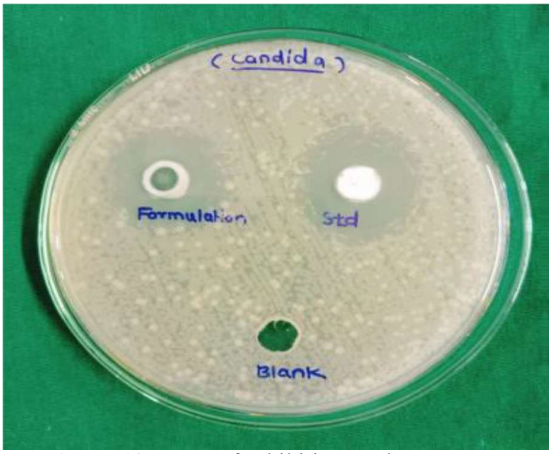


Figure 12: Zone of Inhibition study outcomes

In Vivo analysis

HPLC method validation for determination plasma samples

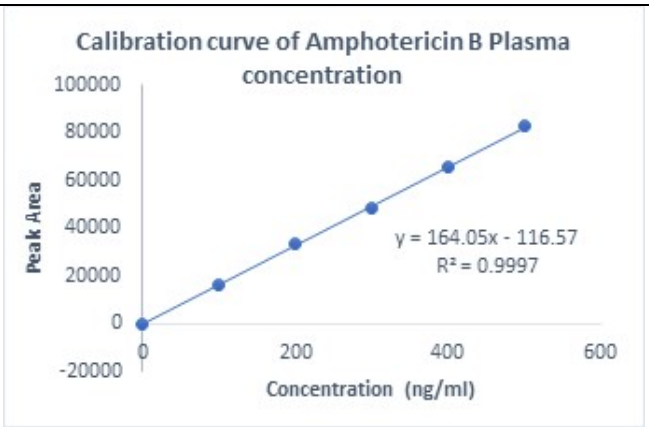
The estimation of drug concentration in plasma samples was carried out by validated HPLC method. The peak area with corresponding concentration has been summarized in the table below along with the linearity of plasma sample concentration from both formulations. The respective calibration curve data shown below figure.

Linearity of Luliconazole plasma sample concentration	Calibration curve of Luliconazole plasma

Conc (ng/ml)	Peak Area
0	0
100	12852
200	25310
300	37205
400	51025
500	62164

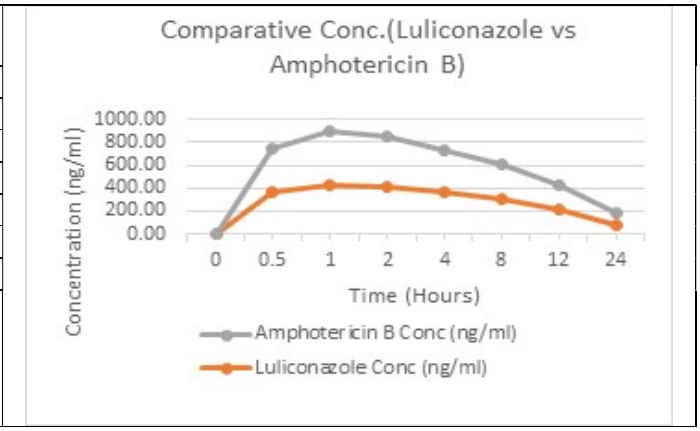
Linearity of Amphotericin B plasma sample concentration

Conc (ng/ml)	Peak Area
0	0
100	16294
200	33119
300	48257
400	65305
500	82401



The peak area values to corresponding plasma concentration by HPLC analysis for both the formulation as below,

Time	Luliconazole Conc (ng/ml)	Amphotericin B Conc (ng/ml)
0	0.00	0.00
0.5	365.09	387.39
1	423.49	470.74
2	409.99	434.54
4	357.61	377.60
8	300.87	314.19
12	208.26	220.13
24	81.14	96.87



Pharmacokinetic parameters after IM administration of newly developed nano emulsion (Ampho & Luliconazole) vs Marketed formulations (Ampho- Liposome & Conventional Amphotericin B) was compared to understand the drug pharmacodynamic parameters and summarized as below,

Parameters	*Liposomal Amphotericin B (marketed formulation)	*Conventional Amphotericin B (marketed formulation)	Amphotericin B Nanoemulsion	Luliconazole Nanoemulsion
C max (ng/mL/hrs.)	7300	1700	470.74	423.49
T max (hrs.)	1	1	1	1
$T_{½}$ (hrs.)	10.7	24 - 48	10.12	8.54
AUC0-t (ng*hr/ml)	27000	18650	5930.38	5544.38
AUMC	NA	NA	105997.18	83564.28
MRT (hrs.)	7-10	8-10	14.43	12.77

The PK comparison table shows that both newly prepared nanoemulsions (Ampho B and Luliconazole) produced much lower peak plasma levels (Cmax) and overall exposure (AUC0–t) than the marketed Amphotericin B formulations, while Tmax remained 1 h for all groups. The half-life of the nanoemulsions (8.54–10.12 h) was closer to liposomal Ampho B (10.7 h) and shorter than conventional Ampho B (24–48 h). Despite lower exposure, nanoemulsions showed higher MRT (12.77–14.43 h), indicating a more prolonged residence time compared with marketed formulations.

4. CONCLUSION

This study developed and evaluated Luliconazole nanoemulsions for fungal infection treatment. Using systematic screening and DoE optimization, castor oil and a 2:1 Smix ratio produced stable nanoemulsions with optimized homogenization parameters. Characterization confirmed suitable globule size, high zeta potential, low PDI, high drug content, and good transmittance. Stability was maintained under accelerated conditions. The nanoemulsions improved drug solubility and stability, offered efficient encapsulation, and present a promising delivery system for poorly water-soluble antifungal agents.

ABBREVIATIONS

Abbreviation	Full Form
API	Active Pharmaceutical Ingredient
BCS	Biopharmaceutical Classification System
CCD	Central Composite Design
DSC	Differential Scanning Calorimetry
DLS	Dynamic Light Scattering
DOE	Design of experiment
FTIR	Fourier-Transform Infrared Spectroscopy
NE	Nanoemulsion
PDI	Polydispersity Index
RH	Relative Humidity
Smix	Surfactant and Co-surfactant Mixture
XRD	X-Ray Diffraction

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to the publication of this manuscript.

AUTHOR CONTRIBUTIONS

Pravin N. Kirdat: Conceptualization, experimental design, data collection, manuscript writing; **Meenakshi B. Patel**: Supervision, critical review, data interpretation, and final approval of the manuscript. All authors have read and approved the final manuscript.

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