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FORMULATION AND EVALUATION OF FLUCYTOSINE-LOADED NANOEMULGEL FOR ENHANCED ANTIFUNGAL ACTIVITY THROUGH IN VITRO AND IN VIVO STUDIES

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ABSTRACT

Background: Fungal skin infections constitute a prevalent global health issue, accompanied by increasing resistance to traditional antifungal medications. Flucytosine, a pyrimidine analogue exhibiting potent antifungal properties, is constrained in topical use due to inadequate skin absorption and fast elimination. Nanoemulgels, which integrate nanoemulsions with gels, enhance solubility, penetration, stability, and prolonged release, representing a viable approach for topical antifungal administration. **Methods:** The nanoemulgel was prepared utilizing Carbopol 940 as the gelling agent. The formulations (NEG1–NEG8) were evaluated for physical appearance, pH, viscosity, spreadability, drug content, and *in vitro* drug release and release kinetics. Antifungal activity was assessed by zone-of-inhibition assays and in vivo using Wistar rats infected with *Candida albicans*. Skin irritation, histopathology, and a three-month stability study were also conducted. **Result and Discussion:** The improved NEG5 formulation had a favorable pH (5.92±0.03), high drug content (95.3±1.83%), and maximum cumulative drug release (92.97±5.91% at 24 hours) with first-order release kinetics (R2= 0.9959). The commercial luliconazole cream and NEG5 showed increased antifungal effectiveness in vivo, with lesion clearance by Day 14. The histopathology showed tissue repair with minimal inflammation. NEG5 surpassed other formulations in zone-of-inhibition assays for antifungal activity. No skin irritation was reported, and the formulation was stable for three months under various storage conditions. The improved physicochemical and therapeutic performance of NEG5 suggests enhanced skin penetration and sustained drug release, addressing the limitations of conventional flucytosine therapies. **Conclusion:** Flucytosine-loaded nanoemulgel (NEG5) offers a promising, effective topical treatment for fungal skin infections with improved drug delivery and patient safety.

INTRODUCTION

Fungal infections are a prevalent category of dermatological illnesses globally, affecting about 150 million individuals who endure significant morbidity or mortality, particularly in poor and underdeveloped countries. While fungal infections do not cause mortality, they are an essential contributor to morbidity

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and healthcare costs. Superficial fungal infections are prevalent globally, with an estimated incidence of 20–25%, and are linked to everyday activities, inadequate hygiene, and substandard healthcare quality [1]. Skin fungal infections constitute a significant and increasing global health problem, exacerbated by the emergence of pathogenic strains resistant to standard antifungal agents. Recent epidemiological studies indicate an increase in the prevalence and severity of these diseases, particularly among immunocompromised individuals and in regions characterized by high humidity and temperature variability [2]. Conventional systemic and topical therapies frequently encounter limitations, including inadequate drug penetration, systemic toxicity, and the development of resistance, underscoring the need for innovative treatment modalities that effectively target the site of infection while minimizing adverse effects [3]. Topical drug delivery methods have been extensively investigated as a means to overcome the limitations of systemic administration, offering the benefit of targeted treatment with less systemic exposure [4]. The efficacy of topical preparations is often constrained by the skin's intricate barrier properties, which impede the absorption of many medicinal substances. In this context, novel delivery vehicles, such as nanoemulgels, have emerged as viable platforms for enhancing drug penetration and retention within the skin layers [5]. Nanoemulgels integrate the beneficial attributes of nanoemulsions, including enhanced solubilization capacity, stability, and improved bioavailability, with the positive features of gels, such as ease of administration, controlled drug release, and improved patient compliance [6]. This hybrid formulation enables encapsulation of both hydrophilic and lipophilic drugs, thereby expanding the range of therapeutic agents that can be efficiently administered to affected skin tissues. Recent advances in nanoemulgel technology have demonstrated efficacy in enhancing the topical distribution of several antifungal drugs, suggesting a potential role in addressing the challenges posed by emerging skin fungal infections [7].

Flucytosine, a fluorinated pyrimidine analogue with antifungal properties, has been utilized for over 70 years as a well-established drug primarily in combination therapy for systemic fungal infections, particularly in the treatment of Cryptococcus infections in HIV-positive patients [8]. Its mechanism, which entails the suppression of fungal RNA and DNA synthesis, establishes it as a potent agent against a wide range of fungal infections. However, when applied topically, flucytosine is limited by poor skin absorption and rapid elimination at the site of infection. Incorporating flucytosine into a nanoemulgel system enhances its stability and regulates its release, resulting in elevated local concentrations at the site of infection and improved therapeutic efficacy. Flucytosine is well established for systemic fungal infections; its topical use is restricted due to skin absorption and rapid clearance [9]. To date, there are no significant publications on topical flucytosine administration, highlighting the originality of our approach. Several studies have shown that nanoemulgel systems can improve topical antifungal treatment. For instance, Kumar et al. developed a miconazole nanoemulgel with better skin penetration and antifungal effectiveness [10]. Sadozai et al. [11] found increased skin penetration and safety of nanoparticle-based topical antifungal solutions in vitro, ex vivo, and in vivo. These findings support the development of a flucytosine nanoemulgel, which may overcome topical distribution issues and meet nanoemulgel effectiveness and safety standards. The amalgamation of nanoemulgel technology with flucytosine is an innovative and promising strategy to address developing cutaneous fungal infections. This study investigates the formulation, characterisation, and therapeutic efficacy of a flucytosine-loaded nanoemulgel to overcome limitations in topical antifungal therapy and enhance treatment strategies. The inquiry is supported by recent findings indicating the potential of nanotechnology-based drug delivery systems to improve the bioavailability and therapeutic efficacy of antifungal agents [12].

MATERIAL AND METHODS

Materials

The materials enumerated below were acquired from the specified sources without further purification. Flucytosine was procured from BLD Pharma, situated in Hyderabad, India. The residual chemicals employed were of analytical reagent quality.

Methods

Preparation of Flucytosine-loaded Nanoemulgel

A flucytosine nanoemulgel was developed using a two-step procedure: preparation of a nanoemulsion followed by incorporation into a gel formulation. A high-speed homogenizer (JHG-54-P100, Shanghai Pulisheng Fusion Machinery Co., Ltd., China) was employed to prepare the nanoemulsion [13]. In a clean, dry beaker, flucytosine was dissolved in a portion of the aqueous phase. Briefly, the required amount of flucytosine was dissolved in distilled water and mixed into the Smix (Tween 80 and propylene glycol in a 1:1 ratio) to produce an aqueous drug phase. The oil phase was prepared by mixing 10 mL of castor oil and 15 mL of liquid paraffin. The phase was gradually added to

aqueous Smix containing dissolved flucytosine at a 30:70 v/v oil: aqueous ratio under continuous stirring to obtain a coarse emulsion. The resulting coarse emulsion was homogenized at 15,000 rpm for 20 minutes, followed by probe sonication for 10 minutes to reduce droplet size and ensure homogeneous o/w nanoemulsion formation [14]. To prepare the nanoemulgel, an accurately weighed amount of carbopol 940P was first dissolved in purified water and left overnight for complete, homogenous swelling. Methyl paraben and propyl paraben, utilized as preservatives, were dissolved in a minimal volume of warm distilled water and integrated into the hydrated gel matrix. The previously formulated flucytosine nanoemulsion was progressively integrated into the Carbopol gel under mild agitation to create a homogeneous gel matrix. The pH of the formulation was ultimately adjusted with triethanolamine, yielding a smooth, homogeneous nanoemulgel suitable for topical administration [15].

Characterization of Flucytosine-Loaded Nanoemulgel

Physical Examination: The prepared nanoemulgel composition was visually inspected for colour, appearance & consistency.

pH: The pH of the formulation was assessed using both the direct and dilution methods. For the direct method, approximately 10 gm of the sample was placed in a glass vial, and the pH electrode was immersed directly into the sample. The pH value was recorded after stabilization. In the dilution method, a 10% dispersion was created by mixing 1 gm of the formulation with 9 g of purified water, followed by thorough shaking. The pH electrode was inserted into this diluted sample, and the measurement was taken once the reading stabilized [16].

Viscosity: A Brookfield digital viscometer (Cone and Plate, Model 2000+, Brookfield Engineering Lab., USA) with a 25 cm^3 sample volume was used to measure the viscosities of the formulations. All samples were tested using spindle number 62, with approximately 100 mg of bulk sample at 100 RPM for 1 minute. The readings on the digital reader were noted [17].

Spreadability

To evaluate spreadability, a stationary female Perspex cone was secured to a wooden board. In contrast, a movable male Perspex cone was positioned over the fixed female cone on the wooden board (Spreadability Fixture Texture Analyser TA-SF, CT3, Brookfield). The mass of the movable male Perspex cone was measured to be 20 grams. A 5 g sample of the gel was placed in the female Perspex cone, and the surface was uniformly spread with a knife before testing. The male Perspex cone, with a specified weight, was incrementally advanced towards the female Perspex cone, traversing a distance of 6 cm. The duration of each 6 cm movement was documented for each sample [18,19]. In this study, the values were determined in triplicate and calculated using the formula below-

$$\textit{Spreadability} = \frac{\textit{Weight of male perspex conex length moved on fixed female perspex cone}}{\textit{Time is taken to move towards female perspex cone}}$$

Drug content

0.5 g of topical nanoemulgel was measured and dissolved in 50 mL of methanol. Sonication was conducted for 15 minutes to ensure complete dissolution of flucytosine in methanol. The solution was filtered using Whatman filter paper, and the resulting filtrate was subsequently diluted with methanol. The aliquot was analysed at 248 nm using a UV spectrophotometer (Shimadzu 1900i, Japan), and the drug content was determined [20].

In-vitro drug release

A diffusion study utilising a Franz diffusion cell apparatus can effectively assess the *in vitro* drug-release profile of the prepared nanoemulgel, employing egg membrane as the semipermeable membrane. Initially, fresh egg membranes are isolated by immersing the eggs in dilute acetic acid to soften the shell, followed by the careful peeling of the membrane. The membranes are subsequently rinsed with phosphate-buffered saline (PBS) to remove residual debris. The cleaned membrane is positioned between the donor and receptor compartments of the Franz diffusion cell.

The receptor compartment was filled with PBS (pH 7.4), maintained at 37 ± 0.5°C, and stirred continuously using a magnetic stirrer to simulate physiological conditions. 100 mg of nanoemulgel is positioned in the donor compartment. Aliquots are withdrawn from the receptor compartment at predetermined time intervals of 0.5, 1, 2, 4, 6, 8, 10, 12, 18, and 24 hours, with fresh buffer added to maintain sink conditions. The samples are analysed using a spectrophotometer (UV-1800, Shimadzu, Japan) at 248 nm to quantify cumulative drug release [21–24].

***In-vitro* release kinetic study**

The pharmacokinetic principle indicates that the drug-release kinetics of dosage forms can be described by multiple equations corresponding to different kinetic models [25,26].

In-Vivo Study

Animal: Healthy adult male Wistar rats were utilised for the antifungal study. The subjects were aged 2-3 months and weighed approximately 150-200 g. A prescribed atmosphere was maintained for the rats. The specified conditions included a 12-hour light-12-hour dark cycle, with a temperature range of 25 ± 2 °C and humidity levels between 30% and 55%. The rats were given rat pellets and water as their daily diet. The Institutional Animal Ethical Committee, Shambhunath Institute of Pharmacy, Prayagraj, India, approved the study (SIP-IACE/002/07/23).

Skin irritation Study (dermal toxicity): Before initiating an in vivo study with Wistar rats, the formulated nanoemulgel was evaluated for skin sensitivity and irritation. The results indicated a favourable outcome. This study was conducted five days before the initiation of the fungal study, given the drug's washout period after application. Wistar rats were allocated to three groups: Group 1 (Control), Group II (Standard), and Group III (Test), each comprising three rats. The dorsal hair on each rat was removed 24 hours before the study, over an area of approximately 4-5 cm, to evaluate the hypothesis. All three topical formulations (placebo, standard, nanoemulgel) were administered to the shaved area of each group. The area was visually assessed for dermal reactions, such as erythema & edema, at 24, 48, & 72 hours, and the grading scores were recorded according to standard criteria. scores given in tables 1 [27,28].

Antifungal Activity Using Wistar Rats

Procedure: The in vivo antifungal study utilised *Candida albicans* to induce mycosis in Wistar rats. The procedure commenced with the removal of hair from a 4 x 5 cm area on the dorsal surface of the rats. The hair was removed utilising a depilatory cream. The cleaned surface was lightly abraded with sandpaper, and the skin was inoculated with *Candida albicans* using a cotton swab. The animals were monitored for an additional three days to observe the progression of the infection. The animals were categorised into five groups, each comprising six individuals, for treatment application.

Table 1: Standard score grades for skin sensitivity study

Erythema formation grading score	
No erythema	0
Very slight erythema	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema	4
Total possible score formation	8

The groups consisted of a control group (No disease), negative control (Having disease + Untreated), test control 1 (prepared formulation of flucytosine gel), test control 2 (flucytosine nanoemulgel formulation), and standard/positive control (a marketed formulation), (luliconazole cream 1%) was selected as the marketed comparator due to the absence of a topical flucytosine formulation. Luliconazole is an FDA-approved standard topical antifungal treatment for *Candida albicans* infections, providing a clinically relevant benchmark despite differing mechanisms of action. All formulations were applied topically to the cleaned and abraded skin of the rats. The formulations were administered once daily for 14 days. Nevertheless, the control groups received no treatment. Responses from all groups were recorded after 14 days. Daily assessments were conducted to observe physical signs, including swelling, redness in the interdigital regions of the back, and hair loss throughout the therapy period [11].

Qualitative In-Vivo Antifungal Activity

To provide a quantitative assessment of therapeutic efficacy, lesion scoring and colony-forming unit (CFU) enumeration were performed on *Candida albicans*-infected rat skin following 14 days of treatment

Table 2: Formulation samples and animal group

Group	Details	Formulation	Animals
Group I	Control Group	No disease	6 Wistar Rats
Group II	Negative Control	Having a disease + Untreated	6 Wistar Rats
Group III	Test Control 1	Having disease + Testing Flucytosine Gel	6 Wistar Rats
Group IV	Test Control 2	Having disease + Testing Flucytosine Nanoemulgel formulation	6 Wistar Rats
Group V	Standard/Positive Control	Having disease + Luliconazole Cream	6 Wistar Rats

Blinded Lesion Scoring

Lesion severity was evaluated by two independent, blinded observers using a standardized 0-4 grading scale. Grade 0 shows no visible lesion, 1 shows slight erythema, 2 shows moderate erythema with scaling, 3 shows severe erythema with crusting, and 4 shows an ulcerated lesion.

Quantification of Fungal Burden (CFU Counts)

On day 14, animals were euthanized, and a skin biopsy (1 cm2) from the infected region was aseptically excised, weighed, and homogenized in sterile phosphate-buffered saline. Serial dilutions were plated on sabouraud Dextrose Agar and incubated at 35°C for 48 hrs. Fungal growth was quantified as CFU/gm of tissue.

Histopathological Assessment of Treated Rat Skin

A segment of rat skin was subjected to various treatments, specifically a control group, simple flucytosine gel, flucytosine nanoemulgel, and a marketed product, to assess potential topical toxicity. The animals were sacrificed using an overdose of ketamine/xylazine, and skin samples were obtained using excision, separated with phosphate buffer, and preserved in 10% formalin. Sample preparation and sectioning were executed utilizing a microtome. Additionally, the sectioned specimens were stained with haematoxylin and eosin dye. The staining of the samples facilitated the visibility of the cross-sectioned specimens under microscopy. Each slide was viewed and photographed under a Leica microscope at 10x magnification [28,29].

Antifungal activity by zone inhibition study

The antifungal activity of the prepared gels was assessed against *Candida albicans* utilising the nutrient agar cup method. Nutrient agar cups were prepared under aseptic conditions and inoculated with the fungal suspension by evenly spreading it across the agar surface. The plates were incubated at 35 °C for 24 hours, after which the zones of inhibition were observed and measured using an antibiotic zone reader. Antifungal efficacy was validated by comparing zones of inhibition produced by a plain flucytosine solution (drug control), a marketed formulation, and the optimised nanoemulgel formulation using the agar disc diffusion technique [30].

Stability study

In compliance with ICH Q1A (R2) guidelines, the optimised nanoemulgel formulation was packaged in aluminium laminated tubes (15 g) and subjected to stability studies under various storage conditions: room temperature at 25°C ± 2°C and 60% ± 5% RH, intermediate temperature at 30°C ± 2°C and 65% ± 5% RH, and accelerated temperature at 40°C ± 2°C and 75% ± 5% RH for a duration of 3 months. The samples were analysed for colour and appearance, Phase separation, homogeneity, medication content, spreadability, and pH levels [31,32].

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (Version All 10.6.0). The data are expressed as mean ± standard deviation (SD) from n=3. Data were analyzed using one-way ANOVA, followed by Tukey's multiple comparisons test, with $P < 0.05$ considered statistically significant.

RESULT AND DISCUSSION

Physical Examination

The nanoemulgel was characterised by a white colour and a distinct odour. The transparency of the nanoemulsion within the gel matrix suggests a high degree of homogeneity and uniform dispersion. No phase separation was observed during the investigation, indicating the formulation's exceptional physical stability. The gel's occlusive properties may enhance drug penetration and skin hydration by forming a protective layer on the skin. Additionally, the formulation was found to be washable, indicating that it can be readily removed from the skin's surface, thereby enhancing patient comfort & compliance.

pH: The pH values for all formulations are approximately between 5.54±0.06 and 5.92±0.03, exhibiting minimal variation. NEG5 exhibited the highest pH (approximately 5.92±0.03), whereas NEG6 had the lowest pH (approximately 5.54±0.06). All values are within the acceptable range for topical formulations, indicating minimal irritation and favourable skin compatibility, as shown in Table 2 and Figure 1.

Viscosity: Viscosity is essential in ascertaining the consistency and application characteristics of topical gels. The viscosity readings exhibited considerable variation, spanning from 4.7±0.71 mPa.S to 16.9±0.35 mPa.S. Formulations with elevated viscosity include NEG5 (16.9±0.35 mPa.S), NEG1 (14.5±0.52 mPa.S), and NEG7 (9.7±0.45 mPa.S). Conversely, NEG4 had the lowest viscosity at 3.6±0.42 mPa.S, whereas increased viscosity typically results in prolonged retention on the skin, which may impede spreadability. Elevated viscosity at NEG5 indicates a concentrated gel matrix, which may aid in regulating drug release, as shown in Table 2 and Figure 1.

Spreadability

Spreadability is crucial for ensuring patient compliance and facilitating ease of application. The spreadability values varied from 9.2 ± 0.3 g.cm/s for NEG5 to 16.1 ± 0.5 g.cm/s for NEG8. Formulations NEG3 (15.1±0.5g.cm/s), NEG4 (14.5±0.4g.cm/s), and NEG6 (14.8±0.5g.cm/s) demonstrated favourable

spreadability. NEG5 exhibited the lowest spreadability, presumably attributable to its elevated viscosity. In contrast, NEG8 exhibited the greatest spreadability, alongside a relatively low viscosity of 5.2±0.56 Pa.S, suggesting it may provide both user-friendliness and effective application, as shown in Table 2 and Figure 1.

Drug content

The drug content ranged from 72.1±2.31% (NEG1) to 95.3±1.83% (NEG5). Elevated medication concentration is essential for therapeutic effectiveness. NEG5 and NEG8 had the highest drug concentrations at 95.3±1.83% and 93.43±2.67%, respectively, indicating effective drug loading and minimal loss during production.

NEG3 and NG4 exhibited satisfactory drug content, exceeding 85%. Reduced results in NEG1 (72.1±2.31%), NEG6 (75.5±3.21%), and NEG7 (76.1±2.26%) indicate potential problems with medication integration or stability, as shown in Table 3 and Figure 1.

Table 3: Characterization of gel formulations (n=3)

Formulation Code	pH	Viscosity (mPa.S)	Spreadability (g.cm/s)	Drug Content (%)
NEG1	5.61±0.05	14.5±0.52	14.2±0.5	72.1±2.31
NEG2	5.76±0.04	4.7±0.71	11.8±0.6	83.4±3.13
NEG3	5.82±0.06	8.9±0.69	15.1±0.5	90.2±2.81
NEG4	5.87±0.05	3.6±0.42	14.5±0.4	85.12±3.11
NEG5	5.92±0.03	16.9±0.35	9.2±0.3	95.3±1.83
NEG6	5.54±0.06	6.9±0.51	14.8±0.5	75.5±3.21
NEG7	5.78±0.04	9.7±0.45	12.9±0.6	76.1±2.26
NEG8	5.61±0.05	5.2±0.56	16.1±0.5	93.43±2.67

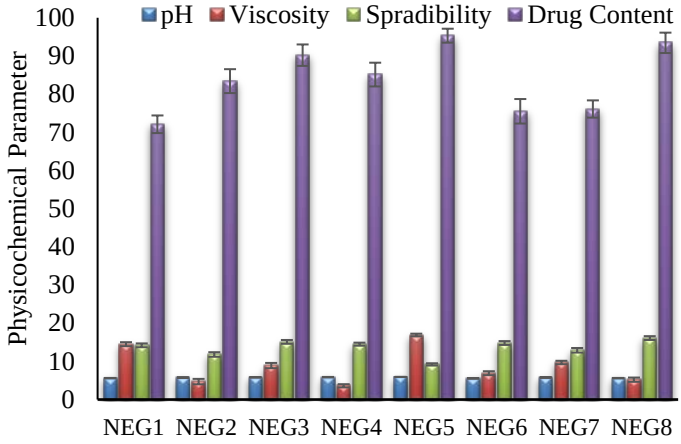


Figure 1: Graphical representation of the characterization of Gel formulation

In-vitro drug release

The in vitro drug release of Flucytosine from eight distinct nanoemulgel formulations (NG1–NG8) was assessed over 24 hours, revealing a time- and formulation-dependent release profile. All formulations exhibited a steady, sustained increase in drug release over time, indicating that the nanoemulgel technology successfully extended the release of Flucytosine. Among the evaluated formulations, NEG5 exhibited the highest cumulative drug release (92.97±5.91%) over 24 hours, followed by NEG6 (82.65±6.98%) and NG4 (80.06±7.33%). The results indicate that NEG5 exhibited the most advantageous formulation features, likely attributable to optimal proportions of oil, surfactant, co-surfactant, and gelling agents, which enhance drug solubilization and diffusion. Conversely, NEG1 and NEG2 exhibited the lowest drug release rates of 54.90±8.73% and 61.35±8.44%, respectively, at 24 hours, suggesting slower release profiles potentially due to increased viscosity or suboptimal emulsification, which impede drug diffusion. NG3, NEG7, and NEG8 exhibited intermediate drug-release rates of 67.16±8.87% to 76.19±6.80%, indicating moderate efficacy in drug maintenance and delivery. The initial burst release observed within the first hour, particularly in NEG5 (25.23±2.96%) and NG4 (16.19±7.82%), suggests rapid drug availability at the application site, which is advantageous for immediate therapeutic effect. In contrast, the sustained release over 24 hours facilitates prolonged action & diminishes application frequency. Statistical analysis confirmed significant differences among formulations. One-way ANOVA revealed a robust treatment effect ($F= 32.97$, $p< 0.0001$). Post hoc Tukey's test demonstrated that NEG 5 released significantly more drug than all other formulations ($p < 0.002$), with additional statistically significant differences observed among several different pairs. The findings indicate that the nanoemulgel formulations, specifically NEG5 and NEG6, provide a regulated, prolonged release of Flucytosine, positioning them as attractive options for topical antifungal treatment, as shown in Table 4 and Figure 2.

Table 4: % cumulative release of various formulation (n=3, $p < 0.05$)

Time (hr)	% Drug Release							
	NEG1	NEG2	NEG3	NEG4	NEG5	NEG6	NEG7	NEG8
0.5	3.29±3.35	5.23±1.94	7.81±4.03	14.26±6.22	4.58±2.23	10.37±5.12	9.09±3.87	7.81±4.03
1	8.45±4.03	9.10±6.98	11.03±5.81	16.19±7.82	9.09±1.93	12.32±7.82	10.39±12.89	9.10±8.44
2	11.68±4.03	15.55±4.87	16.84±8.87	23.29±4.03	18.12±6.79	17.48±4.03	14.26±4.03	13.61±6.80
4	19.42±6.22	20.06±8.06	22.65±8.44	28.45±8.87	34.90±4.02	25.87±6.80	23.94±7.33	19.42±6.80
6	24.58±5.81	29.10±5.91	32.97±13.73	45.87±5.12	44.58±5.91	32.32±5.81	29.74±7.33	25.87±6.80
8	31.03±7.82	40.71±7.33	45.87±7.74	52.97±4.03	54.25±2.95	52.97±7.82	45.23±5.91	41.35±4.03
12	42.65±6.22	50.39±4.03	54.90±13.17	65.23±5.81	71.03±5.12	67.81±6.80	61.35±5.81	57.48±8.87
24	54.90±8.73	61.35±8.44	67.16±8.87	80.06±7.33	92.97±5.91	82.65±6.98	76.19±6.80	68.45±9.93

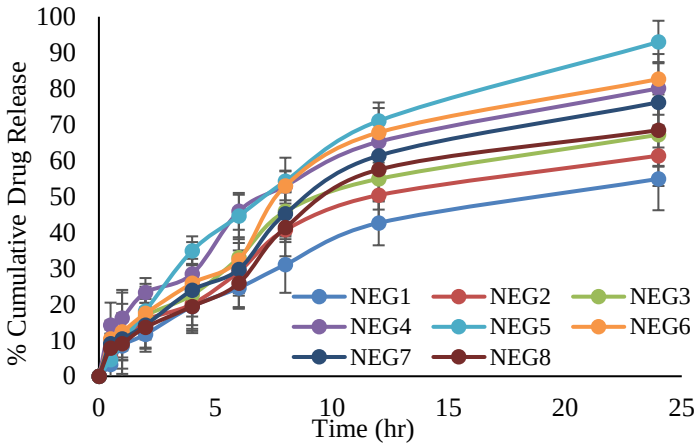
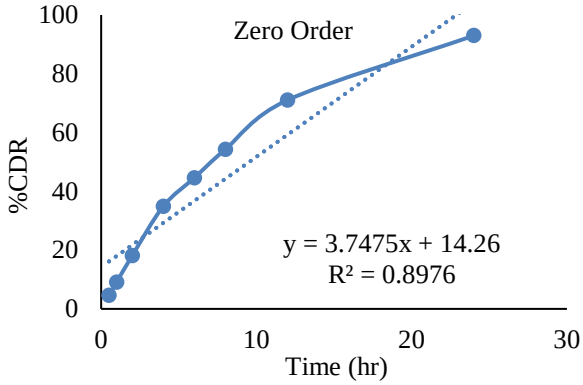


Figure 2: *In vitro* drug release profile of various batches of nanoemulgel formulations

Release kinetics-models

In vitro release data were subjected to several kinetic models, including zero-order, first-order, Higuchi-diffusion, and Korsmeyer-Peppas models. The regression coefficient (r) and 'n' values for all kinetic models are included in Table 5 for the nanoemulgel formulation NEG5. The plots are shown in Figure 3. The higher the correlation coefficient, the more appropriate the kinetic model governing drug release. As shown in the aforementioned table, flucytosine release from NEG5 is best described by the first-order model, consistent with concentration-dependent release, which exhibited the highest correlation coefficient among the kinetic models.



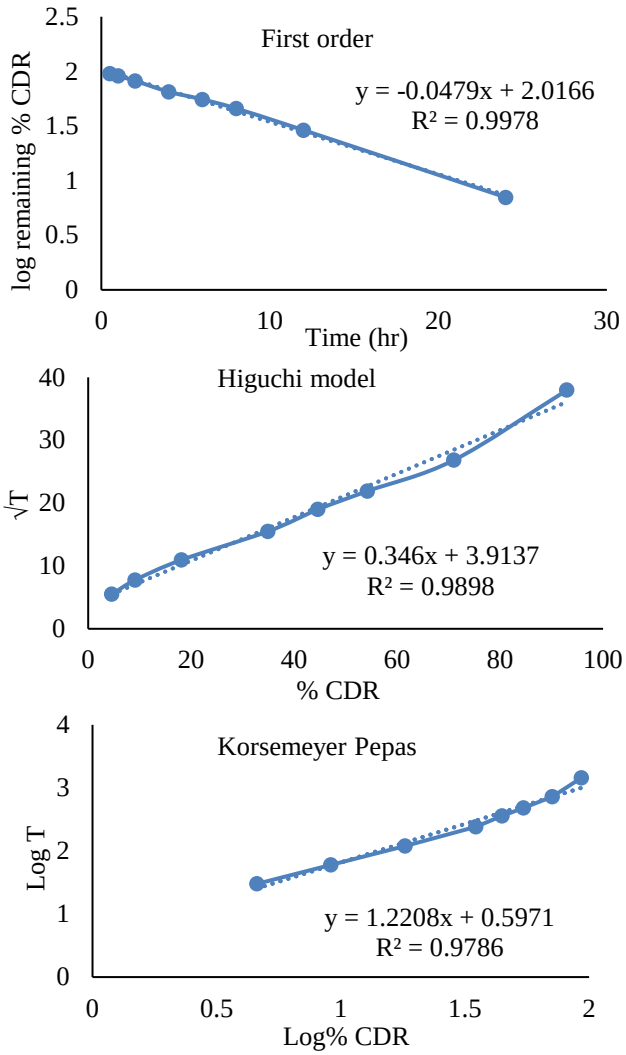


Figure 3: *In vitro* release kinetics of Optimized Flucytosine nanoemulgel

Table 5: Kinetic values obtained from *in-vitro* release of optimized nanoemulgel formulation (NEG5)

Kinetic Model	Slope(n)	Regression Coefficient (r)
Zero Order	3.7475	0.8976
Frist Order	0.0479	0.9978
Higuchi Model	0.346	0.9998
Korsmeyer-Peppas	1.22	0.9786

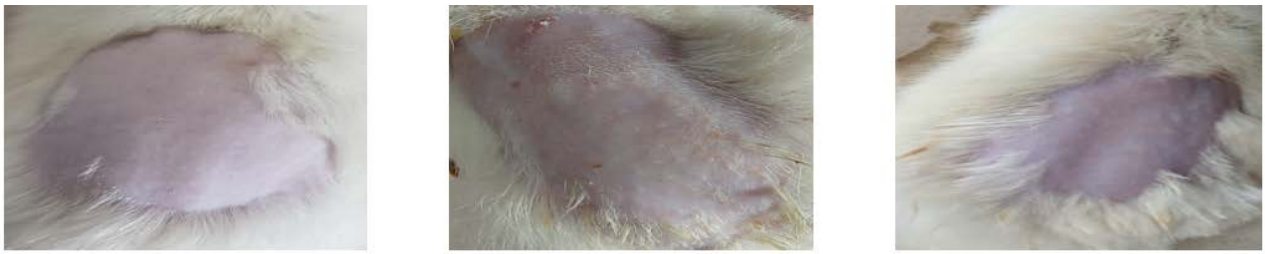
***In-Vivo* Study**

Skin irritation Study (dermal toxicity)

Wistar rats were used as an animal model for an in vivo skin irritation study to assess the irritation potential of the improved formulation compared with standard and control groups. The grading scores for erythema & edema in the animals across all groups were recorded based on visual observation. It was found that animals in the standard group exhibited slight erythema and edema (score 1), and all remaining animals showed no signs of irritation & conformed to conventional grading score ranges. The study results demonstrated no edema or erythema in any of the animals, confirming the safety of the prepared nanoemulgel for topical use, shown in Table 6 and Figure 4.

Table 6: Skin irritation score grading for nanoemulgel

Irritation score grading for parameters		
	Erythema	**Edema**
Group I	0	0
Group II	1	1
Group III	0	0



a b c

Figure 4: Image of skin irritation study (a- control, b- standard, and c- test)

Antifungal Activity Using Wistar Rats

Figure 5 demonstrates the therapeutic effectiveness of several formulations on *Candida albicans*-infected skin in animal models during a 14-day duration. The groups consist of a control group (G I), a negative control (untreated) (G II), a test formulation I (G III) including Flucytosine Gel, a test formulation II (G IV) including Flucytosine Nanoemulgel, and a reference formulation of Luliconazole cream (G V). On Day 1, all groups exhibit clear signs of infection and inflammation, characterized by erythema and lesions. The skin of the control group (G I) remained intact and normal throughout the study, with no apparent lesions, thereby confirming baseline healthy skin. Group II demonstrates lesions that have deteriorated over time, characterized by erythema, crusting, and ongoing infection, with no indications of healing by day 14.

Conversely, Group III (Flucytosine Gel) demonstrated partial healing. By day 7, inflammation had diminished marginally, and lesions remained apparent. By Day 14, modest progress was observed; however, the lesions remained unresolved, suggesting limited antifungal effectiveness. Group IV, which received Flucytosine Nanoemulgel, exhibited notable improvement. By Day 7, the erythema and lesions have markedly decreased, indicating skin repair, and by Day 14, the skin appears nearly normal, with minimal scarring or signs of infection. This results in enhanced antifungal activity and superior skin absorption, likely due to the nanoemulsion delivery method increasing the drug's bioavailability. The Reference (Luliconazole Cream) (G V) group demonstrated gradual healing comparable to that of the nanoemulgel group. By Day 14, the treated skin appeared predominantly normal, indicating significant treatment success consistent with its classification as the standard group. The findings suggest that nanoemulgel-based delivery of Flucytosine (G IV) significantly enhances antifungal efficacy compared with the conventional gel (G III) and achieves therapeutic outcomes comparable to the standard Luliconazole cream (G V). This highlights the potential of nanoemulsion technology to improve transdermal drug delivery and the efficacy of antifungal treatments.

Qualitative In-Vivo Antifungal Activity

The quantitative data (Table 7) clearly demonstrate the superior antifungal performance of the optimized flucytosine nanoemulgel. The mean lesion score for flucytosine nanoemulgel-treated animals group is 0.42±0.11, which was significantly lower than that of the flucytosine gel group, 1.95±0.24, and the untreated infected group, 3.85±0.27. Similarly, CFU counts were markedly reduced in the Flucytosine nanoemulgel group $2.1 \times 10^3 \pm 0.7 \times 10^3$ CFU/g, compared to the flucytosine gel group $3.8 \times 10^4 \pm 1.1 \times 10^4$ CFU/g and the infected control group $5.2 \times 10^6 \pm 0.8 \times 10^5$ CFU/g. The nanoemulgel efficacy was comparable to that of the standard luliconazole cream group ($1.9 \times 10^3 \pm 0.6 \times 10^3$ CFU/g; lesion score 0.38 ± 0.09).

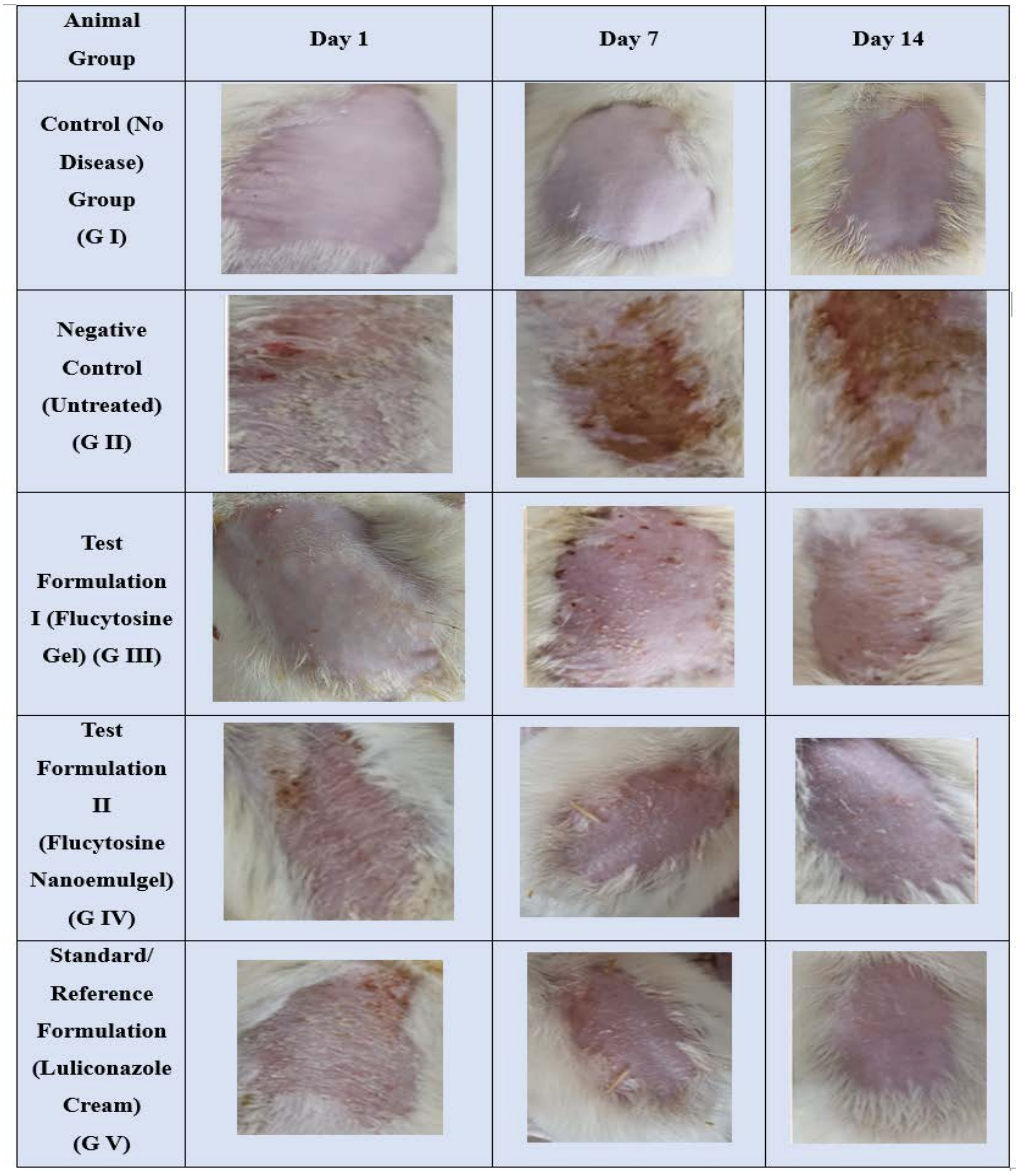


Figure 5: Comparative assessment of antifungal effectiveness of several formulations on *Candida albicans*- infected dermal tissue in animal models during 14 days.

Table 7: Quantitative analysis of in vivo antifungal efficacy after 14 days of treatment

Group	Lesion Score (Mean ± SD)	Fungal Load (CFU/g tissue, Mean ± SD)
I	0.00 ± 0.00	–
II	3.85 ± 0.27	$5.2 \times 10^6 \pm 0.8 \times 10^5$
III	1.95 ± 0.24	$3.8 \times 10^4 \pm 1.1 \times 10^4$
IV	0.42 ± 0.11	$2.1 \times 10^3 \pm 0.7 \times 10^3$
V	0.38 ± 0.09	$1.9 \times 10^3 \pm 0.6 \times 10^3$

Histopathological Study

A histopathological study was conducted to assess the potential toxicity of an improved formulation. The study evaluated rat skin exposed to formalin (test control 1), the enhanced formulation (test control 2), and a standard control, compared with a control group and a negative control. The control group (Figure 6A) demonstrated normal skin structure, characterized by an intact epidermis, organized dermal architecture, and the absence of inflammation or damage. The negative control group (Figure 6B) exhibited considerable pathological changes, including compromised epidermal integrity, pronounced inflammatory infiltration, and tissue edema, suggestive of a fungal infection. The formalin-treated group (test control 1) (figure 6C) exhibited partial restoration of epidermal structure and diminished inflammation, indicating limited therapeutic efficacy. The optimized formulation (test control 2) (Figure 6D) showed significant histological improvements, with the epidermis and dermis closely resembling the healthy control, minimal inflammation, and restored tissue architecture, indicating superior antifungal efficacy and safety for topical

application. The standard control group (Figure 6E) achieved complete restoration of skin structure with minimal residual damage & the lowest inflammatory response, thereby validating its excellent efficacy. Histopathological findings indicated substantial disease-related skin changes in the negative group, moderate improvement with test control 1 & remarkable therapeutic efficacy with test control 2 and positive/standard control. The nanoemulgel formulation exhibited superior antifungal efficacy and improved skin healing relative to the conventional gel, although luliconazole remained the benchmark for optimal tissue recovery. Flucytosine, a recognized antifungal medication, is often associated with systemic side effects such as hepatotoxicity and bone marrow suppression due to elevated plasma levels. In contrast, topical treatment aims to minimize systemic exposure while providing targeted therapeutic effects. This study presents a nanoemulgel formulation designed to enhance skin retention and control drug release, thereby reducing systemic absorption while maintaining effective local concentrations at the site of infection. The formulation's local safety and acceptability are supported by observations that treated animals showed no erythema, edema, or histological abnormalities.

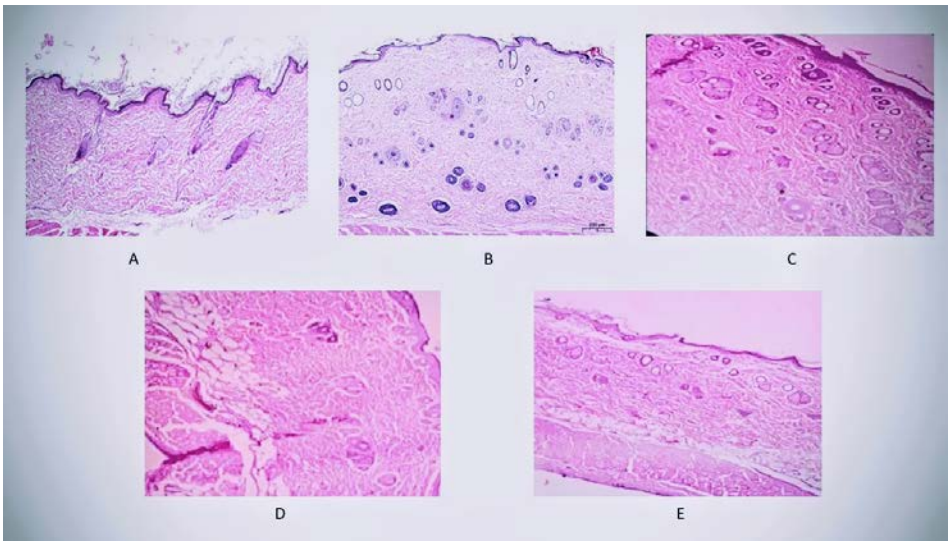


Figure 6: Representation of histopathological characteristics of the rat's skin in different groups. (A) Control Group (B) Negative Control (C) Test Control 1 (D) Test Control 2 (E) Positive Control [standard], ***Images captured at 10× magnification, stained using Hematoxylin and Eosin (H&E).***

Antifungal activity by zone inhibition study

Figure 7 depicts the antifungal efficacy of three formulations, Control Formulation, Marketed Formulation, and Flucytosine Nanoemulgel Formulation, against *Candida albicans*, as evidenced by their corresponding zones of inhibition on the culture media. The Flucytosine Nanoemulgel formulation exhibits the largest zone of inhibition, indicating enhanced antifungal activity. The improved impact may be attributed to greater drug solubility, increased skin penetration, and prolonged-release characteristics provided by nanoemulgel technology. Conversely, the Marketed Formulation exhibits a considerable zone of inhibition, whereas the Control Formulation shows the smallest zone, indicating relatively lower antifungal efficacy. These data demonstrate that nanoemulgel administration markedly enhances the bioavailability and therapeutic efficacy of flucytosine, providing a viable approach to the successful topical treatment of *Candida albicans* infections.

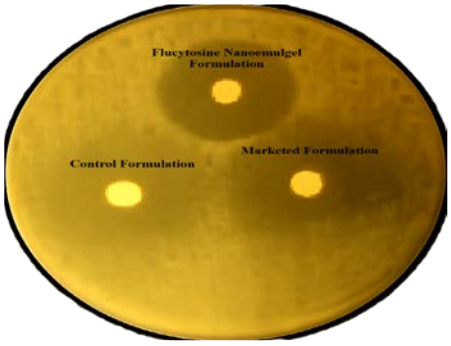


Figure 7: Antifungal activity of various formulations against ***Candida albicans*** **as demonstrated by the zone of inhibition.**

Stability Study

Each batch was assessed according to the evaluation criteria. Batch NEG5 underwent three-month stability testing after optimization, with the maximal release observed within 24 hours. Table 8 presents the results for the first, second, and third months. The Nanoemulgel formulation of flucytosine, which was exposed to 25°C/60% RH, 30°C/65% RH, and 40°C/75%

RH for three months, demonstrated good stability, as indicated by the stability study results. The rationale for selecting Flucytosine for topical treatment is its antifungal properties and systemic application, despite the scarcity of cutaneous studies. The integration into a nanoemulgel system improves solubility, stability, and skin permeability, overcoming challenges in topical distribution. This method seeks to provide localized, prolonged drug administration while reducing systemic exposure, which may lead to undesirable consequences such as hepatotoxicity and bone marrow suppression. In vivo investigations demonstrated favorable local tolerability, with no skin irritation. A clinical examination is necessary to verify therapeutic cutaneous concentrations and low systemic absorption.

Table 8: Stability studies results of optimized formulation (NEG5)

Stability Period	Physical Appearance	Phase Separation	pH Value	Spreadability	Drug Content
25 ± 2°C/ 60 ± 5% RH					
Initial	White smooth Cream	No	5.12	9.02	95.31
1st Month	White smooth Cream	No	5.15	9.12	94.12
2nd Month	White smooth Cream	No	5.41	9.61	94.78
3rd Month	White smooth Cream	No	6.01	10.01	96.21
30°C ± 2°C/65% ± 5% RH					
Initial	White smooth Cream	No	5.12	9.12	95.59
1st Month	White smooth Cream	No	5.18	9.27	96.32
2nd Month	White smooth Cream	No	5.68	9.52	96.78
3rd Month	White smooth Cream	No	6.32	10.32	97.21
40°C ± 2°C and 75% ± 5% RH					
Initial	White smooth Cream	No	5.12	9.12	95.78
1st Month	White smooth Cream	No	5.21	9.42	96.82
2nd Month	White smooth Cream	No	5.92	9.82	97.31
3rd Month	White smooth Cream	No	6.58	10.17	96.45

CONCLUSION

The development and thorough assessment of the flucytosine-loaded nanoemulgel, NEG5, exhibited significant progress in the topical management of fungal dermal infections. The optimized formulation (NEG5) demonstrated favourable physicochemical properties, including suitable pH, high drug content, appropriate viscosity, good spreadability, and prolonged drug release with first-order release kinetics. *In vivo* investigations in Wistar rats demonstrated the formulation's safety, as no erythema or edema was observed in skin irritation assays. Studies on antifungal efficacy demonstrated that the flucytosine nanoemulgel exhibited markedly superior therapeutic efficacy compared with traditional flucytosine gel and was comparable to the commercially available luliconazole cream, as indicated by enhanced lesion healing, improved histopathological recovery, and a wider zone of inhibition against *Candida albicans*. Furthermore, stability experiments confirmed that the enhanced formulation retained its integrity, drug content, and efficacy across diverse storage conditions for three months. Nonetheless, the constraints of this investigation include a relatively brief stability-testing duration and an antifungal evaluation restricted to *Candida albicans*. Comprehensive strain testing and prolonged stability investigations are necessary to assess long-term therapeutic efficacy thoroughly. These findings collectively indicate that integrating flucytosine into a nanoemulgel delivery system can overcome the limitations of traditional topical treatments by improving drug solubility, penetration, and retention in skin tissues, while reducing systemic exposure. This novel method underscores the therapeutic promise of flucytosine nanoemulgel as a viable option for the safe, effective, and patient-centric treatment of superficial fungal infections. Nonetheless, more clinical studies are needed to validate its effectiveness and safety in human participants. With the increasing incidence of cutaneous fungal infections and the emergence of resistance to traditional antifungal treatments, this nanoemulgel formulation is a timely and promising development in topical antifungal drug delivery that could significantly improve patient outcomes and adherence.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

The study was conceptualized & designed by Rajat Srivastava, who also performed the experimental procedures, gathered & analysed the data, & drafted the initial version of the manuscript. AKS Rawat provided support in developing the methodology, oversaw the project's progress, contributed to data interpretation, & thoroughly revised the manuscript for critical intellectual content. All authors have reviewed & approved the final manuscript & take full responsibility for the integrity & accuracy of the work.

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