

## *In vitro* Antifungal Activity of a Novel Allylamine Antifungal Nanoemulsion Gel

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### Abstract

Terbinafine HCl (TBH) is a broad spectrum antifungal agent used against dimorphic fungi, yeast, molds, dematiaceous fungi and dermatophytes. However, the poor solubility of this potential molecule renders its efficacy limited. So, the combinations of oral and topical doses are prescribed. Hence, the aim of the current study was to increase the solubility of TBH by incorporating into Nanoemulsion gel (NEG) and to investigate its *in vitro* antifungal activity using *Candida albicans* as a fungal inoculum. The TBH nanoemulsion was prepared using high speed homogenization technique and evaluated for its micromeritics. The particles results revealed that acceptable globule size of 11.47 nm with a PDI of 0.556. The obtained nanoemulsion was converted into NEG and evaluated for *in vitro* permeation and *in vitro* antifungal activity. The permeation studies revealed that NEG (76.23 ± 2.81%) has higher permeation, when compared to marketed cream (MC 29.37 ± 1.82%). *In vitro* Antifungal studies showed that the prepared NEG (0.66 ± 0.17 mm) has high zone of inhibition (p<0.01) almost to that of pure drug solution (0.91 ± 0.07 mm) compared to MC (0.13 ± 0.05 mm). Hence, the formulated NEG of TBH may be best suitable for delivery of poorly soluble drugs like TBH, which are intended for topical antifungal application.

**Keywords:** Nanoemulsion; Antifungal activity; Allylamine; Terbinafine HCl

### Introduction

Terbinafine HCl (TBH) is an orally administered, topically viable allylamine antifungal agent [1]. TBH possesses broad spectrum *in vitro* potential against dimorphic fungi, yeast, molds, dematiaceous fungi and dermatophytes. TBH is active *in vitro* against a wide range of dermatophytes, dimorphic fungi, molds and yeasts. However, previous studies suggest that the TBH had little activity against *Candida albicans* (*C. albicans*) [2]. *Candida species* are considered as the most important fungal human pathogens, causing a variety of infections, ranging from superficial, cutaneous-mucosal to deep-seated infections [3]. *Candidiasis* or yeast infection is the most common skin infection caused by the *C. albicans*. Among the various types of *candidiasis*, cutaneous *candidiasis* skin fungal infections will most often reoccur and is rarely cured [4]. TBH is the most successful compound in the category of antifungals, when compared to other antifungals *viz.*, butenafine, ketoconazole and itraconazole etc. [5]. However, the poor solubility of this potential molecule renders its efficacy limited [6]. Hence, the combinations of oral and topical doses are prescribed. The development of nanoparticles (1-100 nm) for topical application has been emerging in the past decade and offer potential applications in topical drug delivery [7]. These systems increase the drug permeability of poorly soluble drugs and provide enhanced drug availability at the targeted site. They also create a drug depot at the site of action [8,9]. The controlled release properties of nanoparticles facilitate pulsatile release of drug molecules minimizing the frequent and total number of dosages, thereby offering increased patient compliance. Potent bactericidal activity against gram positive and negative bacteria has

been seen in nanoparticles encompassing antibiotics [10,11], which can be extrapolated for possible antifungal efficacy too. Silver nanoparticles, an example of antimicrobial NPs have been recently developed [12,13], to counteract the constantly evolving bacterial resistance to regular antibiotics. Another advantage of using NPs as a carrier for antifungal and antibiotic drugs lies in the fact that NPs exhibit bactericidal action at low concentrations (units of mgL<sup>-1</sup>) [14], thereby reducing the toxic burden of these drugs on human cells. In the present study, an attempt was made to prepare a nano particulated drug delivery system for TBH and its antifungal efficacy was evaluated by *in vitro* microbial assay using *C.albicans* fungal inoculum.

### Materials and Methods

TBH was purchased from Hetero labs, Hyderabad, India. Liquid paraffin was purchased from Unicorn (Chennai, India). Polysorbate 80 was purchased from N.O.F Corporation (Mumbai, India). Milli Q water was prepared by Milli-Q plus 185 purification system (Bangalore, India), which was used throughout the study. Marketed cream (MC) containing 1% of TBH dispersed in cream base was used as standard for all the studies.

### Development of calibration curve by HPLC

Calibration curve of TBH was performed by Liquid chromatographic analysis as previously described [15]. Briefly, HPLC system (Shimadzu, Japan) comprising LC-20AD solvent pump and a SPD-M20A PDA detector were used in this study. The LC solutions (ver. 1.11) software was used for data analysis and processing. Chromatographic separation was performed on Luna C<sub>18</sub> (Phenomenex, California, USA) (250×4.6 mm) column using a mobile

phase comprising 25 mM potassium dihydrogen orthophosphate buffer at pH 4.0 adjusted with orthophosphoric acid and methanol in the ratio of 20:80 at 222 nm. The mobile phase was delivered at 1 mL min<sup>-1</sup>.

### Preparation of nanoemulsion gel

TBH nanoemulsion was prepared by the high speed homogenization method (PT-3100D, Kinematica, Schweiz) reported elsewhere [14]. The nanoemulsion contains two phases i.e., aqueous phase and oily phase. Sodium acetate, disodium edate, glycerin and

polysorbate 80 were dissolved in purified water (continuous phase). TBH was dispersed in liquid paraffin (dispersed phase). The dispersed phase was added to continuous phase by high speed homogenization. The rpm was maintained in the range of 4000-5000 for 1 h. After homogenization, the resulting nanoemulsion was incorporated into carbomer 970 gel base (1.5%). The carbomer 970 gel base was prepared in advance by addition of 1.5 g of carbomer 970 into water with continuous stirring. Subsequently, the pH of prepared nanoemulsion gel (NEG) of TBH was adjusted to 5.5-6.0 using 2 N NaOH. The detailed composition of NEG is described in Table 1.

Formula	Category/Role	Concentration range (%Wt/Wt)
TFH	Active Pharmaceutical Ingredient	1%
Liquid paraffin	Carrier	5%
Polysorbate 80	o/w Emulsifier	2%
Glycerin	Humectant	1%
Sodium acetate	Buffering agent	0.20%
Edetate disodium	Anti-Oxidant	0.02%
Benzyl alcohol	Preservative	2%
TFH	Active Pharmaceutical Ingredient	1%

**Table 1:** Composition of TBH-NEG formulation.

### Drug content determination

Each one gram of NEG was dissolved in methanol. The solution was sonicated for 2 h using Ultrasonicator (Equitron, Mumbai, India). The resulting suspension was centrifuged at 25,000 rpm (Model-R8C, Remi-Centrifuge, India) for 1 h and the resulting supernatant was filtered through 0.45 µm filter to obtain clear solution. The drug content was estimated by HPLC as described under HPL analysis.

### Characterization

**Size and zeta potential:** Dynamic light scattering technique was employed to measure mean droplet size and poly dispersity index of nanoemulsion using Malvern Zetasizer (Malvern, UK). The globule size and polydispersity index of the emulsion was assessed after each cycle of high speed homogenization.

**Transmission electron microscopic (TEM) analysis:** To study the external morphology by TEM (100s, JEOL Ltd, Japan), a drop of nanoemulsion was placed on the copper grids and dried over night to remove the moisture completely. The analysis was performed at 200 kV.

**Viscosity:** The viscosity of NEG was determined by using Brookfield viscometer DV-II+ model having a T-bar spindle in combination with helical path.

**In vitro permeation studies [16]:** *In vitro* permeation studies of NEG and MC were carried in Franz diffusion cells with a diffusion area of 3.8 cm<sup>2</sup>. The receptor compartment was filled with 15 mL of phosphate buffer (pH 7.4) and stirred continuously using magnetic stirrer at 300 rpm [17]. Excised pork ear skin was fixed between donor and receptor compartment with the stratum corneum positioned towards the donor compartment [18]. 200 mg of NEG and MC was placed separately on the skin. 1mL of sample was withdrawn from each

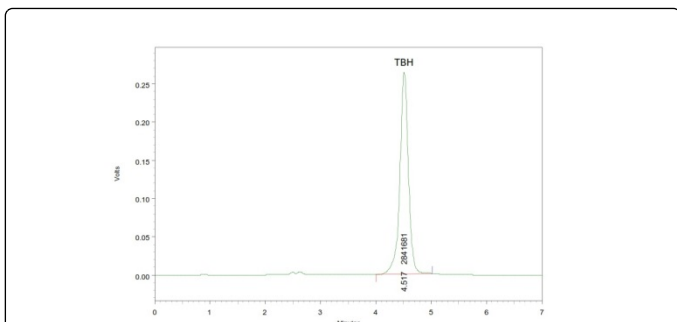
cell at predetermined intervals of 0, 0.25, 0.5, 0.75, 1, 2, 4, 6 and 9 hours and replaced with equal volume of buffer media [19]. At the end of study, the amount of permeated TBH, retained on skin and within the skin was determined by HPLC analysis.

**Microbiological assay:** The microbiological assay of NEG and MC was carried out on *C.albicans* species using cup plate method. Petri-dishes containing 20 mL of medium (Sabouraud dextrose agar) were seeded with 100 µL of the fungal inoculum (*C.albicans*). Consequently, each well in 2cm diameter was cut out of the agar. 2 g of NEG (1%) formulation and marketed cream (MC, 1%) were placed into each well. For positive control, methanolic solution of TBH (1%) equivalent to 2 g of formulation was used. Plates of *C. albicans* were incubated at 25°C for 2 days. After 48 h, the zone of growth inhibition were observed and measured [1,20]. Statistical analysis using one-way ANOVA with Tukey's post-hoc test was performed using Graph Pad Prism® software, version 6.0 (Graphpad Inc., USA).

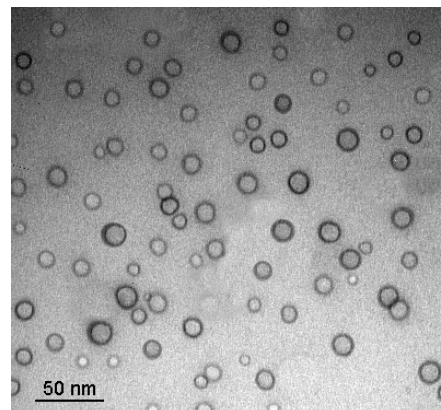
## Results

### Development of calibration curve

Development of the calibration curve was performed to find out the linearity between concentration of the drug in the solution and its absorbance. The obtained data revealed that linearity was observed in the concentration range of 10-500 µg/ml. The correlation coefficient obtained was found to be 0.999 with a slope 10702x and y intercept value of 46723 as shown in Figure 1.



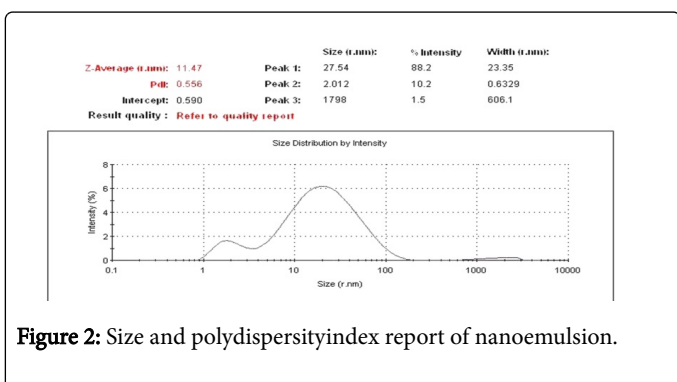
**Figure 1:** Chromatogram of terbinafine HCl in nanoemulsion gel formulation.



**Figure 3:** Transmission electron microscopic image of nanoemulsion.

### Preparation and characterization of nanoemulsion

Nanoemulsion was prepared using HSH with different parameters as described above. The nanoemulsion obtained is having a globule size of 11.47 nm with a polydispersity index of 0.556 as shown in the Figure 2. Nanoemulsion was converted into nanoemulsion gel found to be homogenous without any difference in color intensity, separation of phase, aggregates, with smooth feel and non-greasy for ease of application with a pH of 5.8. pH was considered as the acceptable range as pH of the skin was 5.5 almost near which won't cause any irritation upon application on skin. The drug content of nanoemulsion gel using plotted calibration curve was found to be 98.09%.



**Figure 2:** Size and polydispersity index report of nanoemulsion.

### TEM analysis

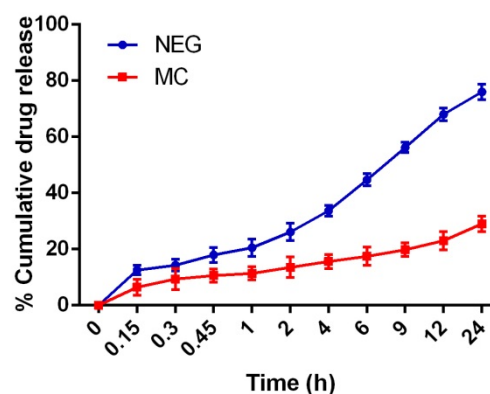
External morphological studies (TEM) revealed that particles in the nanoemulsion appeared as numerous, scattered, spherical dark stained circles possessing a size of <20 nm as shown in Figure 3. The size of the nanoparticles observed by TEM correlated well the particle size measured by zeta sizer.

### Viscosity

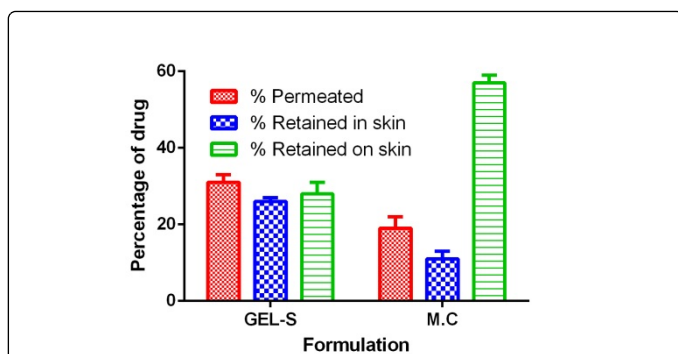
The viscosity of the formulation helps to show good spreadability ensure that a suitable dose is applied or delivered to the site of target. Viscosity of NEG was found to be in the range of 3,500- 4,300 cps, while that of MC was found to be in the range of 5,200-6,000 cps. The viscosity is inversely proportional to efficacy, less the viscous more the spreadability which increases the drug delivery topically. On this basis it was concluded that NEG could be more efficient than MC.

### *In vitro* permeation study

The percentage cumulative release of TBH permeated from NEG and MC until 24h is shown in Figure 4. The percentage of TBH permeated (receptor chamber), retained in the skin and on the skin (donor chamber) at the end of the study is presented in Figure 5. The total amount of TBH permeated was observed to be high with NEG ( $76 \pm 0.9\%$ ) than MC ( $29 \pm 0.8\%$ ). The MC had retained a large amount of drug ( $57.2 \pm 3.1\%$ ) on the skin.



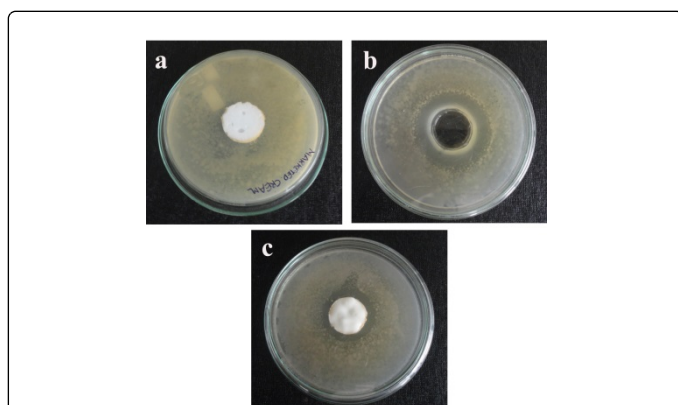
**Figure 4:** Permeation profiles of tested formulations (mean  $\pm$  SD, n=3).



**Figure 5:** TBH in different compartments of skin after the *in vitro* permeation study (mean  $\pm$  SD, n=3).

### *In vitro* microbial assay

The microbial assay for zone of inhibition was performed on fungal strain *C.albicans*. The maximum zone of inhibition was found to be  $0.66 \pm 0.17$  mm for NEG in comparison with MC  $0.13 \pm 0.05$  mm as shown in Figure 6. These results suggest that NEG could be more efficient than MC. This was attributed to greater penetration of TBH present in the size of nano in nanoemulsion.

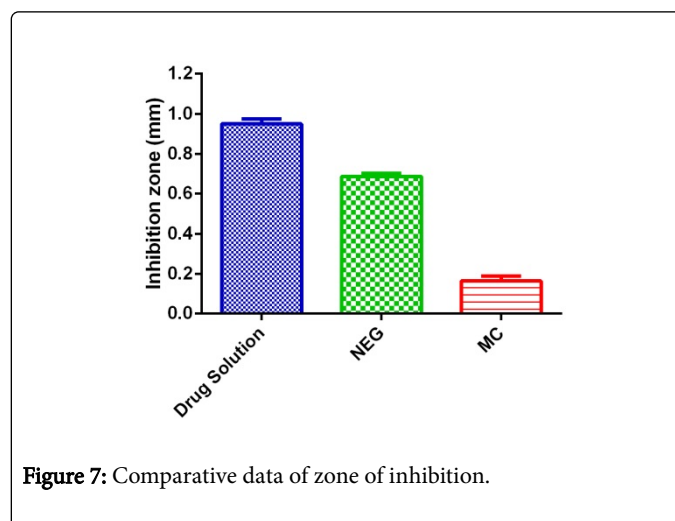


**Figure 6:** Plates showing zone of inhibition of (a) MC (b) pure drug solution (c) NEG.

### Discussion

The prepared NEG was found to be homogenous and did not contain aggregated particles. The pH of NEG was found to be 5.8, which is nearly to the pH of normal skin (pH-5.5) that ensures it will not produce any local irritation on skin. Extremes of pH on acidic and basic side can always cause irritation with redness at the site of application [21]. Topical application of particles requires less than 20 nm size [7] for high concentration of drug to penetrate the skin effectively into the stratum corneum. Hence, the obtained globule size (11.47 nm) was found to exist within the acceptable range for topical application i.e., <20 nm. The viscosity of prepared NEG was comparatively less than MC, since gels have lesser viscosity compared to creams. As viscosity is inversely proportional to spreadability, with the increase in viscosity, the spreadability and efficacy of the formulation is expected to decrease. Gelshave less viscosity with

highest spreadability. This characteristic feature of gels helps in ensuring greater contact of drug with skin and has higher efficacy rates. Therefore, NEG is expected to have higher efficacy compared to MC. *In vitro* permeation studies revealed that the prepared NEG showed high penetration and retention in the skin compared to MC; this may be attributed to the lower globule size and lesser viscosity of NEG. Low size globules or particles have greater penetrability towards the stratum corneum and functionally create a large drug depot in the skin. The lipophilic nature of prepared nanoemulsions will have greater retention time in epidermis (lipophilic) and have more chances of sustained release patterns [22]. MC has less permeation, which may be attributed to its slower release (high viscosity) and poor permeation [23,24]. In the microbial assay, one-way ANOVA followed by Tukey's multiple comparison test revealed NEG to be significantly effective ( $p < 0.01$ ) compared to MC in the zone of inhibition, which may be due to the high lipophilic nature of carrier (liquid paraffin), present as an o/w nanoemulsion in NEG. No significant differences were observed between NEG and pure drug solution as shown in Figure 7. Hence, in the current study NEG is highly lipophilic compared to MC. As fungal strains have an innate specificity towards lipophilic compounds, the formulated NEG with distinct lipophilic characteristics is readily taken up by them, thereby resulting in high zone of inhibition.



**Figure 7:** Comparative data of zone of inhibition.

### Conclusion

The present TBH is available in market in different dosage forms like tablets, creams, gels, and sprays. But each having its own disadvantages like tablets with poor bioavailability and should not be given for hepatic and cardiac patients, in case of creams and gels they are having poor penetration, hence require long term of therapy for curing and to minimize the relapse rates. Usually a combination of topical and oral antifungal is preferable with a minimum 14 days of therapy which increases the cost of therapy and decreases the patient compliance. Hence in the present study a novel dosage form for TBH is designed to increase its penetrability and reduce the treatment time there by increasing the patient compliance and reducing the cost of therapy. Accordingly the prepared NEG has overcome the problems associated with these poorly soluble drugs by enhancing the drug penetration and showing a maximum antifungal activity *in vitro* as that of drug. To conclude, the present study confirms that NEG of TBH

may be the best one for delivery of poorly soluble drugs like TBH, which are intended for topical antifungal application.

## Future Perspective

Future studies should be done on large number of animals and humans to evaluate the safety and efficacy of the prepared TBH-NEG. Since, these NEG technologies have greater scale up capabilities compared to other nano drug delivery systems, feasibility of product transfer from research and development to large scale has to be determined with the help of technology transfer.

## Conflict of Interest

None declared.

## Note

The content of this article is the subject of Indian Patent Application number 1186/CHE/2013 dated 20/03/2013 titled "Composition of Terbinafine HCl topical nanoemulsion gel for treating fungal diseases" by the Author.

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