10. Summary

10.1 Introduction

Fungal infections, such as ringworm and athlete's foot, along with yeast infections and nail afflictions, can cause significant discomfort and have a prolonged duration. These infections are particularly dangerous for individuals with weakened immune systems, potentially leading to severe health complications. A small number of fungal species are responsible for the majority of human fungal infections, affecting over a billion individuals globally and resulting in more than 1.5 million deaths annually. Infections by Candida species, whether in the vagina, eye, mouth, skin, or nails, are especially problematic due to their ability to form biofilms. Other infectious agents like *Pseudomonas aeruginosa* and *Aspergillus niger* also pose serious health risks.

Symptoms of skin fungal infections typically include redness, itchiness, scaly patches, and sometimes painful pus-filled areas. Onychomycosis is a chronic nail fungus infection that often goes unrecognized in countries with limited healthcare resources.

While oral antifungal medications are effective, they can cause adverse reactions due to their systemic distribution. Topical treatments are preferred for their targeted action and reduced risk of side effects. Luliconazole is an imidazole antifungal agent approved in Japan since 2005 for treating skin mycoses. Tavaborole is an oxaborol antifungal agent that inhibits protein synthesis in fungi by binding to leucyl tRNA.

Nanoemulsions and nanostructured lipid carriers (NLCs) are gaining attention for their ability to deliver drugs effectively. They offer advantages like enhanced drug solubility, high loading capacity, and controlled release, which improves patient adherence by reducing the frequency of administration.

10.2 Analytical method development

Developing an analytical method is crucial for the convenient analysis of drugs at various stages of experimental work, such as during formulation development, *in-vitro* and *in-vivo* studies, ex vivo skin deposition/permeation, and stability testing. UV spectrophotometry and HPLC methods have been validated for the quantitative analysis of Luliconazole and Tavaborole across different experimental setups. These methods are employed to create calibration curves in methanol and determine drug entrapment percentages in nanocarriers.

10.3 Preformulation studies

Preformulation studies involve verifying the authenticity of Luliconazole and Tavaborole and their compatibility with excipients. This is done by comparing FTIR spectra, melting points, and lambda max with literature values. The solubility of these drugs in various lipids, oils, and surfactants is also assessed to select suitable components for nanoemulsion and NLC formulations.

For the formulation of Luliconazole and Tavaborole nanoemulsions, solubility tests are conducted in different oils and surfactants. The process involves adding incremental amounts of the drug to 1 ml of solvent until saturation is reached. After centrifugation at 14000 rpm, the supernatant is analyzed using the aforementioned analytical methods. Capmul MCM C8 was selected as the oil phase due to its high solubility capacity. To boost antifungal efficacy, a combination of Capmul MCM C8 and coconut oil was chosen for Luliconazole nanoemulsions. For Tavaborole, despite high solubility in Acconon MC8-2, stability issues led to the selection of the same combination as for Luliconazole. Cremophore EL and Pluronic F127 were chosen as surfactant and co-surfactant respectively.

Solubility of Luliconazole and Tavaborole in lipids such as compritol ATO 888, precirol ATO 5, dynasan 118, softemul AS, softemul SE, glyceryl tristearate, monegyl GN05 and surfactants such as span 40, span 60 were determined. Luliconazole and Tavaborole were added individually to 100 mg of solid lipid at 70°C in a test tube for solid lipid screening. By confirming the lack of drug crystals, it was possible to visually determine the drug's solubility in molten lipid. If the additional drug was soluble, more drug was added until saturation was reached. Solid lipids which can solublize higher amount of Luliconazole and Tavaborole were selected respectively. From the result it was found that Softemul AS and Pluronic F 127 showed highest solubility for Luliconazole so selected as liquid lipid and surfactant respectively. From the result it was found that Softemul SE and Pluronic F 127 showed highest solubility for Tavaborole so selected as liquid lipid and surfactant respectively. Capmul MCM C8 was selected as liquid lipid based on solubility. To determine the optimal lipid blend for Luliconazole and Tavaborole solubilization, selected solid and liquid lipids were combined in varying weight-to-weight ratios (50:50, 60:40, 70:30, and 80:20). These mixtures were stirred at 100 revolutions per minute for half an hour at a temperature of 70ºC. After cooling to ambient temperature (25±1°C), the mixtures were allowed to solidify. The compatibility of the lipid components was assessed by applying a small amount

of the solidified mixture onto filter paper and inspecting it visually for any signs of separation or inconsistency. The 70:30 w/w ratio of solid to liquid lipid emerged as the most suitable combination for NLC formulation, as evidenced by the homogenous appearance of the sample on filter paper.

10.4 Formulation development of Nanoemulsion of Luliconazole and Tavaborole

Optimization of process and formulation parameters was conducted using Box Behnken Design in Design Expert® software. A systematic approach based on Quality by Design (QbD) principles, incorporating statistical experimental designs, was adopted to thoroughly investigate how different materials and process variables influence the key attributes of the formulation. The LZNE and TBNE were synthesized using a high-energy technique. The variables associated with the production of a LZNE and TBNE via a high-energy method were delineated using an Ishikawa diagram. In establishing a reliable, accurate, and repeatable production process, globule size, %EE and %drug release from nanoformulations were considered essential for the Quality Target Product Profile (QTPP). Among them globule size and %EE were selected as critical quality attributes (CQA) based on extensive literature review and preliminary experiment findings.

10.5 Characterization of Nanoemulsion of Luliconazole and Tavaborole:

Globule size, zeta potential, %Entrapment efficiency and Drug loading:

Globule size of the optimized formulation of Luliconazole loaded nanoemulsion and Tavaborole loaded nanoemulsion were found to be 237.26±0.35 nm and 234.32±0.81 nm respectively. Zeta potential of the optimized formulation of Luliconazole loaded nanoemulsion and Tavaborole loaded nanoemulsion were found to be -28.54±0.94 mV and -26.12±1.32 mV respectively. PDI of the optimized formulation of Luliconazole loaded nanoemulsion and Tavaborole loaded nanoemulsion were found to be 0.182±0.04 and 0.202±0.06 respectively. Entrapment efficiency of the optimized formulation of Luliconazole loaded nanoemulsion and Tavaborole loaded nanoemulsion were found to be 80.28±1.14% and 79.82±0.72% respectively.

Centrifugal stability test:

The physical stability of the nanoemulsion was evaluated through a centrifugal stability test. This involved observing the formulation for phase separation after undergoing centrifugation at speeds ranging from 4000 to 10000 rpm for 20 minutes. The nanoemulsion displayed slight

variations in globule size when centrifuged at 4000 to 8000 rpm after 15 days but remained stable with no signs of instability up to 8000 rpm.

Transmittance: Transmittance was observed by using UV spectrophotometer (UV 1900, Shimadzu, Japan) at 630 nm. Transmittance of the optimized formulation of Luliconazole loaded nanoemulsion and Tavaborole loaded nanoemulsion was found to be 98.34±0.21% and 99.16±0.11% respectively.

10.6 Incorporation of Nanoemulsion into gel:

The nanoemulsion was formulated into a gel using Carbopol 974P as a gelling agent, with pH adjustments made using triethanolamine. The resulting gel underwent characterization for its viscosity, gel strength, and pH.

10.7 Formulation development of NLCs of Luliconazole and Tavaborole

The study used a Quality by Design (QbD) approach, incorporating statistical experimental designs, to explore how material attributes and process variables impact the formulation's key properties. Identification of the formulation, process, and environmental aspects that may affect the end product's qualities is crucial before any formulation is created. These variables were thoroughly categorized using an Ishikawa diagram for the high-energy generation of LZ NLCs and TB NLCs. Critical quality attributes (CQAs) that were determined were particle size and %EE based on a thorough examination of the literature and initial experimental runs.

10.8 Characterization of NLCs of Luliconazole and Tavaborole:

Particle size, zeta potential, %Entrapment efficiency and Drug loading:

Particle size of the optimized formulation of Luliconazole loaded NLCs and Tavaborole loaded NLCs were found to be 242±1.38 nm and 235±1.44 nm respectively and Zeta potential of the optimized formulation of Luliconazole loaded NLCs and Tavaborole loaded NLCs were found to be -14.32±1.76 and -23.12±1.43 mV respectively. PDI of the optimized formulation of Luliconazole loaded NLCs and Tavaborole loaded NLCs were found to be 0.231±1.04 and 0.176±0.52 respectively. Entrapment efficiency of the optimized formulation of Luliconazole loaded NLCs and Tavaborole loaded NLCs were found to be 80.32±0.41% and 79.98±1.28 respectively. GC analysis was carried out to check presence of residual solvent present in formulations. Optimized batch of Luliconazole loaded NLCs and Tavaborole loaded NLCs were evaluated for estimation of acetone. The result showed there is no acetone present in the optimized batches.

10.9 Incorporation of NLCs into gel:

NLCs were converted into gel by using 1% Carbopol 974P as gelling agent. The pH was adjusted by using triethanolamine. Gel was characterized for viscosity, gel strength and pH.

10.10 *In-vitro* and ex-vivo studies

The optimized formulations of Luliconazole and Tavaborole were subjected to a series of tests, including *in-vitro* drug release, cytotoxicity and cellular uptake studies, *in-vitro* antibacterial assays, as well as ex-vivo drug permeation and skin retention studies. *In-vitro* drug release study showed that drug release from Luliconazole loaded gel, Luliconazole loaded Nanoemulsion based gel and Luliconazole loaded NLCs based gel was found to be 98.39±1.12%, 95.97±1.96% and 96.61±1.73% respectively. Permeation of drug from Tavaborole loaded gel, Tavaborole loaded Nanoemulsion based gel and Tavaborole loaded NLCs based gel was found to be 99.93±0.74%, 97.61±2.34% and 98.13±1.44% respectively.

Utilizing full-thickness rat skin, the penetration and deposition characteristics of the newly developed formulations were assessed. A Franz diffusion cell, equipped with a 7 ml receptor chamber, served as the evaluation apparatus. The receptor chamber was filled with a phosphate buffer at pH 7.4, and a water bath maintained a constant temperature of 37°C. Samples were collected from the diffusion cell's sampling arm at predetermined time points—1 through 8 hours and at 12 hours—each with a volume of 1 ml. To maintain a consistent volume within the system, an equivalent amount of fresh diffusion media was introduced after each sample collection. Following a 24-hour period, the skin sample was removed from the diffusion cell and washed thrice with 5 ml of diffusion media to calculate drug adherence to the skin. The skin was then minced and immersed in methanol. This mixture was homogenized for five minutes in a chilled environment and sonicated for an additional 15 minutes. Centrifugation at 5000 RPM for 10 minutes facilitated drug extraction from the skin for quantification purposes. A syringe filter with a pore size of 0.2 µm was used to filter all samples before drug quantification using an optimized HPLC method.

Fluorescence microscopy aided in visualizing the permeation behavior of the developed formulations. Formulations containing FITC, both as a suspension and within nanoemulsions and NLCs, were prepared for this study. A confocal laser scanning microscope was employed to observe fluorescence on prepared slides.

The antimicrobial efficacy of Luliconazole and Tavaborole formulations was evaluated against *Candida albicans* and *Trichophyton rubrum* to determine their minimum inhibitory concentration (MIC). The Luliconazole formulations demonstrated MIC values ranging from 0.063-0.07 µg/ml against *Candida albicans* and 0.0018-0.0024 against *Trichophyton rubrum*. Tavaborole formulations showed MIC values between 0.8-1 µg/ml against *Candida albicans* and 1-2 µg/ml against *Trichophyton rubrum*, indicating potent antifungal activity.

The safety profile of Luliconazole-loaded Nanoemulsions, NLCs, as well as Tavaborole-loaded Nanoemulsions and NLCs, was confirmed through their non-toxic effects on 3T3-fibroblast cells, validating their suitability for dermal delivery. Cellular uptake studies revealed that both Luliconazole and Tavaborole formulations achieved higher cellular uptake compared to their free drug counterparts after four hours, suggesting an enhancement in therapeutic efficacy.

In vitro studies focusing on nail fungal infections demonstrated the effectiveness of these formulations in treatment applications.

For in vivo analysis, both pharmacokinetic and pharmacodynamic studies were conducted. The pharmacokinetic study indicated minimal systemic drug absorption, thereby reducing potential side effects. Pharmacodynamic evaluations showcased the superior efficacy of these formulations compared to existing market products.

10.11 Stability study

At intervals of 1, 2, and 3 months, the developed formulations underwent evaluation for parameters such as globule/particle size, zeta potential, and %EE. Stability assessments were conducted at a controlled environment of 30±2°C with a relative humidity of 65±5%. Monthly sample analysis revealed minor increases in globule/particle size and marginal decreases in zeta potential and entrapment efficiency. Nonetheless, the measurements at the three-month interval remained within an acceptable range, affirming the stability of the formulations.

10.12 Conclusion

The development and optimization of lipid-based nanoformulations of Luliconazole and Tavaborole, including nanoemulsions (NEs) and nanostructured lipid carriers (NLCs), have demonstrated significant advancements in the treatment of fungal infections, particularly those affecting the skin and nails. These formulations address the common limitations of traditional topical treatments, such as poor penetration and suboptimal antifungal activity, by utilizing nanocarriers to enhance drug delivery and efficacy.

Through rigorous characterization and optimization, the nanoformulations were successfully developed with optimal drug encapsulation, desired particle size, and uniform distribution. Key parameters, such as zeta potential, pH, viscosity, spreadability, and sustained release profiles, were also found to be within the ideal range for effective topical application. Notably, the formulations exhibited good cellular uptake, with no signs of cytotoxicity, indicating their safety for use on the skin. *In-vitro* and ex-vivo studies showed that LZ NE, LZ NLCs, TB NE and TB NLCs showed sustained drug release compare to free drug containing gel. Skin deposition studies indicated higher amounts of drug(s) deposited in the skin layers in case of NE and NLC in comparison to the free drug. *Ex-vivo* studies further confirmed that the gel-based nanocarrier formulations significantly enhanced the penetration of the active ingredients into the skin layers, thereby improving the therapeutic potential of the drugs.

In vitro antifungal activity, as assessed through the zone of inhibition, showed that the nanoemulsion formulations of Luliconazole and Tavaborole exhibited superior antifungal efficiency compared to their NLC formulations. This suggests that the nanoemulsions may offer a more potent approach for combating fungal infections. However, the difference between the two formulations was not drastically significant, indicating that both approaches could be effective depending on specific treatment needs. Pharmacokinetic analysis demonstrated that the drugs predominantly remained in the skin layers, with very low concentrations reaching the systemic circulation, ensuring that the formulations are likely to be free from systemic toxicity, a key consideration for patient safety. However, the plain drug formulations also showed low plasma concentrations. The superiority of the NE and NLC based formulations is evident from the fact that majority of the drug(s) are deposited in the skin, which is a desirable property in order to treat deep fungal infections. Contrary to this finding, the plain drug formulations have low skin deposition while maximum amount of the drug(s) are retained on the surface of the skin, which makes them unable to treat the deep fungal infections. Pharmacodynamic studies indicated the faster onset of therapeutic response of Tavaborole nanoemulsion (TB NE) compared to Tavaborole NLCs (TB NLCs), but the overall difference was minimal, reinforcing the efficacy of both types of formulations. Similarly, Luliconazole nanoemulsion (LZ NE) and NLCs (LZ NLCs) demonstrated comparable antifungal activity.

In conclusion, the developed lipid-based nanocarrier formulations of Luliconazole and Tavaborole, whether in the form of nanoemulsions or NLCs, offer a promising solution for the

treatment of skin and nail fungal infections. These formulations meet the essential quality criteria outlined in their Quality Target Product Profile (QTPP) and exhibit strong antifungal activity, favorable pharmacokinetic profiles, and a good safety profile. They represent a significant step forward in the development of more effective and safer topical antifungal therapies, particularly for immunocompromised individuals who may be at greater risk of fungal infections. These findings highlight the potential of lipid-based nanoformulations as a viable and advanced therapeutic option in the management of skin and nail fungal infections, paving the way for further clinical exploration and development of nanomedicine in dermatology.