

# Colloidal nanocarriers for the enhanced cutaneous delivery of naftifine: characterization studies and in vitro and in vivo evaluations

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**Abstract:** In topical administration of antifungals, the drugs should pass the stratum corneum to reach lower layers of the skin in effective concentrations. Thus, the formulation of antifungal agents into a suitable delivery system is important for the topical treatment of fungal infections. Nanosized colloidal carriers have gained great interest during the recent years to serve as efficient promoters of drug penetration into the skin. Microemulsions are soft colloidal nanosized drug carriers, which are thermodynamically stable and isotropic systems. They have been extensively explored for the enhancement of skin delivery of drugs. This study was carried out to exploit the feasibility of colloidal carriers as to improve skin transport of naftifine, which is an allylamine antifungal drug. The microemulsions were formulated by construction of pseudoternary phase diagrams and composed of oleic acid (oil phase), Kolliphor® EL or Kolliphor® RH40 (surfactant), Transcutol® (cosurfactant), and water (aqueous phase). The plain and drug-loaded microemulsions were characterized in terms of isotropy, particle size and size distribution, pH value, refractive index, viscosity, and conductivity. The in vitro skin uptake of naftifine from microemulsions was studied using tape stripping technique in pig skin. The drug penetrated significantly into stratum corneum from microemulsions compared to its marketed cream ( $P < 0.05$ ). Moreover, the microemulsion formulations led to highly significant amount of naftifine deposition in deeper layers of skin than that of commercial formulation ( $P < 0.001$ ). Microemulsion–skin interaction was confirmed by attenuated total reflectance – Fourier transformed infrared spectroscopy data, in vitro. The results of the in vivo tape stripping experiment showed similar trends as the in vitro skin penetration study. Topical application of the microemulsion on human forearms in vivo enhanced significantly the distribution and the amount of naftifine penetrated into the stratum corneum as compared to the marketed formulation ( $P < 0.05$ ). The relative safety of the microemulsion formulations was demonstrated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability test. This study indicated that the nanosized colloidal carriers developed could be considered as an effective and safe topical delivery system for naftifine.

**Keywords:** antifungal drug, tape stripping, in vivo skin penetration, microemulsion, colloidal carriers

## Introduction

Naftifine HCl (naftifine) is an allylamine that is effective in the management of superficial dermatomycoses. It is a fungicidal compound against a broad spectrum of dermatophyte fungi and provides good activity against *Candida* and *Aspergillus* species. Naftifine causes interruption of fungal ergosterol synthesis by inhibition of the enzyme squalene epoxidase and leads to accumulation of intracellular squalene in fungal organisms.<sup>1</sup> Currently, its conventional topical preparations are commercially available in market.

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In general, cutaneous delivery of antifungal compounds is preferred since the potential risks of systemic side effects are diminished and the drugs are also delivered into the site of infection. However, the efficacy of topical delivery of drugs strongly depends on the formulation characteristics as well as physicochemical properties of the drugs. The lipophilic compounds can partition into stratum corneum (particularly, lipid rich domain), but the enhancement of drug release into skin is required. Naftifine is expected to demonstrate a high affinity for the stratum corneum following topical application. However, taking into account its highly lipophilic characteristics (logP: 5.4) and low aqueous solubility, the optimization of its colloidal carriers seems to be feasible to enhance its solubility and cutaneous delivery. The other difficult aspect in the skin delivery of drugs is to overcome the barrier of stratum corneum. The therapeutic efficacy of applied antifungal drugs topically depends on the ability of the formulation to overcome the stratum corneum barrier and improve the uptake in deeper skin layers. Therefore, a delivery system aiming to increase the topical penetration of naftifine is important to enhance its local antifungal efficacy. So far, topical niosomes of naftifine are investigated within the context of their entrapment efficiency and stability.<sup>2</sup>

There has been increased interest during the recent years in the optimization of nanosized carriers to serve as efficient promoters of drug localization to skin.<sup>3</sup> Microemulsions are one of the soft colloidal nanosized carriers with a dynamic microstructure that form spontaneously by combining appropriate amounts of oil, water, surfactant, and a cosurfactant.<sup>4,5</sup> They are thermodynamically stable, isotropic systems prepared mostly by the phase titration method and can be depicted with the help of pseudoternary phase diagrams. Microemulsions have been extensively explored for a variety of pharmaceutical applications, including dermal and transdermal drug delivery.<sup>6-9</sup> The use of these nanocarriers in skin drug delivery has been frequently exploited and has proven highly successful for the delivery of both hydrophilic and lipophilic compounds.<sup>10</sup> Most of the studies demonstrated that more pronounced cutaneous drug localization in skin layers rather than percutaneous permeation can be obtained with microemulsions.<sup>11</sup> Numerous potential enhancement mechanisms have been proposed to elucidate the action of microemulsions on improving the transport of drugs to the skin. The ingredients of microemulsions can modify the diffusional barrier of the stratum corneum either by perturbation/fluidization of intercellular lipid bilayers or by denaturation of intracellular keratin or modification of its confirmation. The partitioning and solubility of drugs in stratum corneum could be increased depending on microemulsion composition, and

due to the high solubilization capacity of microemulsions, an increased concentration gradient toward the skin can be reached. Furthermore, the ultralow interfacial tension and the continuously fluctuating interfaces of microemulsions can facilitate drug penetration into deeper skin layers compared to conventional formulations.<sup>5,6,11-15</sup>

The aim of the present study was to formulate, characterize, and evaluate microemulsion-type novel nanocolloidal carrier formulations for effective skin delivery of naftifine. Two microemulsion formulations consisting of oleic acid (oil phase), Kolliphor® EL or Kolliphor® RH40 (surfactant), Transcutol® (cosurfactant), and water were developed by constructing phase diagrams and characterized by isotropy, particle size and size distribution, pH, conductivity, refractive index, and viscosity. The enhanced in vitro and in vivo human skin penetration of naftifine from microemulsions was studied by means of tape stripping experiments. The skin interaction of microemulsions on a molecular level was studied by attenuated total reflectance – Fourier transformed infrared (ATR-FTIR) spectroscopy. Finally, the in vitro cytotoxic activity of the microemulsions was evaluated in comparison to that of sodium lauryl sulfate (SLS), which served as a severe irritant.

## Materials and methods

### Materials

Naftifine was kindly provided by Zentiva (Istanbul, Turkey), and polyoxyl castor oils (Kolliphor® EL and Kolliphor® RH40) were kind gifts of BASF (Limburgerhof, Germany). Oleic acid was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Diethylene glycol monoethyl ether (Transcutol®) was kindly provided by Gattefossé (Lyon, France). Cell culture media were purchased from Wisent Bioproducts (St Bruno, QC, Canada), and sterile plastic materials were purchased from Greiner (Frickenhausen, Germany). SLS was obtained from Merck Millipore (Billerica, MA, USA). All other chemicals and reagents used were of analytical grade.

### Preparation of formulations

The concentration range of components necessary for the formation of microemulsions was determined by construction of pseudoternary phase diagrams based on water titration method at ambient temperature ( $25^{\circ}\text{C}\pm 0.5^{\circ}\text{C}$ ).<sup>4,16</sup> Briefly, oil in water microemulsions was prepared by mixing surfactant (Kolliphor® EL or Kolliphor® RH40), cosurfactant (Transcutol®), and oil (oleic acid), followed by gentle titration of distilled water. The weight ratio of surfactant to cosurfactant was fixed at 1:2. The weight ratios of oil phase (oleic acid) to the mixture of surfactant and cosurfactant

ranged from 9:1 to 1:9. The microemulsion formed spontaneously at room temperature as a clear monophasic liquid. Naftifine was solubilized in the oil phase before preparation of microemulsions. The drug (naftifine) was loaded into the microemulsions at the concentration of 1% (w/w).

## Characterization of formulations

### Polarized light microscopy

In order to verify the isotropic nature of the prepared microemulsions, cross-polarized light microscopy (Olympus BX51 U-AN360; Olympus Corporation; Tokyo, Japan) imaging was performed. A drop of the freshly prepared microemulsion was placed between a coverslip and a glass slide and observed under cross-polarized light. It is expected that an isotropic material, such as a microemulsion, will not interfere with the polarized light, and the field of view will remain dark.<sup>17</sup>

### Particle size measurements

The particle size and the polydispersity index of the microemulsions were determined using dynamic light scattering (Zetasizer Nano ZS; Malvern Instruments, Malvern, UK) after prefiltering (0.45  $\mu\text{m}$ , Millex; Merck Millipore) of the microemulsions. Microemulsion samples were loaded into disposable cuvettes having a volume of 1 mL without diluting, and triplicate measurements were taken at 25°C. The particle size was expressed as an average size of droplets in the system, and polydispersity index indicated the width of the size distribution.<sup>16</sup>

### Electric conductivity, pH, and refractive index measurements

The electric conductivity of the microemulsions was measured using a conductometer (EuTech PC 700; Eutech Instruments, Landsmeer, the Netherlands) at a frequency of 94 Hz. The measurements were made in triplicate at 25°C. The pH of the microemulsions was measured using a pH meter (EuTech PC 700), and triplicate measurements were done at 25°C. The refractive index of the microemulsions was measured by a digital Abbe refractometer (Atogo Co., Ltd, Tokyo, Japan) by placing one drop of microemulsion on the slide. The measurements were carried out in triplicate at 25°C.

### Rheology

In order to measure the viscosity of the plain and drug-loaded microemulsions, a Brookfield rheometer (Brookfield DV3THACJ0; Brookfield Engineering Laboratories, Middleborough, MA, USA) with a cone plate measuring device was used. All measurements (shear stress, shear rate,

and apparent viscosity) were performed in triplicate in a temperature-controlled environment at 25°C, and rotational speed ranged from 5 to 100 rpm.

## In vitro skin penetration study

Pig skin (obtained from a local slaughterhouse) was carefully cleaned from fat and muscle and then dermatomed to a thickness of 750  $\mu\text{m}$  (Zimmer Electric Dermatome, Warsaw, IN, USA). The integrity of the skin was confirmed by transepidermal water loss (TEWL) measurement with the open chamber device (Tewameter TM 300; Courage – Khazaka Electronic, Cologne, Germany) as is stated in the literature.<sup>4</sup>

Skin penetration of naftifine was studied using Franz-type diffusion cells (diffusion area of 1.76  $\text{cm}^2$ , PermeGear V6A stirrer; PermeGear Inc., Hellertown, PA, USA). The skin was placed on the receiver chambers with the stratum corneum facing upward, and then the donor chambers were clamped in place. The receptor chamber was filled with 12 mL of phosphate-buffered saline (PBS, pH 7.4). The receptor phase maintained at 37°C under constant stirring (300 rpm) with a magnetic bar. Microemulsion formulation (2 mL) was placed in the donor compartment of diffusion cells up. The donors were sealed by Parafilm M® (Bemis, Oshkosh, WI, USA) immediately after the addition of the formulation in order to prevent evaporation. The penetration of naftifine from microemulsions was followed up to 8 hours. At the end of the experiment, skin pieces were gently wiped to remove excess formulation, and the stratum corneum was separated from the underlying tissue by sequential tape stripping as described in our previous study.<sup>18</sup> Extraction was carried out with 5 mL of methanol at an ambient temperature for 12 hours (Thermo Forma 420 Orbital Shaker; Thermo Fisher Scientific, Waltham, MA, USA). Samples were filtered through 0.45  $\mu\text{m}$  Millex syringe filters (Merck Millipore) prior to high pressure liquid chromatography (HPLC) analysis. The concentration of naftifine in tapes and in the rest parts of skin was used as indices of cutaneous delivery. Data were determined from the average of at least six experiments. Since naftifine is a topically used and marketed antifungal compound, the obtained in vitro skin penetration results were related to the dermal delivery of naftifine from its marketed topical formulation (Exoderil). This formulation also contained 1% of naftifine.

## ATR-FTIR spectroscopy

At the end of the in vitro penetration study, the effect of microemulsions ME1 and ME2 and marketed formulation (Exoderil) on the pig skin was investigated by ATR-FTIR spectroscopy. Moreover, the microemulsion components

(oleic acid, Kolliphor<sup>®</sup> EL, Kolliphor<sup>®</sup> RH40, or Transcutol<sup>®</sup>) were applied to the pig skin under the identical *in vitro* conditions, and their effect on skin was studied on a molecular level by ATR-FTIR spectroscopy. The ATR-FTIR spectra were taken in the frequency range of 4,000–650  $\text{cm}^{-1}$  with a spectral resolution of 4  $\text{cm}^{-1}$  using an FTIR spectrometer (Perkin Elmer Spectrum 100 FT-IR Spectrometer, PerkinElmer Inc., Waltham, MA, USA). The internal reflection element was a ZnSe crystal with a trapezoidal cut at 45°C. The peak positions were determined using Perkin Elmer Spectrum Version 6.0.2 software. To ensure reproducible contact between the sample and the crystal, always the same pressure on the top of skin was applied (force gauge 80 N). In order to minimize inter sample variation, the same piece of skin before treatment was used for normalization, and a normalization procedure was performed according to the literature.<sup>19,20</sup> The frequency shifts of the C–H symmetric and asymmetric stretching absorbances, at 2,850 and 2,920  $\text{cm}^{-1}$ , respectively, which are sensitive to perturbations in the conformational order of the stratum corneum intercellular lipids, are investigated.

### In vivo skin penetration study

The *in vivo* human skin penetration of naftifine was studied by using the tape-stripping technique.<sup>21,22</sup> Four healthy volunteers (female, age 23–44 years), with no history of dermatological disease, participated in the study, which was approved by the Local Research Ethics Committee (Cerrahpaşa Medical Faculty Ethics Committee, Istanbul, Turkey). Written informed consent was obtained from each subject. The *in vivo* studies were carried out in an examination room under constant environmental conditions of 40%±5% relative humidity and 25°C±1°C. The volunteers were allowed to acclimatize (30 minutes) before application of the formulations on the internal region of both their forearms, which were cleaned with ethanol and water and dried. Briefly, the tape-stripping process involved the following steps: a single dose of 500  $\mu\text{L}$  of the microemulsion ME1 or 500 mg of Exoderil was applied to a delineated area (2×2 cm) on the forearm, and the treated area was covered by an occlusive tape (3M Tegaderm, St Paul, MN, USA). At the end of the 1 hour treatment, excess formulation was removed, and the stratum corneum surface was blotted completely dry with absorbent tissue. The site was then left unoccluded for 30 minutes before stripping.<sup>23</sup> Then the stratum corneum was sequentially stripped from the same area, 20 times with adhesive tapes (Scotch Book Tape; 3M Tegaderm) in accordance with the published studies.<sup>21–24</sup> The adhesive tapes were applied to the skin with vigorous rubbing by

the gloved operator and then removed. To check the skin barrier function, TEWL measurements were recorded using an open chamber device (Tewameter) before the first tape strip (baseline value) and during the tape stripping procedure. The number of tape strips allowed collecting the majority of stratum corneum (in other words, the majority of drug in the upper and lower stratum corneum) as showed by the TEWL value, which reached four to five times the baseline value at the end of the tape stripping procedure.<sup>23</sup>

Naftifine in the tape strips was subsequently extracted with 1 mL of methanol at an ambient temperature for 12 hours (Thermo Forma 420 Orbital Shaker). Samples were filtered through 0.45  $\mu\text{m}$  Millex syringe filters (Merck Millipore) prior to HPLC analysis. The first two tape strips were combined and analyzed separately from the remaining strips, which were combined into four groups of sequentially ordered tapes (strips 3–5, 6–10, 11–15, and 16–20). A separate analysis of tapes containing untreated stratum corneum and known amounts of drug was also conducted to evaluate the efficiency of the extraction procedure.

### HPLC analysis

For the HPLC (Shimadzu Model LC 20AT; Shimadzu Corporation, Kyoto, Japan) analysis of naftifine, we used a reversed phase C18 column (4.6×150 mm, 5  $\mu\text{m}$ ; EMD Millipore, Billerica, MA, USA) preceded by a guard column (4×4 mm, 5  $\mu\text{m}$ , Merck). The mobile phase consisted of acetonitrile:tetrahydrofuran:tetramethyl-ammonium hydroxide buffer (pH 7.8) (62:10:28), and the flow rate was fixed at 1.2  $\text{mL}\cdot\text{min}^{-1}$ . The wavelength of detection was set at 280 nm. The limit of quantification was found to be 0.025  $\text{mL}\cdot\text{min}^{-1}$ . The method was validated for selectivity, linearity, accuracy, and precision. It was found to be linear between the concentration range of 0.025  $\mu\text{g}/\text{mL}$  and 100  $\mu\text{g}/\text{mL}$  with a high correlation coefficient ( $r^2 > 0.999$ ) and was precise (intra- and interday variation <2%) and accurate (mean recovery >99%). Comparison of the chromatograms of samples from the extracted pig and human skin and blank tapes did not reveal any interfering peaks with naftifine confirming the selectivity of the method.

### Evaluation of cellular viability

To evaluate the relative safety of the plain microemulsions, we compared their cytotoxic effects to that of SLS, which is considered as a moderate-to-severe irritant.<sup>18,25</sup> For this purpose, mouse embryonic fibroblast cell line (BALB/3T3, clone A31) was obtained from American Type Culture Collection (ATCC<sup>®</sup> CCL-163<sup>™</sup>; Manassas, VA, USA). The cell line

was cultured at 37°C and 5% CO<sub>2</sub> atmosphere in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and additional penicillin and streptomycin (1%). For the cellular viability assay, cells were seeded in 96-well flat-bottom tissue culture plates (10<sup>4</sup> cells/well) and treated for 8 hours with either ME1, ME2, oleic acid, Transcutol®, Kolliphor® EL, Kolliphor® RH40, or SLS at concentrations ranging from 0.15% to 5% (v/v) in the cell culture medium. PBS was used as the negative control at 1% concentration (v/v), while SLS was used as the positive control for all assays.

Cell survival was assessed using (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) assay based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations, the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of various compounds on cell lines.<sup>26,27</sup> Briefly, cells were washed with PBS immediately after treatment, and 200 µL of cell culture medium and 20 µL of MTT solution (5 mg/mL in PBS) were added to each well, and the plates were incubated for 2 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in an incubator. After removing the unreduced MTT and culture medium, 200 µL of dimethyl sulfoxide was added to dissolve the MTT formazan crystals. Plates were shaken for 10 minutes, and the absorbance was recorded at 570 nm using a microplate reader (Epoch, Bad Friedrichshall, Germany). The cytotoxic activity was expressed as an IC<sub>50</sub> value, the concentration of extracts that caused a 50% inhibition of enzyme activity in the cells. All experiments were performed in triplicate using cells between passages 3 and 15.

## Statistical analysis

All results are the mean ± standard deviation of at least three to six experiments. The statistical analysis was performed using the Student's *t*-test with *P*<0.05 as the level of significance (GraphPad Software, Inc., La Jolla, CA, USA).

## Results and discussion

### Preparation and characterization of formulations

The optimization of the appropriate components to be used in the formulation of microemulsions has great impact to solubilize drugs and to enhance skin penetration. The design of biocompatible microemulsion system is also important since they are composed of relatively high concentrations of surfactants and cosurfactants. On the basis of preliminary

screening studies (data not shown), oleic acid as an oil phase, Kolliphor® EL or Kolliphor® RH40 as a surfactant, and Transcutol® as a cosurfactant were chosen as the best components of the microemulsions. Kolliphor® EL and Kolliphor® RH40 have been reported as nonionic surfactants with good emulsification capacity, and they have been proven to enhance drug permeation through biological membranes.<sup>28</sup> Transcutol® was chosen as a cosurfactant in the microemulsion formulations because of its nonirritating and nonvolatile capacity. The use of Transcutol® in topical and transdermal drug delivery has been examined widely due to its nontoxicity and optimal solubilizing parameters for a number of drugs.<sup>29–32</sup>

It is well known that microemulsion structures are strongly dependent on the ratio of the constituents. Pseudoternary phase diagrams can be used to note the physical boundary conditions (microemulsion, liquid crystalline, micelles) of oil, surfactants, and water necessary to formulate stable formulations.<sup>12,33–35</sup> The pseudoternary phase diagrams with different weight ratios of oil, water, and surfactant/cosurfactant are shown in Figure 1. The shaded area of the pseudoternary phase diagrams refers to the microemulsion region, while the outside area indicated multiphase turbid regions. In this study, a surfactant/cosurfactant weight ratio ( $K_m$ ) of 1:2 was selected to formulate naftifine-loaded microemulsion systems because this ratio is the most stable formulation. The isotropic nature of the optimized formulations and the absence of liquid crystalline phases also were confirmed by polarized light microscopy (Olympus BX51 U-AN360). Based on these phase diagrams, two microemulsion formulations (referred to as ME1 and ME2; Table 1) optimized were subjected to in vitro skin penetration studies.

Table 2 shows the physicochemical characteristics of naftifine-loaded microemulsions ME1 and ME2 and their blank counterparts. Dynamic light scattering measurements revealed that the particle size of the investigated microemulsions ranged from 7.34±0.03 to 11.17±0.25 nm at polydispersity indices ranging from 0.15±0.01 to 0.19±0.01. Both of the microemulsions have a single peak in size distribution. The small particle size of microemulsions has been proven to provide more opportunities for drug transporting into the skin as the total surface area of the microemulsion contacting with the skin increases.<sup>12,16</sup> A slight increase in the particle size in drug-loaded microemulsions has been observed (Table 2), which might be due to the naftifine accumulation in the droplets' interfacial layers rather than staying in the continuous phase.<sup>4</sup>

A strong correlation between the microemulsion structure and electrical conductivity has been reported.<sup>17</sup> As the

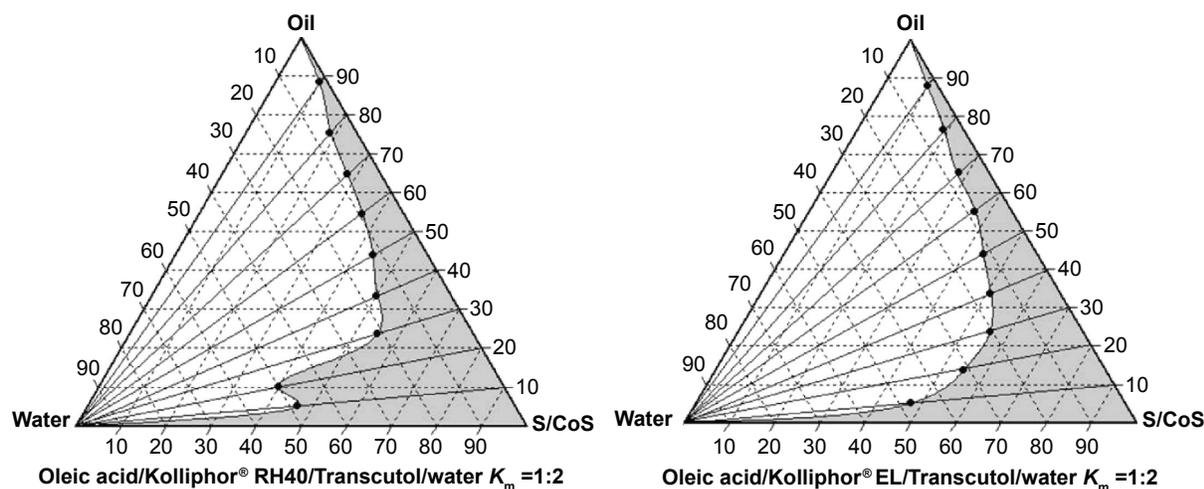


Figure 1 Pseudo-ternary phase diagrams of microemulsion systems under  $K_m = 1:2$ .

percentage of water volume in microemulsion increases, the electrical conductivity increases. The obtained conductivity values revealed that the microemulsions prepared were in oil in water (o/w) form and naftifine in salt form (naftifine HCl) led to an increased conductivity. This finding is in accordance with the expectation that the salt forms dissociate in the presence of water and thereby cause an increase in conductivity of the microemulsions.<sup>36</sup>

The viscosity of a microemulsion is the function of the type of its components (oil, surfactant, cosurfactant, and water) and their concentrations.<sup>37</sup> Both microemulsions (ME1 and ME2) exhibited low viscosities, which are typically observed in microemulsions.<sup>37</sup> The apparent viscosity values of the microemulsions were between  $70.76 \pm 0.12$  and  $83.58 \pm 0.46$  cP, and the correlation coefficients between shear rate and shear stress were equal to 1, indicating Newtonian flow behavior as expected for microemulsion systems (Figure 2).<sup>36</sup> The lower viscosity of ME1 compared to ME2 is in correlation with the finding that increased surfactant content can lower the viscosity of a microemulsion.<sup>28</sup>

Incorporation of naftifine in salt form (naftifine HCl) slightly reduced pH values of the investigated microemulsions. The refractive indices of drug-loaded microemulsions were similar to those of their blank counterparts.

**Table 1** Composition of optimized microemulsion formulations

Formulation components	ME1 (%)	ME2 (%)
Oleic acid	8	8.7
Kolliphor® RH40	19.0	–
Kolliphor® EL	–	17.2
Transcutol®	38.0	34.8
Naftifine	1.0	1.0
Water	34.0	38.1

## In vitro skin penetration study

The cutaneous delivery of colloidal drug carrier systems is of considerable interest to understand in which extent they can be found in the skin.<sup>38</sup> Skin penetration of naftifine across dermatomed pig skin ( $750 \mu\text{m}$ ) was studied using Franz-type diffusion cells in vitro. The quantification of naftifine penetration into the stratum corneum and lower layers of pig skin is shown in Figure 3.

When compared with the commercial formulation of naftifine (Exoderil), approximately 8.8- and 6.9-fold increase in the amount of naftifine ( $\mu\text{g}/\text{cm}^2$ ) deposited in the pig skin was achieved with microemulsion formulations ME1 and ME2, respectively. This finding demonstrated that naftifine penetrated significantly into stratum corneum from ME1 and ME2 compared to its marketed cream ( $P < 0.05$ ). In particular, both microemulsion formulations (ME1 and ME2) led to highly significant amount of naftifine deposition in deeper layers of the skin than that of commercial cream formulation ( $P < 0.001$ ). Additionally, ME1 enhanced significantly the localized amount of drug in deeper layers of the skin in comparison with ME2 ( $P < 0.05$ ), but there is not very high difference between ME1 and ME2 for the amount of naftifine in stratum corneum. ME1 and ME2 are colloidal carriers composed of almost similar components in slightly different quantity (Table 1). The observed difference in the penetration enhancement effect between both formulations could be due to the higher surfactant concentration of ME1, which lead to a disruption in the lipid bilayer organization of the stratum corneum. The lower viscosity of the system may be another factor affecting drug release and delivery. Thus, ME1 formulation, providing much higher drug retention in skin, has been examined further in in vivo human penetration studies.

**Table 2** Physicochemical characteristics of the plain and drug-loaded (ME1 and ME2) microemulsions

Formulations	Droplet size (nm)	PDI	pH	Conductivity ( $\mu\text{S}/\text{cm}$ )	Refractive index	Viscosity (cP)
Plain ME1	10.52 $\pm$ 0.37	0.18 $\pm$ 0.01	5.71 $\pm$ 0.03	34.78 $\pm$ 0.06	1.4135 $\pm$ 0.0002	70.76 $\pm$ 0.12
ME1	11.17 $\pm$ 0.25	0.19 $\pm$ 0.01	4.26 $\pm$ 0.01	289.00 $\pm$ 0.36	1.4150 $\pm$ 0.0001	75.62 $\pm$ 0.24
Plain ME2	7.34 $\pm$ 0.03	0.15 $\pm$ 0.01	4.86 $\pm$ 0.02	26.67 $\pm$ 0.29	1.4090 $\pm$ 0.0003	81.13 $\pm$ 0.30
ME2	8.56 $\pm$ 0.19	0.17 $\pm$ 0.01	3.71 $\pm$ 0.02	340.90 $\pm$ 0.30	1.4100 $\pm$ 0.0001	83.58 $\pm$ 0.46

**Note:** Each value is the mean  $\pm$  SD of at least three experiments.

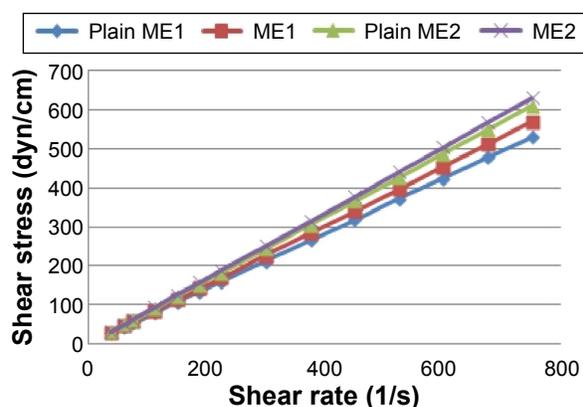
**Abbreviations:** PDI, polydispersity index; SD, standard deviation.

## ATR-FTIR study

ATR-FTIR spectroscopy facilitates an evaluation of the influence of solvents/penetration enhancers on the organization of lipids and proteins within the skin. The symmetric and asymmetric C–H stretching vibrations describe the conformational order of the stratum corneum intercellular lipid chains.<sup>39</sup> The blue shifts of these frequencies have been attributed to the conformational disordering of the lipids, which refers to an increase in motional freedom of the lipid hydrocarbon chain with the introduction of more gauche conformers in the stratum corneum lipids' acyl chains or due to phase separation.<sup>19</sup> Table 3 shows the effect of the microemulsions, their components, and Exoderil on the frequencies of the C–H symmetric and asymmetric absorbances. Compared to untreated control skin, the stretching vibrations were detected at higher wavenumbers in the skin samples treated either with ME1, ME2, or their components (oleic acid, Kolliphor<sup>®</sup> EL, Kolliphor<sup>®</sup> RH40, or Transcutol<sup>®</sup>), an observation indicating a higher barrier permeability. It has been reported that oleic acid fluidizes the stratum corneum intercellular lipids and induces phase separation, and thereby exerts a penetration enhancing effect.<sup>31,37,40,41</sup> Together with the oleic acid reported effects, the partitioning of the surfactant (Kolliphor<sup>®</sup> EL or Kolliphor<sup>®</sup> RH40) and the cosurfactant (Transcutol<sup>®</sup>) may

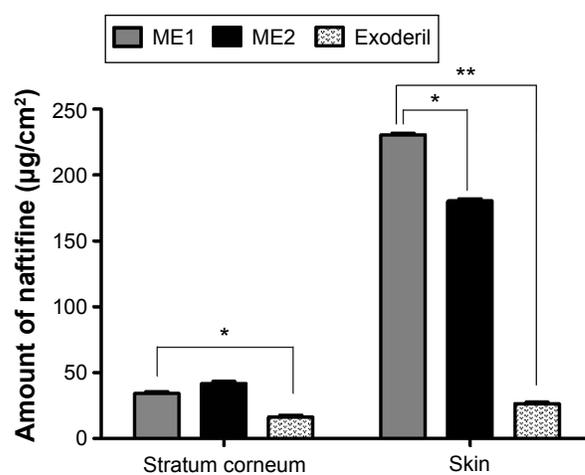
have also contributed to the C–H symmetric and asymmetric blue shifts.

The C–H symmetric stretching frequencies are known to be more susceptible to changes in lipid matrix organization.<sup>42</sup> While values approximately 2,850  $\text{cm}^{-1}$  are indicative of orthorhombic arrangement, a shift approximately 2,852  $\text{cm}^{-1}$  suggests hexagonal phase, and thus a more fluid structure of the intercellular lipid matrix.<sup>39</sup> As can be seen in Table 3, the symmetric stretching vibration shifted from 2,850.4 $\pm$ 0.1  $\text{cm}^{-1}$  (untreated control skin) to 2,854.0 $\pm$ 0.2  $\text{cm}^{-1}$  for Kolliphor<sup>®</sup> RH40 and to 2,852.4 $\pm$ 0.3  $\text{cm}^{-1}$  for ME1, suggesting the domination of a hexagonal arrangement on the skin surface in both cases. These data are in good agreement with our skin penetration experiments and also in accordance with the consideration that microemulsion components can interact with stratum corneum lipids and thereby facilitate drug transport across the skin barrier.<sup>37</sup> The type of the surfactant affected differently the permeability of the skin, and as a consequence, cutaneous drug delivery from the microemulsions. No remarkable shifts of the C–H stretching vibrations could be detected after the application of the marketed cream



**Figure 2** Newtonian flow behavior of the plain and naftifine-loaded (ME1 and ME2) microemulsions.

**Abbreviation:** s, second.



**Figure 3** Comparison between the amounts of naftifine accumulated into the stratum corneum and the rest of skin after removing stratum corneum in vitro for the microemulsions ME1 and ME2 and the marketed topical formulation (Exoderil).

**Notes:** Each bar represents the mean  $\pm$  SD of six determinations. Significant differences were calculated using Student's *t*-test, \* $P$ <0.05, \*\* $P$ <0.001.

**Abbreviation:** SD, standard deviation.

**Table 3** Peak shifts of skin lipids in C–H symmetric and asymmetric stretching absorbances due to the application of microemulsions, microemulsion components, and Exoderil

Treatments	Symmetric C–H stretching (cm <sup>-1</sup> )	Asymmetric C–H stretching (cm <sup>-1</sup> )
Untreated skin	2,850.4±0.1	2,918.4±0.1
ME1	2,852.4±0.3	2,918.8±0.2
ME2	2,851.3±0.2	2,918.5±0.5
Kolliphor® EL	2,851.8±0.3	2,919.8±0.2
Kolliphor® RH40	2,854.0±0.2	2,919.7±0.3
Oleic acid	2,851.8±0.2	2,920.8±0.1
Transcutol®	2,850.8±0.1	2,919.1±0.2
Exoderil	2,850.7±0.2	2,918.6±0.3

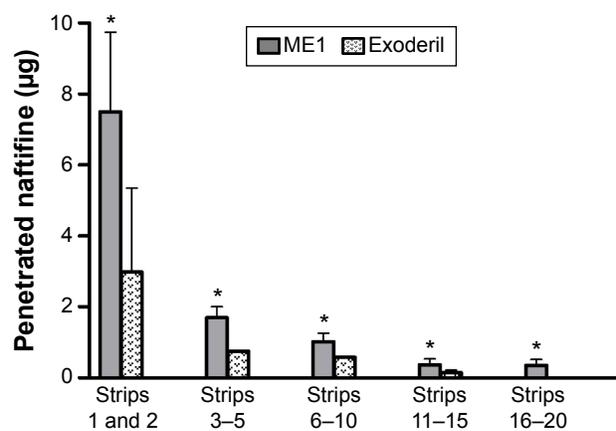
Exoderil, indicating that the cream failed to disturb the stratum corneum lipid organization.

It has been reported that percutaneous penetration is an essential requirement for satisfactory topically applied antifungals in order to achieve therapeutic concentration for deep fungal infections.<sup>43</sup> The ability of a microemulsion to improve the transport of drugs to and across the skin is mainly affected by the composition and concentration of the microemulsion components, as well as the internal structure and type (o/w, w/o, or bicontinuous systems) of the microemulsion used.<sup>25</sup> Oleic acid is one of the most frequently selected oils in microemulsions, and it has been demonstrated that microemulsions containing oleic acid increased the penetration of lipophilic drugs through the skin.<sup>44</sup> The permeation-enhancing effect of oleic acid has been reported to be more pronounced in oil in water (o/w)-type microemulsions, and the superiority of oleic acid to increase the release of lipophilic drugs from microemulsions has been shown.<sup>11</sup> The type and concentration of the surfactant may also modulate drug release from microemulsions. Our results demonstrated a synergistic effect of the added surfactant Kolliphor® RH40 apart from the contribution of oleic acid and Transcutol® in enhancing drug penetration into skin. Additionally, the poor aqueous solubility of drugs is a rate-limiting step for the penetration of drugs.<sup>45</sup> Depending on its physicochemical characteristics and system microstructure, a drug may be incorporated in the dispersed phase, dispersing medium, and/or within the interface in microemulsions. Hydrophobic drugs, incorporated in nonpolar domains of microemulsions, must first partition into stratum corneum to achieve skin penetration of the drug. As emphasized previously, naftifine is a highly lipophilic compound with low aqueous solubility.<sup>46</sup> It would be expected that microemulsion formulations increase the solubility of naftifine, which leads to high concentration gradient toward the skin, and thus greatly improves the drug accumulation in the skin.<sup>5</sup>

## In vivo skin penetration study

The stratum corneum is the main barrier for cutaneous penetration and provides substantial information for comparative evaluation of topically applied compounds in a way that stratum corneum concentration of drugs can be related to deeper skin layers concentration.<sup>38,47,48</sup> From in vivo studies in the literature, it is well known that lipophilic drugs are preferably localized on the skin surface and in the superficial stratum corneum after topical administration.<sup>5</sup> In our study, the in vivo skin penetration of naftifine from microemulsions and from the marketed cream Exoderil was investigated in healthy volunteers. Both formulations were tested on the forearm of same volunteer on the same day. Vehicle-dependent effects on amount and distribution of naftifine within the stratum corneum were studied by means of adhesive tape stripping. After 1 hour of penetration time, the amount of naftifine, measured using the adhesive tapes (2×2 cm), combined in five groups (strips 1 and 2, 3–5, 6–10, 11–15, and 16–20), shown in Figure 4 as a function of strip number (related to stratum corneum depth). The average recoveries of naftifine from the tapes were satisfactory (>95%).

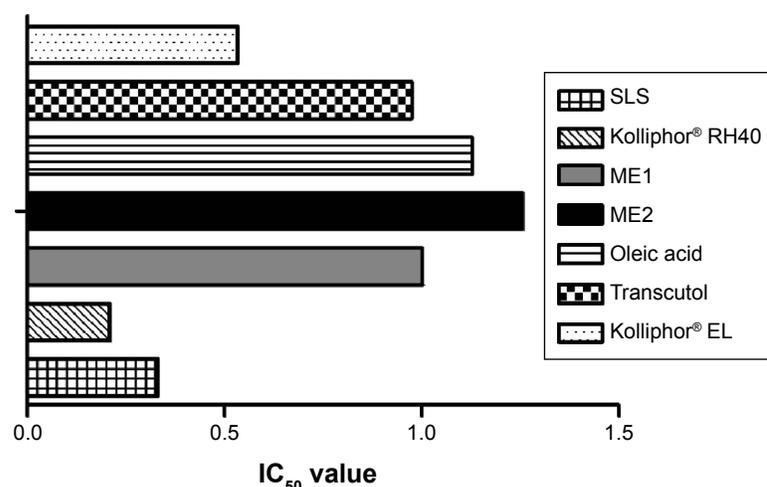
In the case of Exoderil, the main amount of naftifine was localized mainly on the superficial part of the stratum corneum and the drug could not be detected in deeper layers of stratum corneum (strips 16–20). Topical application of the microemulsion ME1 enhanced significantly the distribution and the amount of the drug penetrated in vivo into the stratum corneum (total naftifine amount in the stratum corneum: 10.94±1.12 µg/4 cm<sup>2</sup>) as compared to the marketed formulation (Exoderil) (total naftifine



**Figure 4** Distribution of naftifine amounts in human stratum corneum in vivo after application of microemulsion formulation ME1 and marketed topical formulation (Exoderil).

**Notes:** Each bar represented the mean ± SD of four determinations. Significant differences were calculated using Student's *t*-test, \**P*<0.05 compared to Exoderil.

**Abbreviation:** SD, standard deviation.



**Figure 5** IC<sub>50</sub> values of developed microemulsions, microemulsion components, and SLS.  
**Abbreviations:** SLS, sodium lauryl sulfate; IC<sub>50</sub>, half maximal inhibitory concentration.

amount in the stratum corneum:  $4.46 \pm 0.75 \mu\text{g}/4 \text{ cm}^2$  ( $P < 0.05$ ). The naftifine amount in stratum corneum following application of ME1 and Exoderil for 8 hours in in vitro penetration studies was measured as  $34.11 \pm 1.39 \mu\text{g}/\text{cm}^2$  and  $16.28 \pm 1.34 \text{ cm}^2$ , respectively. Taking into account the applied doses and application durations in in vitro and in vivo human studies, the results of the in vivo tape stripping experiment showed similar trends as the in vitro skin penetration study.

### Evaluation of cellular viability

A common concern related to microemulsion use for topical and transdermal delivery is their potential side effects, mainly the skin irritation potential. This effect is mostly associated with exposure time and the composition and the concentration of surfactants and cosurfactants.<sup>11</sup> In vitro cytotoxicity tests have gained great interest for determining the biocompatibility and tolerability of microemulsions.<sup>18,25,49</sup> Since microemulsions are multicomponent systems and their formation requires high surfactant concentration, the effect of developed microemulsions (ME1 and ME2) and their components (oleic acid, Transcutol®, Kolliphor® EL, and Kolliphor® RH40) on the viability of cultured fibroblasts was evaluated. The cytotoxic activity of microemulsions, each component of microemulsions, and SLS was expressed as IC<sub>50</sub> value and is shown in Figure 5. All of the studied compounds, except Kolliphor® RH40, showed considerably higher IC<sub>50</sub> compared to SLS. Although the IC<sub>50</sub> of Kolliphor® RH40 (0.029) was lower than that of SLS (0.331) and a notable decrease in cell viability was detected, the IC<sub>50</sub> value of ME1 (1.003) was ~3.03 times higher than that of SLS. This finding suggests that ME1 may be a safe colloidal nanocarrier system for naftifine.

### Conclusion

Naftifine-loaded microemulsion type of nanosized colloidal carriers formulated for cutaneous delivery and the in vitro pig skin and in vivo human penetration data obtained in this study showed the impact of microemulsions in the enhancement of topical delivery of naftifine. Microemulsion formulation optimized (ME1) has increased transport of naftifine into deeper layers of the skin with low skin cytotoxicity. Thus, formulated colloidal drug carrier may facilitate relieving for local deep-seated fungal diseases due to localized transport of naftifine. However, to conclude its efficacy, the clinical evaluations of this system should also be performed.

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### Disclosure

The authors report no conflicts of interest in this work.

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