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Formulation and Characterization of Nanoemulsion Containing Terbinafine HCl against Candida albicans Fungal Infections

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Abstract

Background: Transdermal drug delivery of antifungal drugs has always remained very challenging due to poor penetration and retainability of the formulation at the site of infection. Objective: The main objective was to enhance the permeation and bioavailability of Terbinafine HCl-loaded nanoemulsion for better penetration potential and efficacy in treating superficial infections. Method: A Terbinafine HCl-loaded nanoemulsion was formulated using the high-speed homogenization technique and subsequently evaluated through UV spectrophotometric analysis for drug solubility, FTIR spectroscopy for drug compatibility and content confirmation, and HPLC analysis for characterization of the optimized formulation. Furthermore, formulation variables were optimized using Design-Expert software by applying a Central Composite Rotatable Design (CCRD). The zeta sizer measured the particle size, and the heterogeneity index was assessed, while the Malvern Zetasizer was used for the zeta potential assessment. Droplet size was assessed via SEM, and in vitro penetration was studied via the Franz diffusion cell. For in vivo and histopathological examination, albino rabbits were used. Results: The formulation prepared was subjected to various characterization parameters. The average drug content was found to be 98.57%, and the average particle size of the optimized formulation was 57.81 nm with good homogeneity as indicated by a low Heterogeneity index, i.e., 0.08. In vivo and microbiological studies showed that the Terbinafine HCl nanoemulsion significantly reduced the Candida albicans infection in animals; moreover, the microbiological assessment also showed decreased growth of Candida albicans. Conclusion: The present study presents a novel nanoemulsion of Terbinafine HCl, which could be further explored in human subjects for a better understanding of its efficacy and the toxicity effects on human health.

Keywords: Terbinafine HCl-loaded Nanoemulsion, Skin Yeast Infections, Candida Albicans, Heterogeneity.

1. Introduction

The skin, the largest and outermost organ of the human body, functions as a highly specialized protective interface that safeguards underlying tissues from external insults including mechanical trauma, microbial invasion, ultraviolet

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(UV) radiation, and various environmental stressors. Structurally, the skin is composed of three distinct layers: the epidermis, dermis, and hypodermis. Among these, the stratum corneum, the outermost region of the epidermis, constitutes the principal barrier to percutaneous drug permeation. This layer comprises densely packed, keratinized corneocytes embedded within an organized extracellular lipid matrix referred to as lipid lamellae, which collectively restrict the penetration of exogenous substances (Iman, Nadia, & Ebtsam, 2010; Nastiti et al., 2017; Nikam et al., 2018). Transdermal permeation of therapeutic agents occurs predominantly through three pathways: intercellular diffusion across the lipid domains, intracellular (transcellular) transport through corneocytes, and appendageal transport via hair follicles and sweat glands. Advanced drug delivery strategies have been extensively explored, among which transdermal drug delivery systems have emerged as a promising alternative owing to their ability to enhance therapeutic efficacy, improve bioavailability, provide sustained drug release, and facilitate patient compliance while circumventing first-pass hepatic metabolism (Ng, Lau, & effects, 2015; Barakat, Fouad, & Elmedany, 2011) (Figure 1).

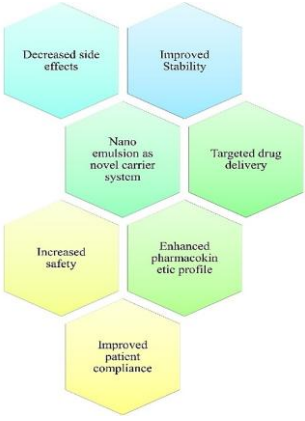

Figure 1. Nanoemulsion as a novel drug carrier

Nanoemulsions are advanced colloidal drug delivery systems composed of two immiscible phases, oil and water, stabilized by optimized concentrations of surfactants and co-surfactants, with droplet sizes typically ranging between 20 and 200 nm. Owing to their enhanced drug solubilization capacity, superior thermodynamic stability, ease of large-scale manufacturing, and remarkable kinetic stability, nanoemulsions have emerged as highly promising carriers for controlled and targeted drug delivery applications. Compared with conventional emulsions, nanoemulsions exhibit several advantageous properties, including improved drug encapsulation efficiency, enhanced dispersibility, greater physicochemical stability, absence of flocculation, prolonged drug release, and increased transdermal permeation (Abolmaali et al., 2011; Shah, Bhalodia, & Shelat, 2010; Suyal & Bhatt, 2017). Terbinafine hydrochloride (TBH), a synthetic allylamine derivative, is a broad-spectrum antifungal agent extensively utilized in the treatment of superficial fungal infections such as tinea corporis, tinea pedis, and tinea cruris. Its antifungal activity is primarily attributed to the selective inhibition of squalene epoxidase, disrupting fungal cell membrane integrity. Despite its potent antifungal efficacy, TBH exhibits poor aqueous solubility, which limits its therapeutic performance. Moreover, oral administration of TBH is associated with approximately 40% drug loss due to extensive first-pass hepatic metabolism, in addition to dose-related systemic adverse effects. Incorporation of TBH into nanoemulsion systems offers a promising strategy to overcome these limitations by enhancing drug solubility, improving physicochemical stability, and promoting efficient transdermal permeation (Elmataeeshy, Sokar, Bahey-El-Din, & Shaker, 2018) (Figure 2).

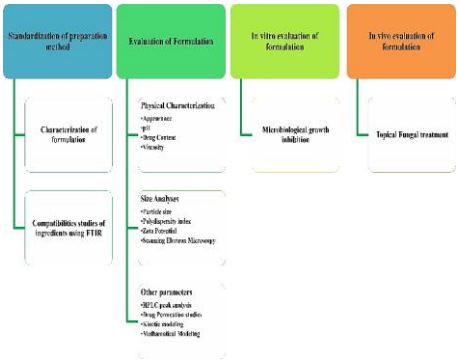

Figure 2. Method of preparation and characterization parameters of Terbinafine HCl nanoemulsion

In the early phase of formulation development, compatibility study of both active ingredient and excipients is essential. Excipients are primarily used to facilitate the administration of a drug substance and enhance the stability of the formulation. They can also improve the drug's taste or appearance and aid in controlling the release or absorption of the active ingredient. In the oil phase, the drug was dissolved using a vortex mixer, then in a separate beaker surfactant was added to water and was homogenized; afterwards, the oily mixture was added to the water phase slowly and mixed at the speed of 15,000 rpm initially, then gradually raised to 20,000 rpm to make Terbinafine HCl nanoemulsion.

2. Materials and Methods

Active drug Terbinafine HCl (Willshire Laboratories, Pakistan), Eucalyptus oil (Flavchem royal, China), Tween 80 (SIGMA-ALDRICH, Germany), Ethanol (Merck KGaA Darmstadt, Germany), and distilled water. Analytical grade chemicals were used for the study.

2.1 Physicochemical Characterization

2.1.1 Suitable Oil Selection

To determine the solubility of Terbinafine HCl in different oils, an excess amount of the drug was introduced into 10 mL of each selected oil, then the mixtures were thoroughly agitated using a vortex mixer and subsequently maintained in an isothermal shaker at 25 ± 1 °C until equilibrium was attained.

2.1.2 Selection of Surfactant

The choice of a suitable surfactant is a key factor in the development of nanoemulsion systems and is largely determined by the hydrophilic-lipophilic balance (HLB) value. In the case of oil-in-water nanoemulsions, surfactants with higher HLB values and pronounced hydrophilic properties are generally preferred. Accordingly, based on an HLB requirement of 15, the non-ionic surfactant Tween 80 was selected for formulation development (Azeem et al., 2009).

2.2 Solubility

The solubility of Terbinafine HCl in water (3 mg/mL) is relatively low compared to organic solvents. In contrast, its estimated solubility in ethanol is 45 mg/mL. Therefore, ethanol is used as a suitable solvent (Shi & Xie, 2017).

2.3 Compatibility Studies

In the initial phase of formulation development, evaluating the compatibility between the active pharmaceutical ingredient and selected excipients is crucial. Although excipients are incorporated to facilitate drug delivery and improve the stability of the formulation, they can sometimes interact with the drug substance, leading to instability, chemical degradation, or altered therapeutic performance. Hence, achieving a safe, effective, and stable formulation depends on the judicious selection of appropriate excipients, which is confirmed through comprehensive compatibility studies (Rojek & Wesolowski, 2019).

2.4 Fourier Transform Infrared (FTIR) Spectroscopy

A non-destructive technique of analysis, namely FTIR spectroscopy, is used to identify the specific chemical characteristics of a substance. It is commonly applied to analyze drugs either alone or in combination with excipients.

Each liquid or powdered sample is placed on the sample port for detection. Liquid material can be analyzed simply by placing it on the sample port, while for powdered material, the material on the sample port is pressed with the tip of the analyzer, characterizing it against an FTIR library, producing a spectrum of the sample (Franca & Oliveira, 2011).

2.5 UV Spectrophotometric Analysis

A standard stock solution of Terbinafine HCl was prepared by accurately dissolving 10 mg of the drug in 10 mL of ethanol, yielding a primary concentration of 1 mg/mL. An aliquot of 1 mL from this solution was diluted to 10 mL with phosphate buffer (pH 6.8), producing a working solution of 0.1 mg/mL (100 µg/mL). Subsequently, 1 mL of this working solution was diluted up to 10 mL using the same phosphate buffer, resulting in a final diluted stock concentration of 0.01 mg/mL (10 µg/mL).

2.6 Calibration Curve

From the stock solution, 2, 4, 6, 8, and 10 mL aliquots were accurately measured into separate test tubes and individually diluted to a final volume of 10 mL with phosphate buffer (pH 6.8), resulting in solutions with concentrations of 2, 4, 6, 8, and 10 µg/mL, respectively. The absorbance of each prepared dilution was recorded at 283 nm, then a calibration curve was constructed by plotting absorbance against concentration, and the slope, intercept, and correlation coefficient (r^2) were obtained through linear regression analysis.

2.7 HPLC Analysis

High-performance liquid chromatography (HPLC) was applied for both identification and quantification of the analyte. The standard and test samples were prepared at an equal concentration of 2 mg/mL, and 10 µL of each solution was injected into the chromatographic system for analysis. Detection was performed at 225 nm. The mobile phase consisted of acetonitrile, methanol, and phosphate buffer in the proportion of 40:30:30 (v/v/v), with the buffer pH adjusted to 3.5 using orthophosphoric acid. Before use, the mobile phase was passed through a 0.45 µm membrane filter and degassed to ensure system suitability, then separation was carried out on a 25 cm L1 column with an internal diameter of 4.5 mm, operating at a flow rate of 1.0 mL/min, while UV detection was maintained at 225 nm. Retention time is used for identification, while the area of the peak is used for quantitative analysis. Assay values or the percentage quantity of the sample can be determined by:

$$\% \, age = Ru/Rs \times Wst/Dst \times Ds/Ws \times potency$$

Where, Ru = Average area of standard, Rs = Average area of sample, Wst = weight of standard, Dst = density of standard, Ds = density of sample, and Ws = weight of sample.

2.8 Method of Formulating TBH Nanoemulsion

To design and optimize the formulation of nanoemulsion containing Terbinafine HCl, Design Expert software using Central Composite Rotatable Design (CCRD) was used. By varying the composition of oil and surfactant, trial formulations were designed. Nanoemulsions using various compositions were prepared using high-speed homogenization. For the optimized formulation, eucalyptus oil, ethanol, and drug (Terbinafine HCl) were added to a glass test tube and placed on a vortex mixer (VELP Scientifica, Italy) for 2–4 min in order to dissolve the drug. In a separate vessel, Tween 80 was dissolved in distilled water to constitute the aqueous phase, followed by homogenization using a high-speed homogenizer (Heidolph Silent Crusher M, Germany). The oil phase was then incorporated slowly in a dropwise manner into the continuously agitated aqueous phase. Homogenization

was initiated at 5,000 rpm and progressively increased to 20,000 rpm, continuing for ten to fifteen minutes to ensure uniform emulsification. The resulting nanoemulsion was subsequently subjected to a range of physical and chemical characterization studies (Karri et al., 2015).

3. Experimental

3.1 Characterization of TBH Nanoemulsion

3.1.1 Appearance

Nanoemulsions containing very fine droplets are transparent and more homogeneous. Turbidity may increase with the increase in droplet concentration.

3.1.2 Particle Size Analysis

Nanoemulsion containing Terbinafine HCl was prepared and analyzed for droplet size using a ZetaSizer (Malvern ZS90). Size was determined at 25°C after mixing 0.1 mL of the formulation in 10 mL of distilled water (Elsheikh, Elnaggar, Gohar, & Abdallah, 2012).

3.1.3 Polydispersity Index

The polydispersity index (PDI) of the prepared optimized formulation indicates a homogeneous system. PDI (size distribution) was established by Zetasizer (Malvern, ZEN90), dispersing 1 mL of the formulation in 100 mL of distilled water. Results should lie within the desired range of PDI; ideally, PDI should be less than 0.7.

3.1.4 Zeta Potential

Zeta potential is a key physicochemical parameter used in nanoparticle characterization because it reflects the electrical charge present at the particle surface and serves as an indicator of colloidal stability. In this study, it was determined using a Malvern Zetasizer ZEN3600. Higher absolute zeta potential values indicate greater electrostatic repulsion between particles and, thus, improved formulation stability.

3.1.5 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a powerful analytical technique used for ultrastructural evaluation because it provides high-resolution, three-dimensional visualization of the surface morphology of both dry and hydrated samples. In this study, SEM (JSM5600LV, JEOL, Japan) was utilized to confirm the nano-sized droplets of the formulation.

3.1.6 pH Measurement

In the literature, variable skin pH values are recorded, all in the slightly acidic range of 4.5–6.5. pH values within this range are considered safe to be applied topically; thus, the formulation pH was then determined (Schott Gerate, CG-820, Germany).

3.1.7 Viscosity

The viscosity of the nanoemulsion formulation was assessed during the consistency study using a Brookfield viscometer maintained at 25 °C, with measurements taken using spindle 1 and spindle 2 at speeds of 30 rpm and 60 rpm, respectively (Qian, Cui, Wang, Wang, & Zhou, 2011).

3.1.8 Drug Content

The amount of drug present in each formulation was determined by UV-visible spectrophotometry through measurement of absorbance at 283 nm, followed by calculation of the concentration using the standard formula:

$$\% \text{ Drug content} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$$

3.2 Drug Permeation Studies

The permeation behavior of the formulation was evaluated using a Franz diffusion cell to determine its drug permeation rate. A cellulose acetate artificial membrane with an approximate pore size of 0.45 µm (Millipore) was used as the diffusion barrier, following established in vitro permeation protocols. The study involved recording and plotting the cumulative amount of terbinafine hydrochloride that permeated through the

membrane over time to compare the release profiles of different nanoemulsion formulations. The optimized nanoemulsion was anticipated to release more than 60% of the drug within the initial 5 hours; furthermore, cumulative drug permeation was calculated for the optimized formulation and expressed as a function of time.

3.3 Kinetic Modelling of Permeation Data

Mathematical modelling contributes to the determination of drug release during formulation development. Nanoemulsions are liquid topical preparations, and within the first 5 hours, 60% of the drug must permeate through the membrane.

3.4 In Vivo Studies of Prepared Formulation

The formulated product in vivo assessment was conducted in accordance with established national ethical and regulatory guidelines for the care and use of laboratory animals, after obtaining ethical approval for the study from the Ethical Committee of the Faculty of Health and Life Sciences, Al Aleem Medical College. Albino rabbits (800–950 g) were randomly assigned to three groups and housed under standard laboratory conditions, including a light/dark cycle of 12 hr, with a temperature controlled at 25 ± 2 °C.

3.4.1 Test Organism and Preparation of Fungal Inoculums

Candida albicans culture was obtained from the Department of Pathology, Al Aleem Medical College. The parent strain was subsequently subcultured in 3% Sabouraud dextrose broth and incubated at 28 °C for three to five days to ensure ideal growth and viability prior to its use in the experiment.

3.4.2 Induction of Cutaneous Candida Infection

All animals were rendered immunosuppressed via intraperitoneal injections of cyclophosphamide at a dose of 33.5 mg/kg body weight, administered in three separate doses on days 5, 3, and 1 before infection induction. The hair on a 4 cm^2 area of the rabbit skin was carefully shaved, after which the site was disinfected using ethyl alcohol. A cotton swab impregnated with 0.1 mL of fungal suspension was then placed onto the prepared skin surface, covered with adhesive tape, and left in place for 3 days to facilitate infection establishment. The successful induction of fungal infection was verified by the development of erythema and visible inflammation at the application site. Animals were allocated into three groups: Group I acted as the untreated control, Group II received a commercially available antifungal cream as the standard reference treatment, and Group III was administered the TBH nanoemulsion formulation. All treatments were applied topically once daily at an equivalent dose of 2 mg of drug per day for five consecutive days (AbdelSamie, Kamel, Sammour, & Ibrahim, 2016).

3.4.3 Ethical Considerations in the Use of Laboratory Animals

All the animal studies were conducted at Al Aleem Medical College. The ethics committee of the institute approved the animal experiment design (MPCH23422).

3.5 Statistics

Data were expressed as mean ± standard error of the mean. Differences between two groups were analyzed using a two-tailed Student's t-test, whereas comparisons among three or more groups were assessed using two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical significance was defined as a p-value <0.05.

4. Results

4.1 Compatibility Study using Fourier Transformed Infrared Spectroscopy (FTIR)

4.1.1 FTIR Spectrum of Terbinafine Hydrochloride

The major peaks of pure Terbinafine hydrochloride present in Figure 3A were well supported by functional groups present in the spectrum available in the literature.

4.1.2 FTIR Spectrum of Eucalyptus Oil

In 2014, Sudhakar and co-workers analyzed the FTIR spectrum of eucalyptus oil to evaluate its purity and reported characteristic peaks similar to those presented in Figure 3B. Likewise, Pant et al. (2014) investigated the FTIR spectrum of eucalyptus oil and identified absorption peaks comparable to those observed in the present study.

4.1.3 FTIR Spectrum of Tween 80

In 2009, Zheng and co-workers prepared a nanoemulsion using Tween 80 as the surfactant and performed FTIR analysis to evaluate its purity, reporting spectral bands similar to those shown in Figure 3C. A strong absorption band observed at 1121.58 cm^{-1} was attributed to C-O stretching vibrations of secondary alcohols.

4.1.4 FTIR Spectrum of Formulation

Drug-excipient interaction was investigated by FTIR analysis. Kuminek et al. (2013) studied the pure Terbinafine HCl spectrum, which showed the same wide peak alkane C-H stretching bands from 2966.89 cm^{-1} to 3305.43 cm^{-1} as present in Figure 3D, indicating the presence of pure TBH in the formulation (Kuminek et al., 2013).

4.2 Physicochemical Evaluation of Formulation

4.2.1 Appearance

Nanoemulsions composed of extremely fine droplets tend to exhibit a transparent appearance, although increasing droplet concentration may lead to a gradual rise in turbidity. Nanoemulsions prepared in this study are shown in Figure 3E.

4.2.2 pH

The negative logarithm (base ten) is defined as pH or the free hydrogen ion concentration in an aqueous solution. As per earlier published literature, variable skin pH values have been recorded, suggesting all variations within the slightly acidic range of pH 4.5–6.5. Table 1 shows pH values of various formulations that agree with those reported in the literature for skin preparations.

Table 1. pH values of different formulations

Formulations	pH values	Formulations	pH values
F1	5.23	F9	5.85
F2	5.65	F10	5.49
F3	5.38	F11	5.56
F4	5.29	F12	5.84
F5	5.26	F13	5.50
F6	5.92	F14	5.63
F7	5.48	F15	5.24
F8	5.13	F16	5.47
OF	5.68		

4.2.3 Drug Content

Drug content of each formulation (Table 2) was interpreted after measuring absorbance at 283 nm, by using the formula:

% Drug content = Absorbance of sample / Absorbance of standard × 100

Table 2. Drug content of various formulations at 283 nm

Formulations	Drug content	Formulations	Drug content
F1	108.32	F9	104.33
F2	104.91	F10	102.59
F3	104.73	F11	105.01
F4	86.71	F12	109.47
F5	105.21	F13	109.97
F6	106.96	F14	106.56
F7	109.30	F15	101.04
F8	102.10	F16	105.69

OF	100.29

4.2.4 Viscosity

Consistency study for Terbinafine HCl nanoemulsion was plotted as graphs (Figure 3F and 3G) that showed viscosity measurements using spindles 1 and 2 at 30 rpm and 60 rpm, respectively. The average viscosity of nanoemulsion with different compositions came out to be 13.2 mPaS to 51.4 mPaS. The terbinafine HCl-loaded nanoemulsion was evaluated using a Brookfield viscometer with temp. set at 25 °C, and the viscosity of the optimized formulation was 17.8–58.7 mPa·s, which is consistent with the results reported in previous studies (Qian et al., 2011).

4.2.5 Size Analysis Results

Nanoemulsion containing Terbinafine HCl was prepared and analyzed for particle size using a ZetaSizer. The results of the optimized formulation showed an average diameter of 57.81 nm, which was comparable with the range stated in the literature. The heterogeneity index of the prepared optimized formulation (Figure 3H) was 0.080, indicating a homogeneous system. The optimized formulation (Figure 3I) showed a zeta potential of −59.1 mV, indicating enough electro-repulsive forces to prevent instability.

4.2.6 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a powerful analytical technique for ultrastructural analysis. Figure 3J shows particle size distribution within the nanoparticle range. The SEM results of the nanoemulsion showed droplet size in the nanometer range, with no evidence of burst release, indicating efficient drug entrapment within the emulsion droplets.

4.2.7 HPLC Analysis

An injection volume of approximately 10 µL was administered for both standard and sample solutions, and detector responses for the main peaks were monitored at a wavelength of 225 nm. The mobile phase comprised a mixture of acetonitrile, methanol, and phosphate buffer. Both standard and sample preparations were formulated at an equal concentration of 0.2 mg/mL. The reference standard of Terbinafine HCl exhibited a retention time of 21.729 minutes with a corresponding peak area of 14,029,592.6 at 225 nm, as presented in Figure 3K. The retention time of sample preparation was 21.736 with the peak area of 13,841,864.5 at 225 nm, as shown in Figure 3L. The results of both the reference standard and sample preparation were comparable and calculated as:

% age = 13841864.5/14029592.6 × 0.2/0.9873 × 0.983/50.5 × 100 = 98.57%

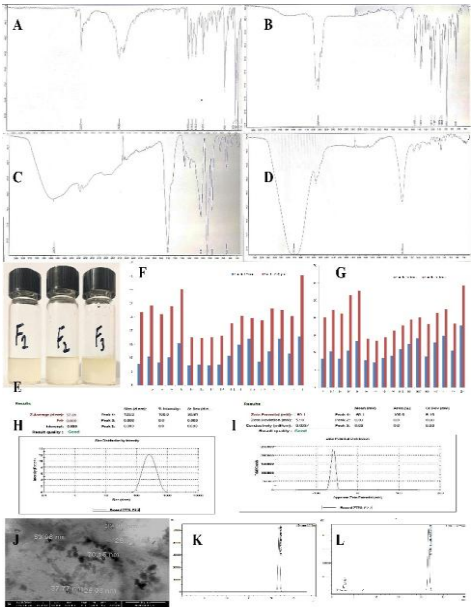

Figure 3. Compatibility study using FTIR and characterization of formulation**.** A, FTIR of Terbinafine HCl; B, FTIR of Eucalyptus oil; C, FTIR of Tween 80; D, FTIR of Formulation; E, Nanoemulsion appearance; F–G, Viscosity graphs; H, Heterogeneity index; I, Zeta potential; J, SEM; K–L, HPLC chromatograms of standard and sample.

Table 3. Permeation study of Terbinafine HCl nanoemulsion

Parameters	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
k0	0.26	0.26	0.15	0.30	0.45	0.13	0.39	0.13	0.24	0.30	0.19	0.301	0.28	0.26	0.38	0.35
Rsqr	0.76	0.89	0.89	0.94	0.84	0.81	0.73	0.84	0.62	0.10	0.51	0.82	0.88	0.56	-0.40	-0.49
k1	0.006	0.006	0.002	0.007	0.01	0.001	0.02	0.01	0.006	0.02	0.003	0.009	0.005	0.008	0.07	0.07
Rsqr	0.90	0.87	0.941	0.80	0.55	0.89	0.53	0.89	0.92	0.72	0.73	0.95	0.68	0.94	0.56	0.606
kH	4.86	4.85	2.84	5.44	8.42	2.53	7.21	2.51	4.56	5.82	3.69	5.56	5.006	4.93	7.29	6.86
Rsqr	0.98	0.93	0.96	0.91	0.96	0.97	0.90	0.96	0.97	0.80	0.92	0.98	0.76	0.95	0.58	0.52
kKP	5.62	2.19	1.57	1.06	5.57	2.23	8.08	1.98	7.17	19.4	7.77	4.6	0.056	8.61	33.7	33.5
Rsqr	0.98	0.95	0.97	0.90	0.96	0.97	0.90	0.96	0.98	0.96	0.97	0.98	0.89	0.98	0.94	0.93

4.3 Drug Permeation Studies

The skin permeation study was carried out using a Franz diffusion cell system. The cumulative quantity of Terbinafine HCl that passed through the diffusion membrane was plotted against time (Figure 4). The optimized nanoemulsion formulation was expected to deliver more than 60% of the drug across the membrane within the first 5 hours. The permeation curve demonstrated a steady, time-dependent rise in drug diffusion with increasing time.

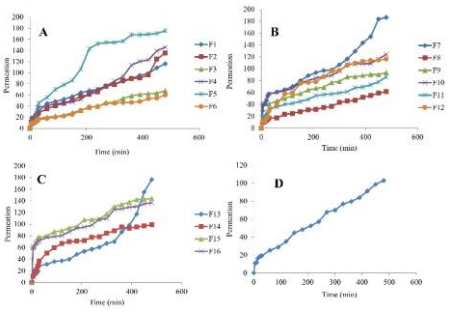

Figure 4. Drug permeation studies (µg/cm²/h) using a Franz diffusion cell**.** A, B, C, and D: Permeation profiles versus time plots showed an increase in drug permeation with increased time.

4.3.1 Kinetic Modeling of Permeation Data

Mathematical modeling contributes to the determination of the drug release from the formulation. Nanoemulsions are liquid topical preparations, and within the first 5 hours 60% of the drug must permeate through the membrane; the kinetic models and the drug content permeation are presented in Table 3. It is pertinent to note that there were 16 formulations that were tested through kinetic modeling.

The release kinetics of Terbinafine HCl from the nanoemulsion showed the best fit with the Korsmeyer Peppas model, exhibiting strong linear correlation and indicating a uniform and controlled drug release behavior.

4.3.2 Mathematical Modeling

Mathematical modeling generated by a design expert for the calculated response and variables:

$$DP = A_0 + AX_1 + BX_2 - CX_3 + ABX_1X_2 + ACX_2X_3 + BCX_1X_3 + A^2X_1^2 + B^2X_2^2 + C^2X_3^2 \quad ....Eq.\ 4.1$$

$$DP = 94.28 + 1.84X_1 + 2.89X_2 - 6.42X_3 + 3.07X_1X_2 + 1.07X_2X_3 + 1.02X_1X_3 + 3.14X_1^2 + 4.04X_2^2 + 0.84X_3^2 \quad ....Eq.\ 4.2$$

Where DP is the response function (Drug permeation), A_0 is a constant; coefficients in Eq. 4.1 are linear (A, B, and C), interaction (AB, AC, and BC), and quadratic (A^2, B^2, and C^2) coefficients, respectively. Response surface analysis of drug permeation data, considering two variables to be compared, showed enhanced permeation of the drug.

4.4 2D and 3D Contour Plots

The contour plots and 3-D response surface plots (Figure 5) demonstrated that both oil and surfactant concentrations positively influenced drug permeation. However, the effect of surfactant concentration on permeation was more significant than that of the oil phase. Surfactants possessing intermediate

hydrophilic-lipophilic balance (HLB) values were found to decrease particle size, which in turn enhanced drug permeation through the skin barrier. Stratum corneum consists of lipophilic membranes; thus, with an increase in solvent, permeation decreases. On the contrary, increased surfactant concentration leads to increased permeation.

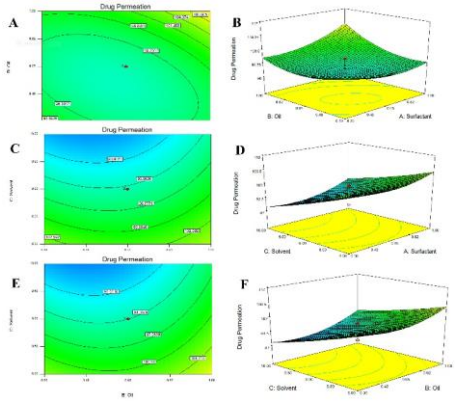

Figure 5. Mathematical modeling was created by a design expert, using 2D contour plots and 3D response surface **plots.** A and B: The 2-D contour plots and 3-D response surface plots graphically illustrate the regression equation and demonstrate the influence of each independent variable on the response.

4.5 Histopathological and Micro-biological Studies

To evaluate the efficacy of the TBH nanoemulsion formulation, its anti-infective potential was further investigated through in vivo animal studies (Figure 6). All experimental groups developed infection before treatment. The untreated control group exhibited more pronounced infection compared to the established brand and TBH-treated groups (Figure 6C). Histopathological assessment was performed on tissue samples collected from the infected sites. The observations indicated improved tissue condition and reduced Candida albicans infection in both the positive control (marketed formulation) and TBH-treated groups compared to the untreated control group (Figure 6F).

Microbiological evaluation was carried out using colony observation and counting. The untreated group showed higher numbers and larger-sized colonies (thirteen small colonies with one large colony), whereas the established brand group demonstrated a reduction in colony number and size (seven small colonies with one large colony). In contrast, the TBH nanoemulsion group exhibited fewer and smaller colonies, with no large colonies observed (Figure 6G).

Overall, the results indicate that terbinafine is effective against Candida albicans, and its antifungal activity appears to be enhanced in the nanoemulsion formulation. Nevertheless, further studies incorporating quantitative CFU determination and appropriate statistical analysis are required to substantiate these findings.

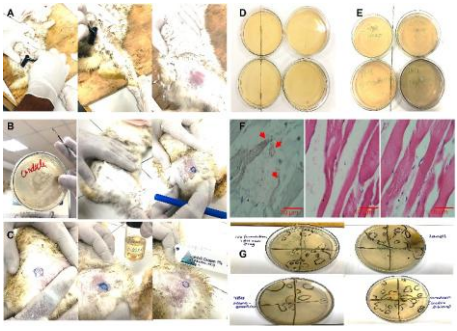

Figure 6. Terbinafine HCl nanoemulsion improves the Candida albicans skin infection in vivo. A: Immunocompromised conditions induced via Cyclophosphamide. B: Hair removal from dorsal skin. C: Untreated infection site. D–F: Histopathological sections. G: Microbiological colony assessment.

Discussion

A transparent TBH Nanoemulsion was prepared, which indicates that the developed formulation has greater stability and homogeneity. According to Zhang and McClements (2018), as nanoemulsions consist of very small droplets (20–200 nm), they are optically transparent products. In the present formulation, Tween 80 was employed as the surfactant.

Use of a surfactant with an intermediate HLB value leads to a clear nanoemulsion. Tween 80 ensured the formation of a clear and homogeneous nanoemulsion with a small droplet size. The prepared TBH nanoemulsion exhibited a PDI value of 0.08, representing a highly even particle size distribution. The pH of the developed TBH formulation was 5.68, which ranges within the normal physiological skin pH range of 4.1–6.5, indicating its suitability for topical application (Proksch, 2018; Das, Nayak, & Nanda, 2013).

Determination of percentage drug content is essential to ensure that each unit dose contains the required amount of active pharmaceutical ingredient for accurate delivery to the infection region (Amichai et al., 2010). The prepared terbinafine hydrochloride nanoemulsion exhibited a drug content of 100.29%, indicating that the formulation falls within acceptable limits. The viscosity of the developed nanoemulsion ranged from 17.8 mPaS to 58.7 mPaS, consistent with findings reported by Qian et al. (2011). The optimized formulation showed a 57.81 nm droplet size, confirming that the nanoemulsion falls within the nanoscale range (<200 nm) (Aqil, Kamran, Ahad, & Imam, 2016). The developed TBH nanoemulsion exhibited a zeta potential of −59.01 mV, indicating excellent stability; generally, zeta potential values >±30 mV are considered desirable as they generate sufficient electrostatic repulsive forces to prevent particle aggregation (Lowry et al., 2016).

Drug release from nanoemulsions is greatly influenced by their surface morphology. SEM analysis of the developed nanoemulsion revealed nanosized particles (<100 nm) with no evidence of burst release, indicating efficient drug entrapment within the emulsion droplets (Divsalar et al., 2012; Saupe, Gordon, & Rades, 2006). The permeation data of the Terbinafine HCl nanoemulsion were analyzed by fitting to multiple kinetic models, including 0-order, 1st-order, Higuchi, and Korsmeyer-Peppas models. The data revealed the Korsmeyer-Peppas model as the best-fitting model. For all data, the correlation coefficient (R^2) was >0.9, indicating good correlation with experimental data (Shah et al., 2010).

Response Surface Methodology (RSM) using a Central Composite Rotatable Design (CCRD) was applied to assess the combined influence of oil, surfactant, and water on drug permeation. The results demonstrated a reduction in drug permeation with increasing solvent concentration, while increased surfactant concentration led to increased permeation (Li & Chiang, 2012; Sood, Jain, & Gowthamarajan, 2014). In vivo studies confirmed the effectiveness of the TBH-loaded nanoemulsion when compared with both the control group and the marketed reference product. While the current study demonstrates the TBH nanoemulsion's effectiveness against fungal infection caused by Candida albicans, the findings also highlighted certain limitations, including the use of animal models that may not fully replicate human skin physiology, predominantly short-term efficacy evaluation, and absence of ex vivo skin permeation studies. Comprehensive long-term stability evaluation and quantitative microbiological analysis with statistical validation will be addressed in future work.

Conclusion

Conclusively, a comprehensive characterization of the Terbinafine HCl nanoemulsion was performed through the systematic evaluation of key physicochemical parameters, including drug content, viscosity, particle size, zeta potential, and surface morphology. The developed nanoemulsion demonstrated favorable physicochemical characteristics, such as high drug loading, optimal viscosity, nanoscale particle distribution, and an appropriate zeta potential, collectively indicating enhanced formulation stability and suitability for

transdermal drug delivery. Scanning electron microscopy confirmed the presence of nano-sized droplets without aggregation, indicating successful drug encapsulation. Permeation studies revealed efficient transdermal delivery of Terbinafine HCl through the nanoemulsion, supported by mathematical modeling showing significant effects of surfactant and aqueous phase on drug permeation. Comparative studies against established brands and controls, including histopathological and microbiological assessments, highlighted the efficacy of the Terbinafine HCl nanoemulsion. In conclusion, the developed Terbinafine HCl nanoemulsion offers a promising platform for topical drug delivery, exhibiting superior characteristics such as stability, permeation enhancement, and efficacy against fungal infections. Further studies may elucidate its clinical applicability and potential for commercialization.

. Ethics Approval and Consent to Participate

In vivo animal experiments were conducted at Al Aleem Medical College. The experimental protocol and study design received approval from the Institutional Ethics Committee under approval number MPH23422.

. Human and Animal Rights

All animal experiments were conducted at Al Aleem Medical College. The study protocol and experimental design were approved by the Institutional Ethics Committee in accordance with the NC3Rs ARRIVE guidelines. Furthermore, all experimental procedures were carried out in strict compliance with the principles described in the "Guide for the Care and Use of Laboratory Animals." No human experimentation was carried out in this study.

. Availability of Data and Material

The present study is original research, and all the relevant data will be made available upon request.

. Funding Declaration

No external funding was obtained for this study.

. Conflict of Interest

All the authors declare no conflict of interest.

. Acknowledgments and Authors' Contributions

Ayesha Rauf conceptualized and designed the study and conducted the experimental work. Nadeem Reyaz performed the histopathological analyses, served as the corresponding author, and prepared the initial manuscript draft. Jureerat Kijsomporn critically revised and corrected the draft, while Tooba Malik finalized the manuscript preparation. Ling Shing Wong reviewed and evaluated the final version of the manuscript. Aqsa Maimoona Malik also served as the corresponding author and contributed to the final editing and refinement of the manuscript. All authors accept responsibility for the integrity, accuracy, and authenticity of the work.

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