

Fluconazole and Curcumin Loaded Nanoemulsion against Multiple Drug Resistance Dermatophytes

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Topical nanoemulsion comprising of fluconazole and curcumin was developed to target multiple drug resistance dermatophytes infection and to facilitate cutaneous delivery of these poorly water soluble drugs. Almond oil, sesame oil and paraffin light were used to formulate nanoemulsions and screened for the stability. The solubility of fluconazole and curcumin in surfactants, co-surfactants and oils was screened to decide the various components of the nanoemulsion. The oil phase was light paraffin whereas tween 80 and span 80 were the surfactants and ethanol was used as a co-surfactant. To identify the area of nanoemulsion existence, a pseudoternary diagram was drawn and optimum systems were developed. Drug-loading efficiency was assessed and the developed nanoemulsions were characterized for globule size, stability, robustness to dilution and pH. The optimized nanoemulsion was further evaluated for drug content, viscosity, skin permeation study (ex vivo) and assay of antifungal activity. The globule size was below 200 nm and uniform for the optimized nanoemulsion formulation. It showed enhanced skin permeation (ex vivo) and better antifungal efficacy as compared to the native form of fluconazole and curcumin suspensions. Antimicrobial assay confirmed the synergistic effect of fluconazole and curcumin combination against multiple drug resistance *Trichophyton rubrum* and *Trichophyton mentagrophytes* as compared to the fluconazole alone. The results clearly indicate an optimized delivery of fluconazole and curcumin in a synergistic way from the nanoemulsion formulation. This resulted in better penetration of these poorly soluble molecules and overall enhanced antifungal activity as compared to these drugs as such against multiple drug resistance dermatophytes.

Keywords: Antifungal; Curcumin; Fluconazole; Multiple Drug Resistance Dermatophytes; Nanoemulsion.

Dermatophytes cover three genera of pathogenic fungi namely, Epidermophyton, Microsporum, and Trichophyton, which illustrate a significant affinity for the keratinized structures present in skin, nails and hair, causing infections (superficial) identified to be dermatophytosis¹⁻³. Keratin is metabolized by these organisms which causes a number of pathologic clinical presentations

such as tinea corporis, tinea pedis, tinea cruris etc^{4,5}. *Trichophyton rubrum* and *Trichophyton mentagrophytes* complex are a few common fungi responsible for dermatophytosis, which is endemic to India^{6,7}. In spite of a plenty of antifungal drugs currently exist in the commercial pharmacies, the propagation of antifungal drug resistance and invasive type fungal infection, particularly caused

by dermatophytes, has significantly increased worldwide and covers around 20%-25% of the world's population^{8,9}. Some identified reasons for resistant are misuse of fixed drug combination (FDC) creams containing very potent drugs like steroids, microbiological resistant of the causative fungi and a transformed immune response of host etc¹⁰.

The mechanism towards antifungal resistant consists of filamentation, formation of bio-film, genomic alterations and over expression of drug efflux pumps (which includes major facilitate or superfamily of few transporters and ATP-binding cassette transporters)¹¹. The dermatophyte showed reduced susceptibility to azole after a prolong use^{6,12,13}. Drug-resistant can be minimized by advanced and better diagnostics for detection of infection at early stage, prophylaxis, multi drug therapy, pre-emptive treatment and identification of new antifungals¹⁴⁻¹⁷.

Fluconazole (FLZ) is a triazole antifungal drug of third generation with broad spectrum activity against superficial and systemic fungal infections. It acts by inhibiting the production of vital elements in the membrane of fungi as ergosterol by limiting the fungal cytochrome P450 enzyme. After prolong exposure of fluconazole, major facilitator superfamily (MFS) of proteins are over expressed in dermatophytes resultant enhanced resistant¹⁸⁻²². The efflux pump inhibitors can be used to sensitize the resistant type of *C. albicans* strains to azoles, which can lower the dose of required antifungal for desired pharmacological benefits and potentially limiting side-effects²².

The solubility of FLZ in water is around 5 mg/mL at 37°C (slightly soluble), with 306.3 Da molecular weight and has a pK_a value of weak base 3.7²³. It is presented as parenteral and oral dosage forms and may exhibit serious side effects as diarrhoea, vomiting, stomach upset, feeling sick, rashes, and reduction in the count of red blood cells. Incidence of hepatotoxicity in patients receiving triazoles has also been reported. Therefore, topical application of FLZ is highly indicated to prevent such adverse effects^{25,26}. Due to the large molecular size of FLZ and hydrophobicity, the development of topical dosage form is challenging. Recently, nano-based formulation strategies are valuable to reduce the adverse effects of FLZ and increase the efficiency²⁷.

The concomitant use of natural chemical compounds co-administered with the conventional chemotherapeutic agents has been recommended as a valuable strategy to overcome multi drug resistance (MDR) in fungi and thus, restoring the susceptibility to anti-fungals²⁸. One natural polyphenol Curcumin, which is found in the roots and rhizomes of *Curcuma longa*. A recent study has reported that it could be used as an important modulator of MDR in *Saccharomyces cerevisiae* strain carrying mutations that has an MDR phenotype²⁹. Curcumin is reported to enhances the activity of fluconazole in many study^{30,31}. Curcumin is poorly water soluble, meaning that it is restricted to the superficial stratum corneum (SC) after topical delivery^{32,33}. Nanoemulsion system improved curcumin permeability³⁴. Nanoemulsions are kinetically stable and isotropic emulsions in which the drug containing oil droplets are stabilized by a very thin layer of emulsifier³⁵. Dissolution rates and bioavailability of poorly water soluble drugs can be improved in the form of nanoemulsions with added advantage of reduced side effects. The high drug loading capacity, enhanced drug solubility, better thermodynamic stability and permeation enhancement through skin without skin irritation makes the nanoemulsion as one of the suitable system for topical delivery^{36,37}.

In this study, we proposed that curcumin can sensitize fluconazole resistant strains of dermatophytes (*T. rubrum* and *T. mentagrophytes*) and potentiate the effect of fluconazole against resistant strains. Further development of this combination into a nanoemulsion formulation may facilitate deep penetration of these poorly soluble drugs into the skin layers.

MATERIALS AND METHODS

Materials

Fluconazole was gifted from Virupakasha organics Ltd, Hyderabad and curcumin (95% pure) from Elixir extract Pvt Ltd, Cochin. Tween 80 and Span 80 were purchased from Nice chemicals Pvt Ltd, Cochin. Ethanol and paraffin light were purchased from Spectrum reagents and chemicals Pvt Ltd, Kochi. Remaining chemicals used were of analytical grade.

Selection of various components of the nanoemulsion

To select the components of nanoemulsion, the solubility of fluconazole and curcumin was determined in various oils and surfactants, such as light paraffin, almond oil and sesame oil and Span 80, Tween 80, ethanol. Slightly excess quantity of fluconazole and curcumin (separately) was mixed to 10 mL of each oils and surfactants. The tubes were shaken for 24 h at 20°C. The supernatant was collected, filtered and assayed spectrophotometrically at 260 and 421 nm respectively for fluconazole and curcumin.

Creation of pseudoternary phase diagram

The pseudo tertiary diagram was constructed using Chemix school software. Paraffin was chosen as oil phase, ethanol and Tween 80 were chosen as co-surfactant and surfactant, respectively. The aqueous phase was distilled water. The surfactant and co-surfactant were used at various mass ratios. These ratios were selected in ascending concentration of co-surfactant with respect to surfactant and an exhaustive study of phase diagram was carried out. Aqueous filtration method was used in order to develop the ternary phase diagram for oil, Surfactant and aqueous phase. To oil and surfactant mixture, aqueous phase is added slowly and titrated. Visual observations were made for emulsions. The physical state of water, oil and a blend of surfactant and co-surfactant at particular set mass ratio were marked on the pseudo ternary phase diagram.

Microbiological assay of fluconazole and curcumin

In vitro antifungal studies were performed against multiple drug resistance *Tricophyton Rubrum* and *Tricophyton Metagrophytes*. These dermatophytes were developed at 37°C and maintained on Sabouraud dextrose agar (SDA) slants at a temperature of 4°C. Dermatophytes were preserved at “20°C in 20% glycerol. The fluconazole’s breakpoint value for dermatophytes resistant was calculated in mg/l. The combinatorial effect of curcumin and fluconazole was evaluated by checkerboard experiments using PRI (protein rich isolate). In brief, BHI (brain heart infusion) medium was used to grow the cells overnight at 37°C with constant shaking. After the incubation period, the cells were harvested, washed in PBS and then suspended at a concentration of 4×10^3

cells/ml. This cell suspension (100 µl aliquot in each well) was placed in a 96-well microtitre plate containing sabouraud without the glutamine medium in the presence and absence various concentrations of curcumin and fluconazole, in combination or alone. The concentration of Fluconazole was 0.5–128 mg/l within the vertical cells whereas curcumin was used at 2–32 mg/l in horizontal lines wells. Curcumin and fluconazole’s interaction was determined by calculating the FICI (fractional inhibitory concentration index). The FICI value ≤ 0.5 represents synergistic interactions whereas the FICI value >4.0 represents antagonistic effect and values in between 0.5 and 4.0 represent no interaction.

$$FICI = FIC \text{ of curcumin} + FIC \text{ of Fluconazole}$$

$$FIC \text{ of Curcumin} = \frac{MIC \text{ of curcumin in presence of fluconazole}}{MIC \text{ of Curcumin}}$$

$$FIC \text{ of fluconazole} = \frac{MIC \text{ of fluconazole in presence of curcumin}}{MIC \text{ of fluconazole}}$$

Where

FICI= Fractional inhibitory concentration index

FIC= Fractional inhibitory concentration

MIC= Minimum inhibitory concentration

Preparation of O/W nanoemulsion

Different nanoemulsion (NE) formulations were developed by ultrasonication as suggested by Ahmad et al., 2019 with some modifications using different concentration of selected oils, surfactants, co-surfactants and distilled water³². Tween 80/SDS was dissolved in water in a beaker (solution A) and ethanol, drug and Span 80 were mixed together in another beaker then added to selected oil phase (solution B). Then aqueous phase (solution A) was kept under magnetic stirrer and oil phase (solution B) was added drop by drop and stirred till 30 min until emulsion was formed. Then the emulsion was sonicated using probe sonicator for 20 min continuously at 24 kHz at power of 400 W. Eleven Different formulations were developed (Table 1).

Characterization of nanoemulsion systems

Visual appearance

The final formulation visually inspected for clarity, colour and transparency. The formulation evaluated for the presence of any gritty particles.

Homogeneity

Homogeneity was tested by visual

inspection. Small quantity of formulation was pressed between thumb and the index finger to find out the homogeneity of formulation.

Measurement of pH

A digital pH meter was used to determine the pH of the formulations which were previously standardized by adjusting the pH in three different buffers (pH 4, 4.5 and 5). After standardization the probe of pH meter dipped in the formulation and the pH was noted³⁸.

Grittiness

Smears of formulation were prepared in glass slide and observed under optical microscope for the presence of any gritty particles.

Stability studies

The nanoemulsions were centrifuged at 4000 g for 30 min to determine the dispersion stability tests. The phase separation was evaluated and the nanoemulsions which didn't show any phase separations were further evaluated by freeze-thaw cycle. Only the surviving formulations in the stability test were selected for further studies³⁹.

Determination of droplet size and zeta potential

Dynamic Light Scattering method at 90° angle was used to determine the polydispersity index and average size and of the nanoemulsion (NICOMP TM 380 ZLS, USA Particles Sizing Systems, Santa Barbara, CA). 100 mg nanoemulsion was diluted with methanol to 10 ml shaking. The instrumental settings were set at viscosity 0.01 poise, temperature 20°C and refractive index 1.333. Along with size, zeta potential of nanoemulsion formulation was also recorded⁴⁰⁻⁴².

Measurement of Viscosity

Viscosity assessment is one of the important physicochemical parameter in the characterization of nanoemulsions. It affirms the type of nanoemulsion system (O/W or W/O). Generally, O/W type systems show low viscosity whereas W/O systems show high viscosity. Viscosity of formulation was determined by rheometer at the temperature 25±2°C⁴³.

Fluconazole and curcumin loaded in optimized nanoemulsion

Optimized formulation was selected drug loading. Fluconazole and curcumin was dissolved in light paraffin by slow addition of surfactants and co-surfactants and distil water using magnetic stirrer for 30 min followed by probe sonication for

20 in continuation min 24 kHz at power of 400 W⁴⁴. (Table 2)

Estimation of drug content

Nanoemulsion formulation (5 mg fluconazole and curcumin equivalent) was stirred with 15 ml methanol in a volumetric flask for 30 min. More quantity of methanol was added subsequently to make up the volume to 50 ml. 2 mL of this solution was then diluted with 5 ml of 0.1 N HCl and 3 ml of methanol to achieve a concentration of 20 µg/ml. 0.45 µm membrane filter was used to filter the solution before measuring the absorbance spectrophotometrically at 260 and 421 nm respectively (UV-1800 UV-Vis Spectrophotometer, Shimadzu Corporation, Japan).

Skin permeation study (ex vivo)

Porcine ear skin was collected from a local slaughter house. An electric shaver was used to remove the hairs and a scalpel along with surgical scissors was used to remove the adherent subcutaneous fatty layer. The skin surface was thoroughly cleaned with freshly prepared Ringer's solution and kept it for drying. The skin piece of suitable size was mounted on Franz diffusion cell. Phosphate buffer pH 5.5 was filled in the receptor compartment. The donor compartment was the epidermal side of the skin whereas the dermal side was facing the receptor solution. The temperature of diffusion cells was maintained as 37°C using an external constant water circulator. The receiver medium was continuously kept on stirring using magnetic bar.

Fluconazole and curcumin loaded nanoemulsion (the composition shown in Table 2), and Fluconazole and curcumin suspensions in distilled water were studied for skin permeation. The nanoemulsion formulation and the drug suspensions were applied on the skin at donor compartment. 3 ml of samples were withdrawn at a predetermined time intervals and same volume of fresh receptor medium was added each time. The samples were analyzed at 260 and 421nm respectively for fluconazole and curcumin using a spectrophotometer. The experiment was performed in triplicate and the steady-state flux (J_{ss} , µg/cm²/h) was calculated from the slope of the linear portion of the plots drawn for cumulative permeated amount of drug versus time in the steady state conditions. The flux was divided with initial

concentration of the drug in the donor compartment to calculate the permeability coefficient (K_p , cm/h). The enhancement ratios were calculated by dividing the flux (J_{ss}) of the nanoemulsion with the flux of the control drug suspensions^{38,45}

RESULTS AND DISCUSSION

Solubility studies

The solubility profile of fluconazole and curcumin in the selected oil and surfactants has been presented in Table 3. The solubility data along with physicochemical properties of fluconazole and curcumin indicated that it has promising potential for development as topical drug delivery system.

Pseudoternary phase diagram

The pseudoternary phase diagram with various weight ratios of water, surfactant, co-surfactant and oil has been presented as Figure 1. This phase study indicated that the maximum proportion of oil was incorporated in nanoemulsion systems when the ratio of surfactant to co-surfactant was equal (1:1) and formulation of nanoemulsion ratio for water, oil and surfactant was 6:4:6 and 6:5:4. The composition of optimized nanoemulsion system is shown in Figure 1

Microbiological assay of Fluconazole and curcumin

Garcia-Gomes et al., (2012) has reported that curcumin exhibit beneficial effects against resistant type of isolated *C. albicans* when combined with fluconazole³⁰. Sharma et al., 2020 has also depicted the role of curcumin in modulating efflux mediated by yeast ABC multidrug transporters and it was synergistic with antifungals²⁹. In the same line, we studied the combined effect of curcumin with fluconazole against two strains of dermatophytes (*T. rubrum* and *T. metagrophytes*) and reporting it first time. The result of antimicrobial assay is presented in Table 4. The FICI value represents synergistic interactions of curcumin and fluconazole against dermatophytes.

Characterization of nanoemulsion

The stable nanoemulsion formulation was further characterized for polydispersity index, average droplet size and zeta potential. Polydispersity is determined by calculating the ratio of standard deviation to the average droplet size, which is directly proportional to the uniformity of the formulation droplet size. Average globule size of nanoemulsion was found as 149.23

Table 1. Composition of various nanoemulsion formulations

Material used	Formulations										
	BNE1	BNE2	BNE3	BNE4	BNE5	BNE6	BNE7	BNE8	BNE9	BNE10	BNE11
Paraffin light (ml)	16	16	16	14	3	12	12	12	-	-	-
Almond oil (ml)	-	-	-	-	-	-	-	-	16	21	-
Sesame oil (ml)	-	-	-	-	-	-	-	-	-	-	16
Span 80 (ml)	0.5	-	1	-	-	-	4	-	-	-	1
Tween 80 (ml)	0.5	0.5	1	0.8	7	8	-	4	2	8	1
SDS (g)	-	0.5	0.5	0.5	-	-	-	-	0.5	-	-
Ethanol (ml)	3	-	-	-	2	2	2	2	-	3	3
Distilled Water (ml)	80	80	80	86	30	80	84	84	80	80	80

Table 2. Composition of optimized nanoemulsion

Ingredients	Quantity (% w/w)
Fluconazole	5
Curcumin	5
Paraffin light	10
Span 80	2
Tween 80	2
Ethanol	1
Water	75

Table 3. Solubility profile of fluconazole and curcumin

Solvents	Fluconazole	Curcumin
Paraffin light	++++	++++
Sesame oil	+++	+++
Almond oil	+++	+++
Tween 80	++	++
Span 80	++	++

++++ = Very soluble; +++ = Freely soluble; ++ = Soluble

nm with the lowest value of polydispersity index of 0.125. In the present study, low polydispersity index indicated the uniformity of nanoemulsion droplets size within the formulation.

The potential stability of any colloidal system depends on the magnitude of zeta potential. When the globules have a large positive or negative charge on that, the globules/droplets repels each other and hence achieve the dispersion stability. Scientifically, zeta potential value beyond +30 or -30 mV is considered optimum to keep the globules away from each other. The Zeta potential was -45 mV for the optimized formulation which confirms that formulation was stable. The pH of the formulation was in the range of 6.5–7.1 as depicted in the Table 5. This pH range was within the acceptable limits for products meant for skin application. The drug content of the nanoemulsion

was in the range of 88.7–93.9 % and 86.5–91.8% for fluconazole and curcumin respectively for the optimized formulation. The viscosity of the formulation was found to be 11.32 ± 1.3 mPa.s.

Ex vivo skin permeation study

The *ex vivo* skin permeation study of the optimized fluconazole and curcumin loaded nanoemulsion formulation and fluconazole and curcumin suspension was performed and permeation of developed nanoemulsion was found better than the respective suspensions of individual drugs. The flux, permeability coefficient and enhancement ratio are shown in the Table 6. The data represents mean value of three replicates. These results clearly indicate that the fluconazole and curcumin loaded nanoemulsion (BNE1) has shown maximum *ex vivo* drug permeation of the fluconazole and curcumin.

Table 4. FICI for fluconazole and curcumin for dermatophytes

Dermatophytes	FIC of fluconazole	FIC of curcumin	FICI
<i>T. rubrum</i>	0.0031 ± 0.0016	0.0239 ± 0.0079	0.0270 ± 0.0095
<i>T. metagrophytes</i>	0.0053 ± 0.0075	0.0143 ± 0.0051	0.0196 ± 0.0126

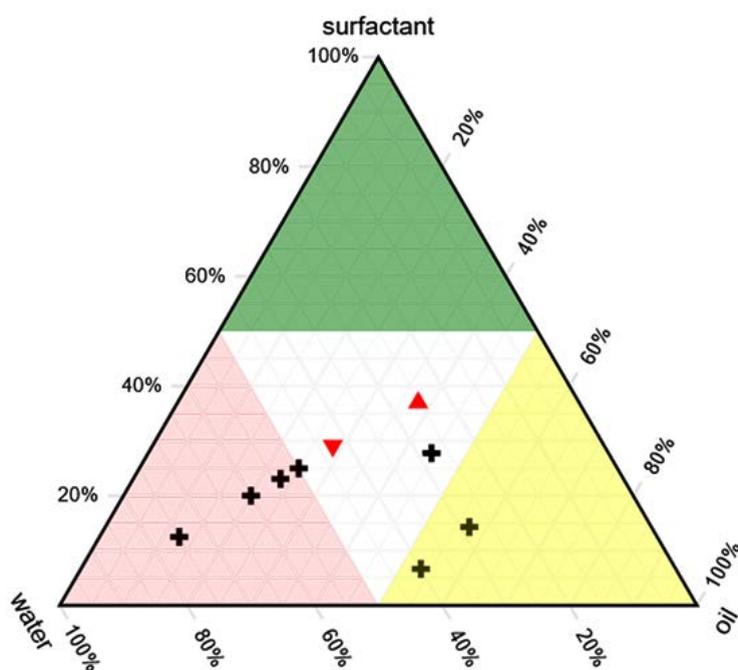


Fig. 1. Pseudoternary phase diagram of nanoemulsion (NE). Various colours indicate different regions of NE: Peach coloured area-micelles and NE; Green area-NE formulation area; Red dots- Optimized NE formation

Table 5. Characterization of nanoemulsion formulation

Parameters	Observations
Clarity	Optically clear
Colour	Slight off white
Transparency	Translucent
Grittiness	No gritty particle
Homogeneity	Homogenous in nature
pH	6.5-7.1
Viscosity	11.32±1.3 mPa.s

The presence of surfactant and co-surfactant in the nanoemulsion formulation might have worked as penetration enhancers and thus affected the structure of stratum corneum and reduced the diffusion barrier. As a thumb rule, nanoemulsions are expected to cross the stratum corneum in its intact form and exist in the whole horny layer without damage to the nanoemulsion system. The nanoemulsion formulation may alter both the lipid and the polar pathways simultaneously after entering into the stratum corneum layer. There

Table 6. Skin permeation parameters

Formulations	Permeability coefficient (cm/h)	Flux ($\mu\text{g}/\text{cm}^2/\text{h}$)	Enhancement ratio
BNE1	4.72×10^{-5}	23.49	1.7
BNE6	4.39×10^{-5}	22.37	1.4
Fluconazole and curcumin suspension	2.56×10^{-5}	13.78	-

are many ways in which interaction occurs between the stratum corneum and the lipophilic domain of the nanoemulsion formulation. The fluconazole and curcumin are present in dissolved form in the lipid domain of a nanoemulsion formulation which helps in increase permeability through lipid layer of stratum corneum. At the same time, the stratum corneum gets hydrated by the hydrophilic domain of the nanoemulsion formulation up to a certain extent.

The percutaneous uptake of poorly soluble drugs largely depends on the degree of hydration of the skin. The interlamellar volume of the lipid bilayer of stratum corneum increases once the aqueous fluid present in the nanoemulsion system enters the polar pathway. This increases the permeation of hydrophobic molecules like fluconazole and curcumin. The combined effects of both the hydrophilic and lipophilic domains of nanoemulsions attributed to the greater drug penetration enhancing activity.

In vitro antifungal activity study against multiple drug resistance *Tricophyton Rubrum* and *Tricophyton Metagrophytes* revealed that the FICI is higher for combination of fluconazole and curcumin as compared to fluconazole and curcumin alone. This may be due to the combination of fluconazole with curcumin is effective in MRD

dermatophyte species. Curcumin has the potential to block "Major Facilitator Superfamily (MFS) transporters" system of fungal cell wall. MFS transporters system is responsible for active efflux of fluconazole from fungal cell. Blocking of MFS transporters system, helps to retain fluconazole in fungal cell and available for action. Nanoemulsion helps in enhancement of deep penetration of drug.

CONCLUSION

In the present study, fluconazole and curcumin were successfully incorporated into a nanoemulsion system under the optimized conditions and adequate use of surfactants, co-surfactants and oil phase which are topically acceptable. The results clearly indicate an optimized delivery of fluconazole and curcumin in a synergistic way from the nanoemulsion formulation. The improved skin penetration of these molecules from the nanoemulsion formulation as compared to the respective drug suspensions can be taken up further for design and development of nanoemulsion based delivery system of these lipophilic drugs for the enhanced antifungal activity against multiple drug resistant dermatophytes, in future.

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Conflict of Interest

The author has no conflict of interest in this research.

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