PhD Synopsis on

"Development of Novel lipid based Topical Formulations for treatment of Skin and Nail Fungal Infection"

by

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1. Introduction

Fungal infections (Athlete's foot, finger and toe nail infection, yeast infection, oral thrust and ring worm) are commonly acquired and are known to persist overtime, causing great discomfort. There are also systemic and opportunistic fungal infections which can result in more serious diseases, particularly in patients with compromised immune system. About 30 fungal species cause 99% of human fungal disease burden. Fungal diseases kill more than 1.5 million and affect over a billion people. Nearly a billion people are estimated to have skin nail and hair fungal infection. Fungal infection is more prevalent in tropical countries like India and it has been reported that there is rising prevalence of dermatophytosis in India which is matter of great concern. (1) Occurrence of skin fungal infections is increasing nowadays and their presence is more prominent in patients suffering from immune compromised diseases like AIDS. Skin fungal infections are a major cause of visits by patients to dermatology clinics. Fungi are parasitic microorganisms which can affect the skin and mucous membrane along with generation of systemic infections of various internal organs.[2] It has been reported that 20%–25% of human populations show presence of skin fungal infections. There are numerous fungal infections that may affect the system, manifest in recurrent infections, and pose a challenge to human existence; some of these can lead to fatal consequences. Infection by *Candida* species in the vagina, eye, mouth, skin, or nails, and its generation of biofilms, are a major cause of concern, as are other major infectious agents like Pseudomonas aeruginosa, Aspergillus niger, Fusarium culmorum, Trichophyton rubrum, Epidermophyton floccosum infection as a secondary, Pseudomonas fluorescens, Aspergillus fumigates, and yeast. Fungal infections in humans are a major challenge and can be a life-threatening disorder in human beings particularly immune compromised individuals.(2)

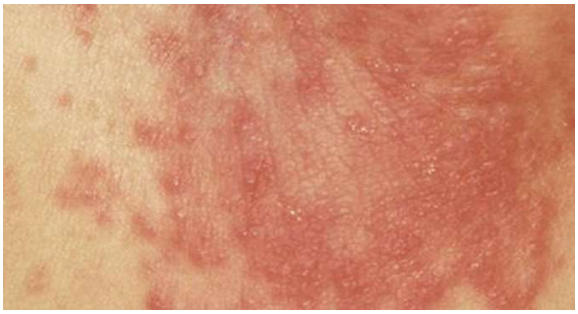

Figure 1: Skin fungal infection[3]

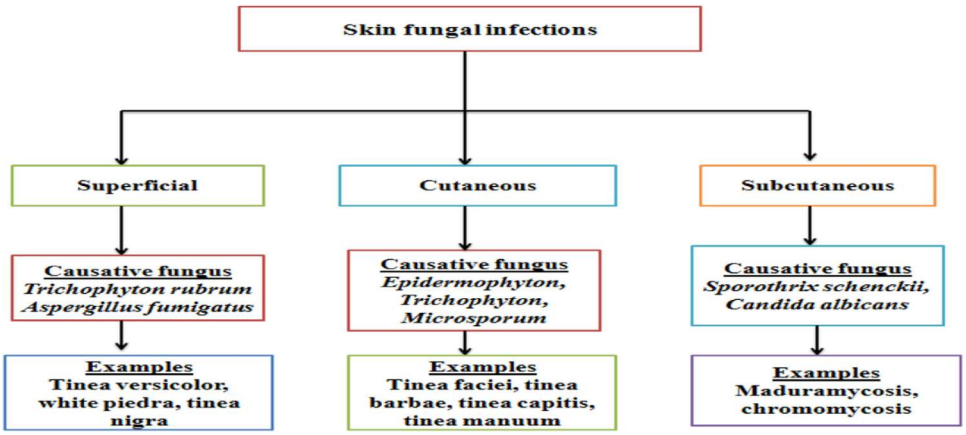

Figure 2: Classification of skin fungal infections depending upon the depth of penetration of parasitic fungus into the skin.[2]

1.1 Nail Fungal Infections:

Onychomycosis is a chronic fungal infection of nails that is largely under diagnosed in developing countries such as India due to poor health care facilities. It affects approximately 5% of the population worldwide and continues to spread and persist. In India, various workers have reported the incidences to vary from 0.5 to 5%. It accounts for approximately 50% of all nail disorders and affects toenails considerably more often than fingernails. The prevalence of onychomycosis has been estimated at around 5% in Western countries and has continued to increase in recent decades.[4] It is most common in adults which may result in infection of feet. People who regularly visit public swimming pool, gyms or shower room have more tendency for nail fungal infection.[5]

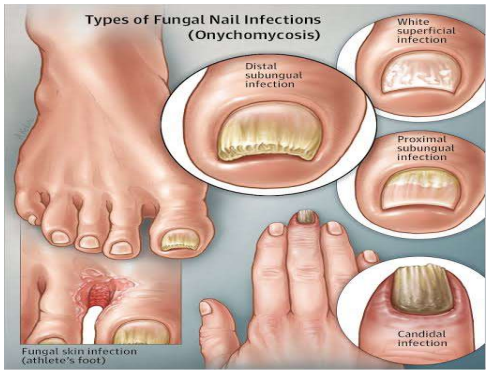

Figure 3: Types of Nail Fungal Infection[6]

1.2 Classification of drugs used for treatment of fungal infection[7]:

1. Antibiotics: Amphotericin b, (amb), Nystatin, Hamcyin, Natamycin, Griseofulvin
2. Antimetabolites: 5-fluorocytosine (5-fc)
3. Azoles:

-Imidazoles: (Topical): Clotrimazole,Econazole, Miconazole, Oxiconazole

(Systemic): Ketoconazole, Luliconazole

-Trizoles: (Systemic) Itraconazole, Fluconazole, Voriconazole

4. Allylamine: Terbinafine
5. Other topical agents: Tolnaftate, Undecylenic acid, Benzoic acid, Quiniodochlor, Ciclopirox olamine, Sodium Thiosulfate

For the treatment of skin as well as nail fungal infection, oral antifungal drugs are prescribed along with topical antifungal drugs. This is because of the poor penetration of the topically applied drugs through the skin and nails resulting in insufficient concentrations for showing the anti-fungal effect. However, orally administered antifungal agents show a large variety of side effects due to non-specific distribution throughout the body. Thus, topical application of anti-fungal drugs is more preferable compared to oral administration due to less chance of systemic side effects and complications because of limited systemic absorption. Moreover, topical preparations used in case of skin and nail fungal infections present the drug in close proximity to the infected site leading to more efficient therapy.[8] Topical preparations are very effective in superficial, cutaneous and sub-cutaneous skin infections as well as onchomycosis, their effectiveness is reduced because of inability of the drug to permeate through the skin and nail. Thus, the present investigation aims at development of improved topical formulation of selected anti-fungal agents.

Luliconazole ($C_{14}H_9Cl_2N_3S_2$) is an imidazole antifungal drug that approved for the treatment of cutaneous mycosis in Japan from 2005 as 1% cream (Luzu).[9] Luliconazole has been shown to have activity against a variety of fungi, including yeast, dermatophytes, and dermataceous fungi, and has significant fungicidal activity against *Trichophyton spp.* The MIC of Luliconazole against *Candida spp.* has been reported to be higher than that against filamentous fungi; however, it is similar to Lanoconazole and greater than that of Bifonazole, Terbinafine, and Amorolfine. The MIC against *C. albicans* was higher than that of Ketoconazole, Clotrimazole, Neticonazole, and Miconazole.[10] Luliconazole has been shown to be many times more effective

than Lanoconazole and Bifonazole in inhibiting 14α demethylase of *C. albicans*. Brown et al recently demonstrated that therapeutic levels of Luliconazole were achieved across full-thickness human nail plate within 7 days of daily dosing with 10% Luliconazole solution in an in vitro *T. rubrum*-infected nail model. Low binding affinity for keratin allows Luliconazole to be released from the keratinous nail plate and be transported across the nail bed. In contrast with many other azoles, its potency remains unaffected by keratin.[11] Luliconazole has excellent tolerability and no systemic side effects were reported when used as topical preparation. It is believed that Luliconazole produces its antifungal effect by inhibiting the synthesis of ergosterol, which is a constituent of the cell membrane of fungi. Nanocrystal loaded hydrogel[12] for skin infection and 5% solution[13] of Luliconazole for nail fungal infection are reported for improvement of dissolution and antifungal activity for skin infection. Marketed formulations of Luliconazole includes LUZU, Luzicon, Luliconaz, Lulisen, Lulivib, Lucinak, Lulifin, Lulix, Lofatin, Lu-gal, Lutoz, Lulicona, Lolyzole, Lunader and Lulibet which all contain Luliconazole 1%w/w cream. Other products are Luzicute and Lilituf (Luliconazole lotion 1%w/v). Marketed formulations have limitations like lower skin permeation and shorter skin retention of drug.[12]

Tavaborole is a synthetic oxaborol antifungal agent. It binds to the editing site through its boron atom to trap leucyl tRNA which prevent its catalytic turnover and inhibit protein synthesis in fungus. It is active against dermatophytes, fungi, molds and yeasts and it is used for topical treatment of mycosis. Based on research, it is concluded that Tavaborole is superior in all aspects like broad spectrum antifungal, low MIC for various fungus species, high mycological cure rates, low molecular weight so penetration through skin is increasing, lower relapse rates in comparison to other class of anti fungals like imidazole, triazoles, polyne antimycotics, pyridine analogues etc. Topical solution (5%) of Tavaborole is commercially available for treatment of fungal infection.

1.3 Topical Drug Delivery System:

Common problem for topical delivery is the poor penetration ability of the drug and low retention time. Different approaches are used for enhancing the penetration and prolonging the retention of the drugs after topical application. Nanotechnology is a modern and rapidly evolving trend in topical drug delivery which includes several forms of nanocarriers such as liposomes, nanoemulsions, nanocrystals, polymeric nanoparticles, lipid nanocarriers and dendrimers. Lipid based drug delivery systems are preferable as nanocarriers for anti-fungal drugs due to the

inherent antifungal activity of some of the lipids, which may give synergistic effect.[18] There are number of essential oils such as lemongrass oil, Eucalyptus oil, Cinnamon bark oil, Fennel oil, Peppermint oil reported to possess antifungal activity[19-20]. In the present investigation, Nanoemulsion and Nanolipid carriers have been selected as the lipid based nanocarriers for loading of the anti-fungal agents.

Nanoemulsions (NEs) and microemulsions are kinetically stable colloidal nanocarriers that exhibit low viscosity and homogenous appearance. They offer a series of advantages: reduced droplet size, improved solubility of poorly water-soluble drugs, high drug loading capacity, high drug permeation rate and low cost preparation which account for their broad utilization in pharmaceutical and cosmetic industries.[21] Nanoemulsion[22] and microemulsion[23] are reported for enhancement of drug permeation through skin and nail. Mechanism of drug release from microemulsion in transangual route is shown in below figure. Nanoemulsion require less concentration of surfactant and cosurfactant compared to microemulsion resulting in less toxicity, Thus nanoemulsion is selected as lipid nanocarrier. However, the crucial feature of NEs is their capacity of improving permeation into the skin by offering high solubilizing potential for lipophilic and hydrophilic drugs, providing large surface area and good skin contact, and a direct permeation-enhancing effect through the *stratum corneum* (SC) due to their composition of oil and surfactants.[22] Release of drug from nanoemulsion through skin is shown in figure. Nanoemulsion of various essential oil such as lemongrass oil, Eucalyptus oil, Cinnamon bark oil, Fennel oil, Peppermint oil also reported for antifungal activity.[19-20]

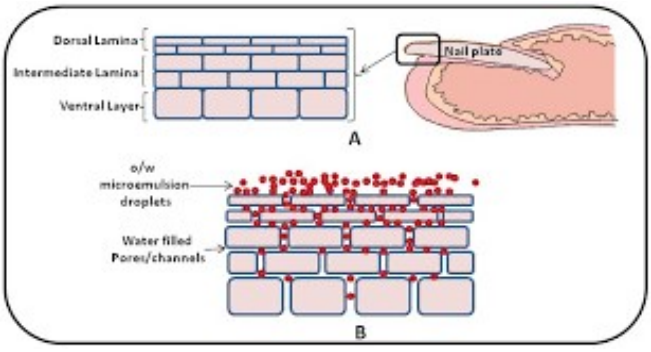

Figure 4: Drug permeation through transungual route from microemulsion[23]

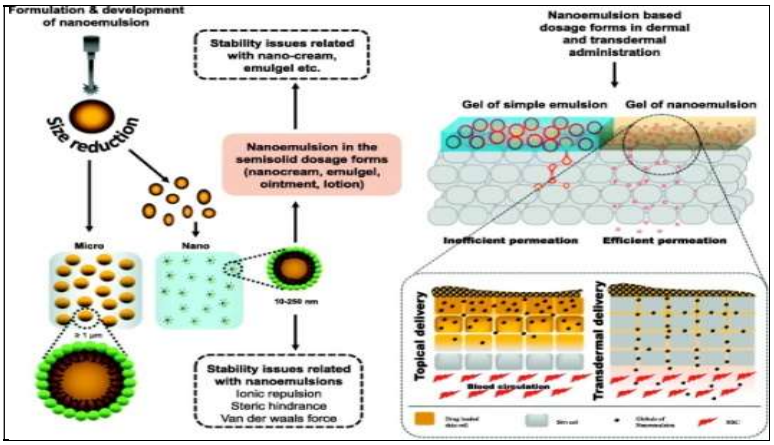

Figure 5: Drug permeation through skin from Nanoemulsion[24]

Lipid nanocarriers like SLN and NLC show essential advantages over conventional drug forms and they are formulated with biodegradable, non-toxic and non-irritant lipids. The small size (from 40 to 800 nm) of lipid nanocarriers allows to adhere them to the lipid film of SC and to increase the number of drug molecules that penetrate into deeper layers of the skin.[25] Solid Lipid Nanoparticles are composed of lipids that are solid at room temperature with a surface covering of surfactant to stabilize them as a nano-dispersion. SLN enhance skin permeation by prolonging contact with the skin surface, providing an occlusive barrier that hydrates the skin, and interacting with the lipids in the stratum corneum bilayers.[26] Nanostructured lipid carriers colloid systems composed of a fluid lipid phase embedded into a solid lipid matrix or localized at the surface of solid platelets and the surfactant layer[27]. The spatial structure of the lipids allows greater drug loading and better stability compared to SLN.[28]

Development, characterization and establishment of efficacy of drug loaded NLCs is now a current topic for the drug delivery and targeting. Since most of the drugs are lipophilic in nature, their solubility in biocompatible liquid lipids is a key factor for NLC development. NLCs are explored in the drug targeting in various diseases.[28] Therefore, less amount of drugs will be required in dosage form. Voriconazole NLC[27] and Terbinafine NLC[28] are reported for enhancing drug penetration in skin and diseased nail plate respectively.

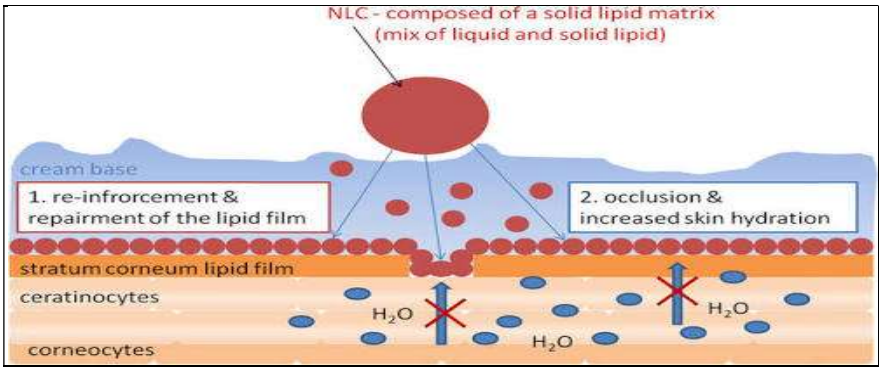

Figure 6: Drug permeation through skin from NLC[29]

2. Objective:

Development of Novel lipid based Topical formulations for treatment of Skin and Nail fungal infection.

3. Analytical Method Development:

3.1 Estimation of Luliconazole by UV Spectrophotometry:

3.1.1 Preparation of Luliconazole drug stock solution:

10 mg of accurately weighed Luliconazole was transferred to 10 ml volumetric flask. Small quantity of methanol was added to the volumetric flask to ensure complete dissolution of drug. Finally, volume was made up to the mark with methanol which resulted in a stock solution of concentration 1000 µg/ml. From the above prepared solution, 1 ml of this stock solution was withdrawn with the help of micropipette and transferred to 10 ml volumetric flask to give a solution of 100µg/ml. From the above prepared solution, 1 ml of this stock solution was withdrawn with the help of micropipette and transferred to 10 ml volumetric flask to give a solution of 10 µg/ml.

3.1.2 Determination of λ_{max}:

The solution thus prepared (10 µg/ml) of Luliconazole, was scanned in the range of 200 to 400 nm using methanol as blank using UV-Visible spectrophotometer. The wavelength at which maximum absorbance was observed, was selected as the analytical wavelength.

3.1.3 Calibration plot of Luliconazole in Methanol:

From the Luliconazole stock solution (100 µg/ml) in methanol, aliquots of 1,1.5, 2, 2.5, and 3 ml were accurately withdrawn with help of pipette and transferred to separate 10 ml volumetric flasks and the volume were made up to the mark with methanol to give final concentration 10, 15, 20, 25 and 30 µg/ml. The absorbance of all the prepared solutions was measured at the absorption maxima of 265 nm, using methanol as a blank.[30]

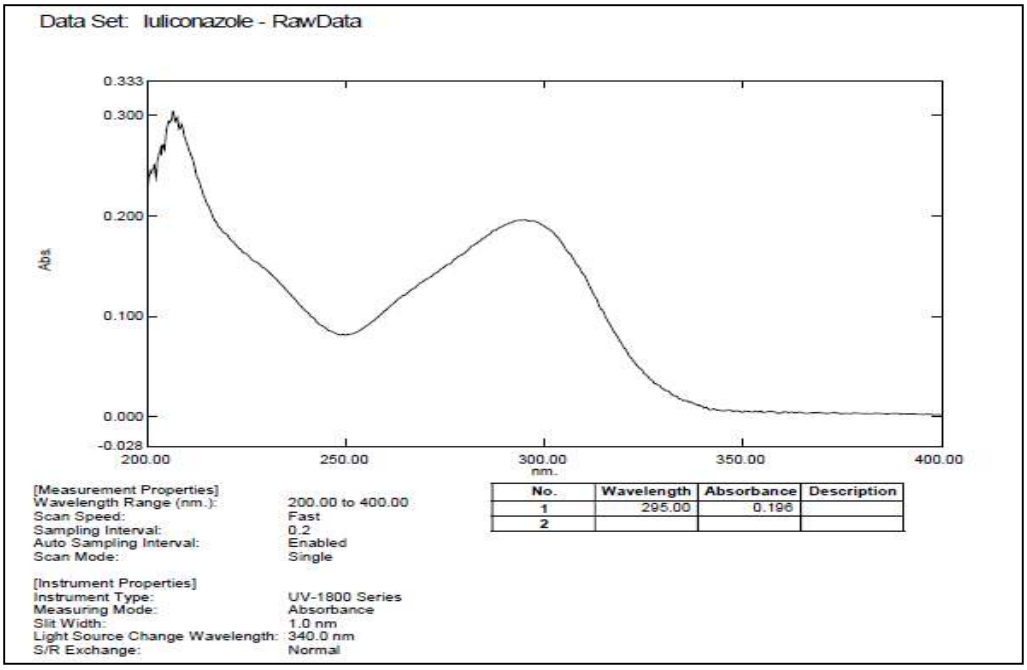

Figure 7: UV Spectrum of Luliconazole in Methanol

Table 1: Calibration data for estimation of Luliconazole in Methanol:

Sr. No.	Conc. (µg/ml)	Absorbance
1	10	0.201±0.001
2	15	0.304±0.001
3	20	0.408±0.001
4	25	0.532±0.007
5	30	0.664±0.025

*All the experiments were performed in triplets

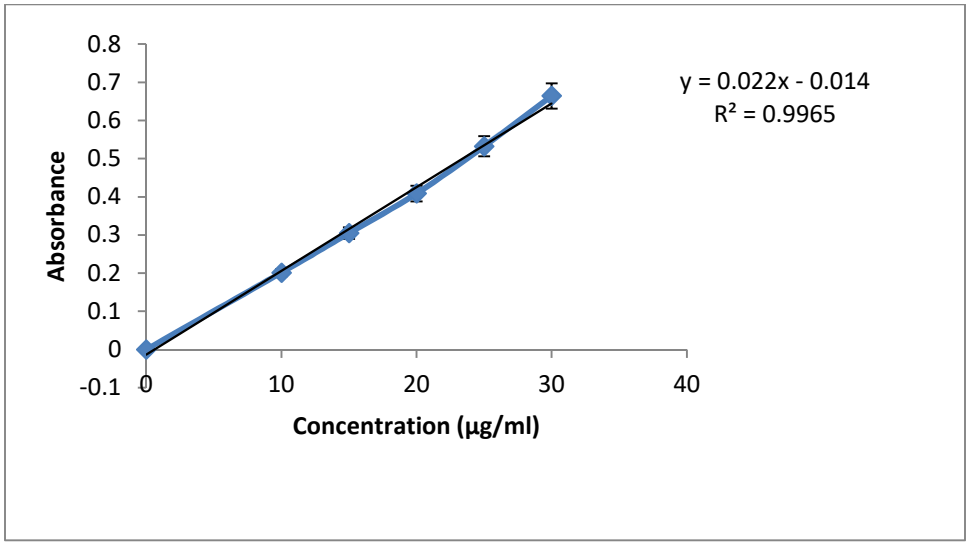

Figure 8: Calibration plot of Luliconazole in Methanol

As shown in the above figure, the calibration plot of Luliconazole in Methanol was found to be linear in the concentration range from 10-30 µg/ml with high value of regression coefficient (R^2 = 0.996) which shows that the Luliconazole obeys Beer's law between 10-30 µg/ml.

Table 2: Parameters from calibration plot of Luliconazole in Methanol

λ_{max}	Solvent	Concentration Range	Regression Equation	Correlation Coefficient
295 nm	Methanol	10-30 µg/ml	0.023x-0.039	0.996

3.2 Analytical method development of Luliconazole on HPLC

Instrument: Agilent binary gradient
Column: Welchrom®, C18, 5 µm, 4.6x250 mm
Mobile phase: Methanol: Water: ACN (75:25:10)
Run time: 10 min
Flow rate: 1 ml/min
λ_{max}: 295 nm
Retention time: 7.1 min

3.2.1 Preparation of Mobile Phase: For the preparation of mobile phase, Methanol (HPLC grade), double distilled water (filtered by 0.45 µm vacuum filter) and ACN were mixed in ratio of 75:25:10. This solution was sonicated for 3 cycles to ensure degassing and complete mixing of both the phases.

3.2.2 Preparation of stock solution: Accurately weighed 10 mg of Luliconazole was transferred to a 10 ml volumetric flask containing 3 ml Methanol (HPLC grade) and was properly shaken to ensure complete dissolution. The volume was made up to 10 ml using Methanol (1000µg/ml). From the above prepared solution, 1 ml of stock solution was withdrawn with the help of micropipette and transferred to 10 ml volumetric flask to make 100µg/ml solution. From the above prepared solution, 1 ml of this stock solution was withdrawn with the help of micropipette and transferred to 10 ml volumetric flask to make 10 µg/ml solution. From this solution, aliquots of 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1 ml and 1.2 ml were withdrawn and transferred to separate 10 ml volumetric flasks. The volume was made up to 10 ml using Mobile phase to make dilutions of 200, 400, 600, 800, 1000 and 1200 ng/ml respectively. 20µl of these samples were then injected in the HPLC system using HPLC syringe.[31]

Table 3: Data of Calibration plot in Methanol:Water:ACN (75:25:10)

Sr. No.	Concentration of sample (ng/ml)	Peak Area±S.D
1	0	0
2	200	1,22,049±135.05
3	400	2,55,080±83.44
4	600	3,95,765±105.63
5	800	5,67,735±95.18
6	1000	6,74,499±125.77
7	1200	8,48,506±100.06

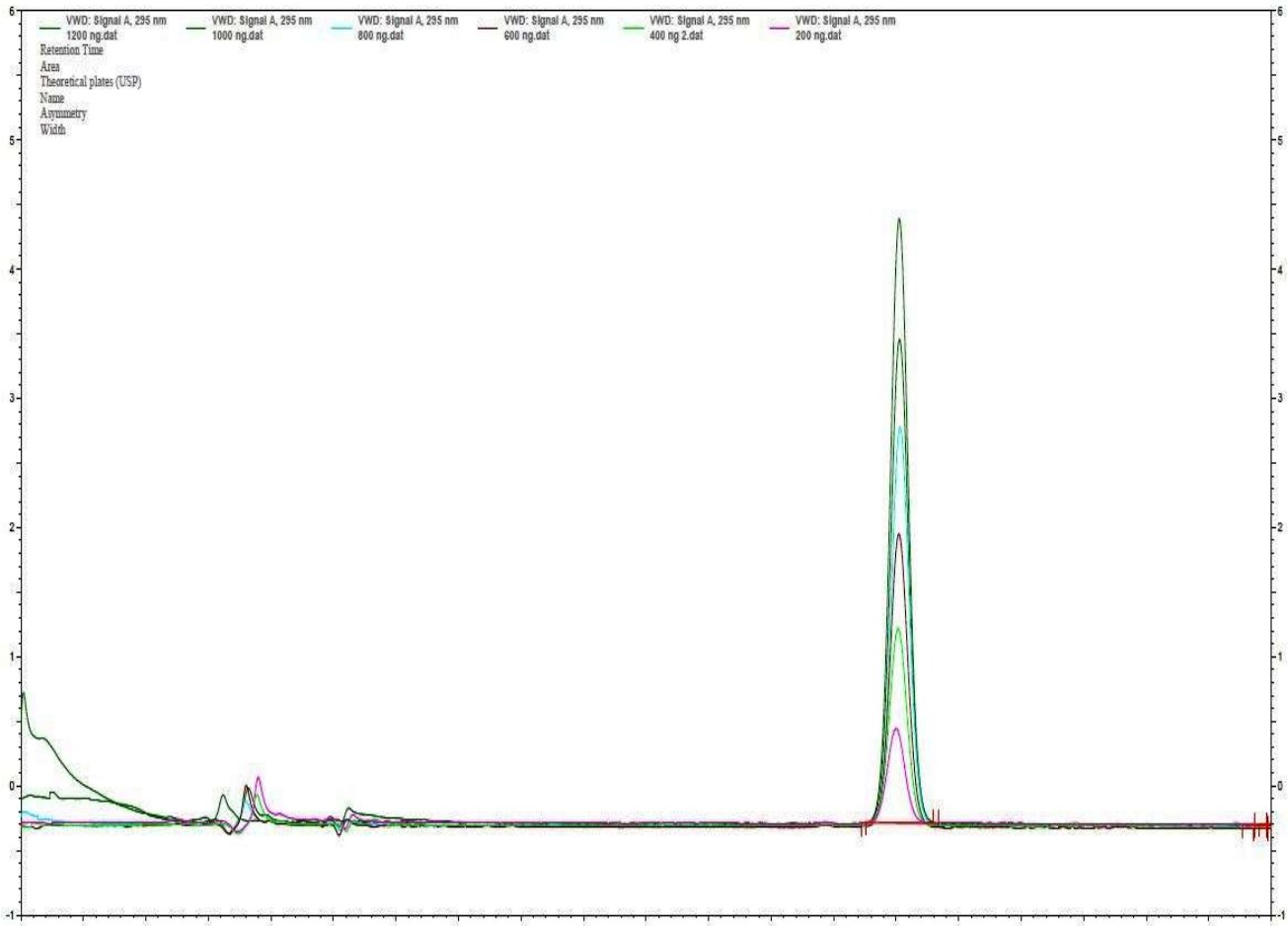

Figure 9: Overlay Chromatogram of Luliconazole by HPLC

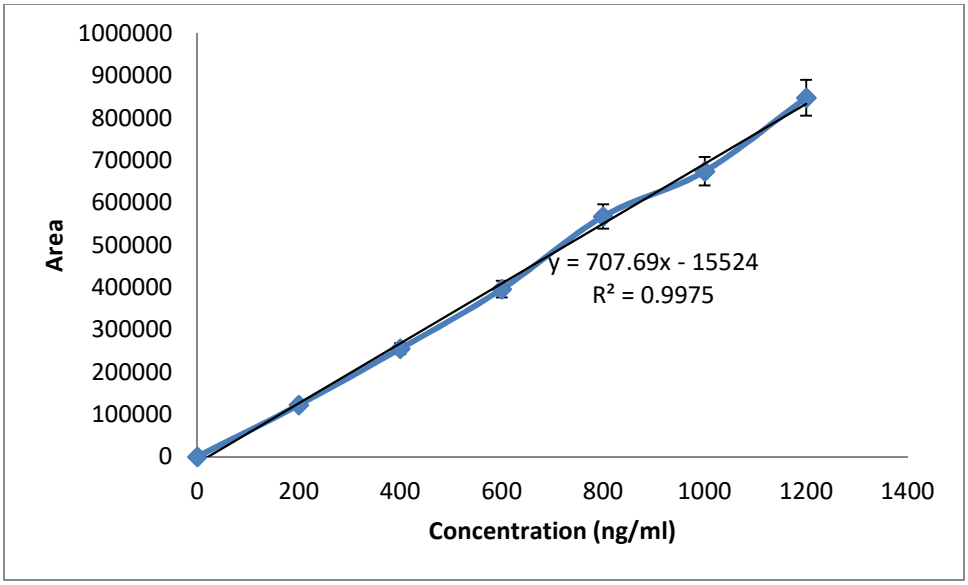

Figure 10: Calibration plot of Luliconazole by HPLC

Table 4: Parameters from calibration plot

λ_{max}	Solvent	Linearity Range	Regression Equation	Correlation Coefficient
295 nm	Methanol: Water: ACN	200-1200 ng/ml	y = 707.6x - 15524	0.997

3.3 HPLC method development for estimation of drug in skin homogenate:

Instrument: Agilent binary gradient
Column: Welchrom®, C18, 5 µm, 4.6x250 mm
Mobile phase: Methanol: Water: ACN (75:25:10)
Run time: 10 min
Flow rate: 1 ml/min
λ_{max}: 295 nm
Retention time: 7.2 min

3.3.1 Preparation of Mobile Phase: For the preparation of mobile phase, Methanol (HPLC grade), double distilled water (filtered by 0.45 µm vacuum filter) and ACN were mixed in ratio of 75:25:10. This solution was sonicated for 3 cycles to ensure degassing and complete mixing of both the phases.

3.3.2 Preparation of stock solution: Accurately weighed 10 mg of Luliconazole was transferred to a 10 ml volumetric flask containing 3 ml Methanol (HPLC grade) and was properly shaken to ensure complete dissolution. The volume was made up to 10 ml using Methanol (1000µg/ml). From the above prepared solution, 1 ml of stock solution was withdrawn with the help of

micropipette and transferred to 10 ml volumetric flask to make 100 µg/ml solution. From the above prepared solution, 1 ml of this stock solution was withdrawn with the help of micropipette and transferred to 10 ml volumetric flask to make 10 µg/ml solution.

3.3.3 Preparation of samples in skin homogenate:

Rat skin was obtained from the animal house of Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Gujarat, India under the IAEC (institutional animal ethics committee) approval. Isolated rat skins were thoroughly cleaned with PBS pH 7.4. Care was taken while separating full thickness of rat skin and was done with the help of forceps and scalpel. Fat present in the skin was cleaned and then the skin was thoroughly inspected for surface and thickness. Afterwards skin was cut into very small pieces and homogenized by using homogenizer. After that 2 ml of skin homogenate was taken into 6 volumetric flasks of 10 ml separately. From the stock solution of Luliconazole (10 µg/ml), aliquots of 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1 ml and 1.2 ml were withdrawn and transferred into 10 ml volumetric flasks accordingly. Afterward 100 µl of methanol was added in each volumetric flask. This mixture was mixed using vortex for 2 minutes. The volume was made up to 10 ml using methanol to make dilutions of 200, 400, 600, 800, 1000 and 1200 ng/ml respectively. All the samples were centrifuged at 3500 rpm for 10 minute at 10°C. Supernant layer was collected and analysed and if necessary do the further dilution.[32]

Table 5: Data of Calibration plot in Methanol:Water:ACN (75:25:10)

Sr. No.	Concentration of sample (ng/ml)	Peak Area±S.D
1	0	0
2	200	1,42,496±135.05
3	400	2,55,080±88.48
4	600	4,77,613±102.26
5	800	6,67,028±86.28
6	1000	7,68,818±105.87
7	1200	10,07,422±95.06

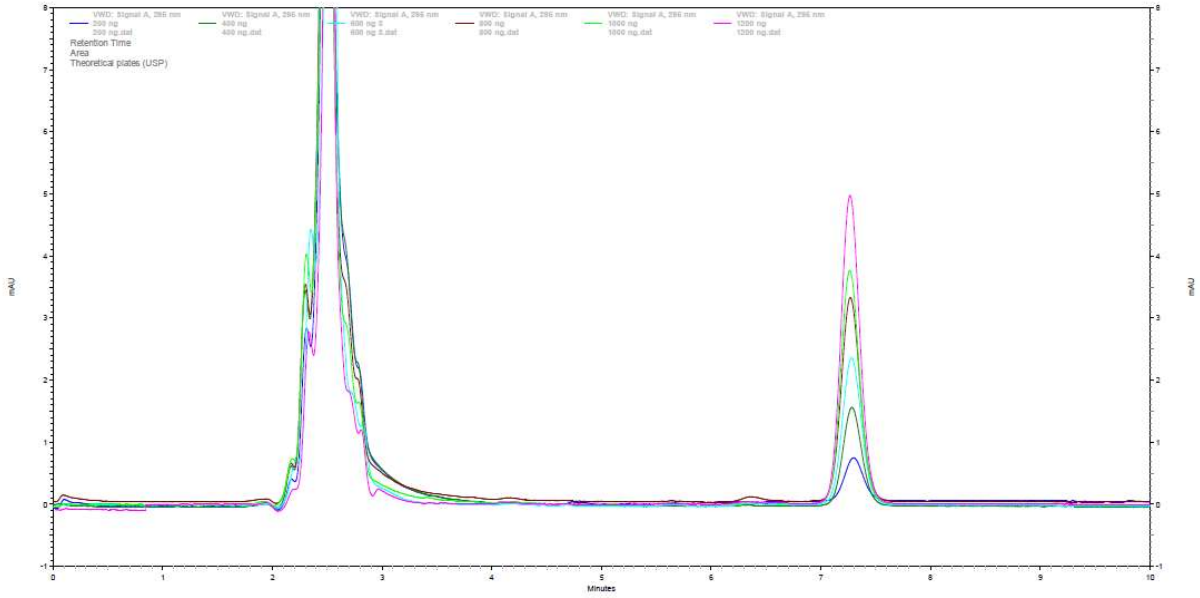

Figure 11: Overlay Chromatogram of Luliconazole by HPLC in skin homogenate

3.4 Analytical method development of Tavaborole on HPLC

Instrument: Agilent binary gradient
Column: Welchrom®, C18, 5 µm, 4.6x250 mm
Mobile phase: Phosphoric acid solution (10 mM, pH 2.0): ACN(60:40)
Run time: 10 minute
Flow rate: 1 ml/min
Retention Time: 7.18 min
λ_{max}: 214 nm

3.4.1 Preparation of Mobile Phase:

For the preparation of mobile phase, 10 mM Phosphoric acid was prepared in double distilled water (pH 2.0) and ACN were mixed in ratio of 60:40. This solution was filtered through 0.45 µ filter by vacuum filter and sonicated for 3 cycles to ensure degassing and complete mixing of both the phases.

3.4.2 Preparation of stock solution:

Accurately weighed 5 mg of Tavaborole was transferred to a 5 ml volumetric flask containing 3 ml ACN (HPLC grade) and was properly shaken to ensure complete dissolution. The volume was made up to 5 ml using ACN (1000µg/ml). From this solution, 0.1ml was withdrawn and transferred to another 10 ml volumetric flask. The volume was made up to 10 ml using mobile phase (10µg/ml). From this solution, aliquots of 0.5 ml, 1 ml, 1.5 ml and 2 ml, 2.5 ml were withdrawn and transferred to separate 10 ml volumetric flasks. The volume was made up to 10

ml using Mobile phase to make dilutions of 0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml and 2.5 µg/ml respectively. 20 µl of these samples were then injected in the HPLC system using HPLC syringe.[33]

Table 6: Data of Calibration plot in OPA:ACN (60:40)

Sr. No.	Concentration (µg/ml)	Area±SD
1	0	0
2	0.5	406760±80.83
3	1	724286±90.91
4	1.5	1150094±112.18
5	2	1499271±85.91
6	2.5	2034394±98.04

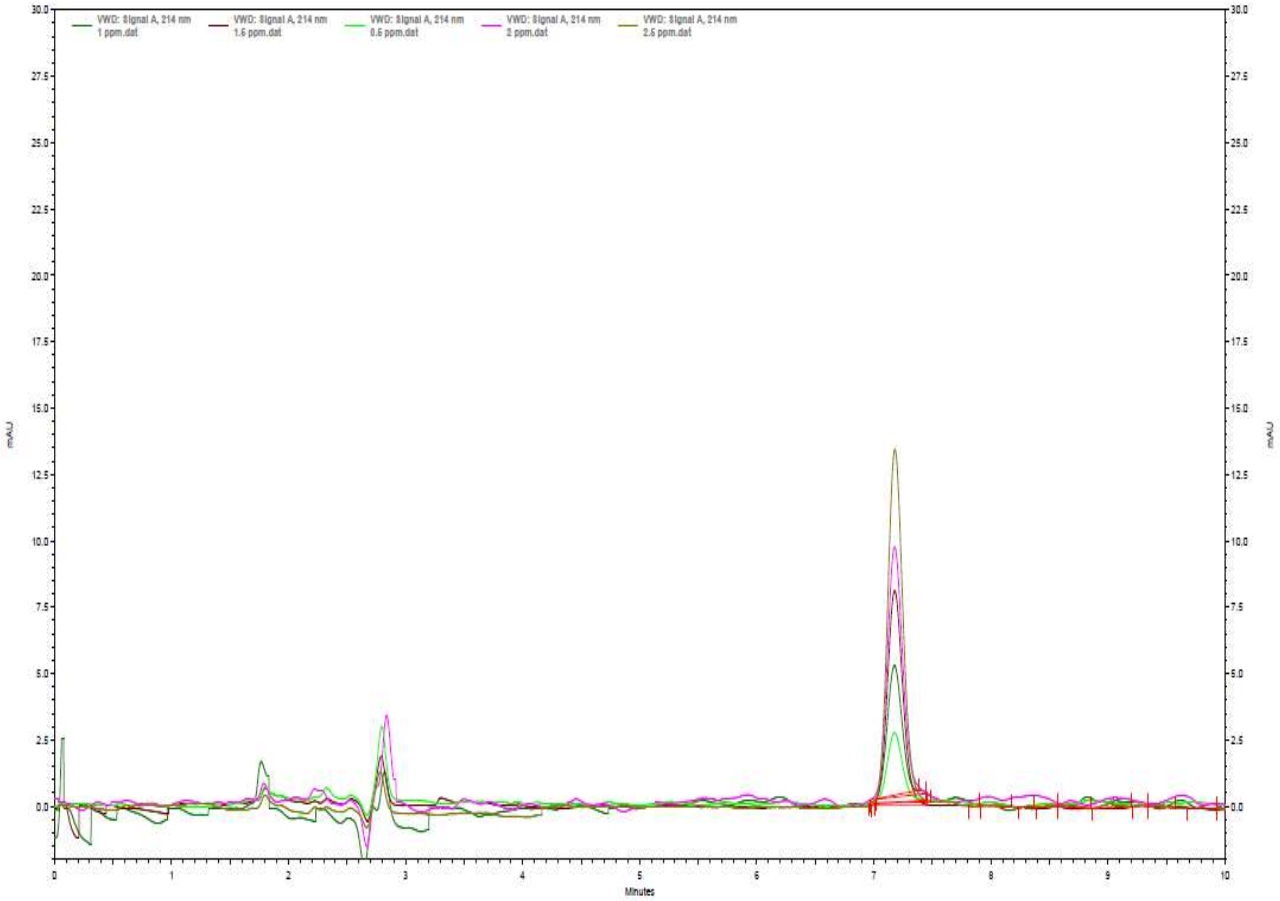

Figure 12: Overlay Chromatogram of Tavaborole by HPLC

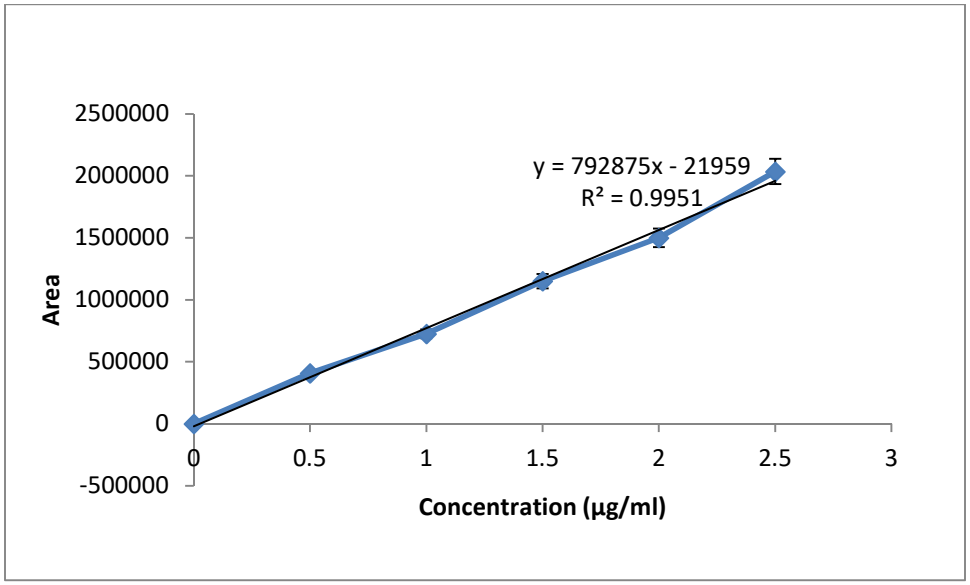

Figure 13: Calibration plot of Tavaborole by HPLC

Table 7: Parameters from calibration plot:

λ_{max}	Mobile Phase	Linearity Range	Regression Equation	Correlation Coefficient
214 nm	Phosphoric acid (10 mM, pH 2.0): ACN (60:40)	0.5-2.5 µg/ml	y = 79287x - 21959	0.995

3.5 HPLC method development of estimation of drug in skin homogenate:

Instrument: Agilent binary gradient

Column: Welchrom®, C18, 5 µm, 4.6x250 mm

Mobile phase: Ortho Phosphoric acid solution (10 mM, pH 2.0): Acetonitrile (60:40)

Run time: 10 minute

Flow rate: 1 ml/min

Retention Time: 7.2 min

λ_{max}: 214 nm

3.5.1 Preparation of Mobile Phase:

For the preparation of mobile phase, 10 mM Ortho Phosphoric acid (OPA) was prepared in double distilled water (pH 2.0) and Acetonitrile (ACN) were mixed in ratio of 60:40. This solution was filtered through 0.45 µ filter by vacuum filter and sonicated for 3 cycles to ensure degassing and complete mixing of both the phases.

3.5.2 Preparation of stock solution:

Accurately weighed 5 mg of Tavaborole was transferred to a 5 ml volumetric flask containing 3 ml ACN (HPLC grade) and was properly shaken to ensure complete dissolution. The volume was made up to 5 ml using ACN (1000µg/ml). From this solution, 0.1ml was withdrawn and transferred to another 10 ml volumetric flask. The volume was made up to 10 ml using mobile phase (10µg/ml).

3.5.3 Preparation of samples in skin homogenate:

Rat skin was obtained from the animal house of Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Gujarat, India under the IAEC (institutional animal ethics committee) approval. Isolated rat skins were thoroughly cleaned with PBS pH 7.4. Care was taken while separating full thickness of rat skin and was done with the help of forceps and scalpel. Fat present in the skin was cleaned and then the skin was thoroughly inspected for surface and thickness. Afterwards skin was cut into very small pieces and homogenized by using homogenizer. After that 2 ml of skin homogenate was taken into 6 volumetric flasks of 10 ml separately. From the stock solution of Tavaborole (10 µg/ml), aliquots of 0.5 ml, 1 ml, 1.5 ml and 2 ml, 2.5 ml were withdrawn and transferred into 10 ml volumetric flasks accordingly. Afterward 100 µl of methanol was added in each volumetric flask. This mixture was mixed using vortex for 2 minutes. The volume was made up to 10 ml using methanol to make dilutions of 0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml and 2.5 µg/ml respectively. All the samples were centrifuged at 3500 RPM for 10 minute at 10°C. Supernant layer was collected and analysed and if necessary do the further dilution.[32]

Table 8: Data of Calibration plot in OPA:ACN (60:40)

Sr. No.	Concentration (µg/ml)	Area±SD
1	0	0
2	0.5	441770±78.23
3	1	752793±89.27
4	1.5	1282153±91.18
5	2	1537159±80.31
6	2.5	2284726±88.17

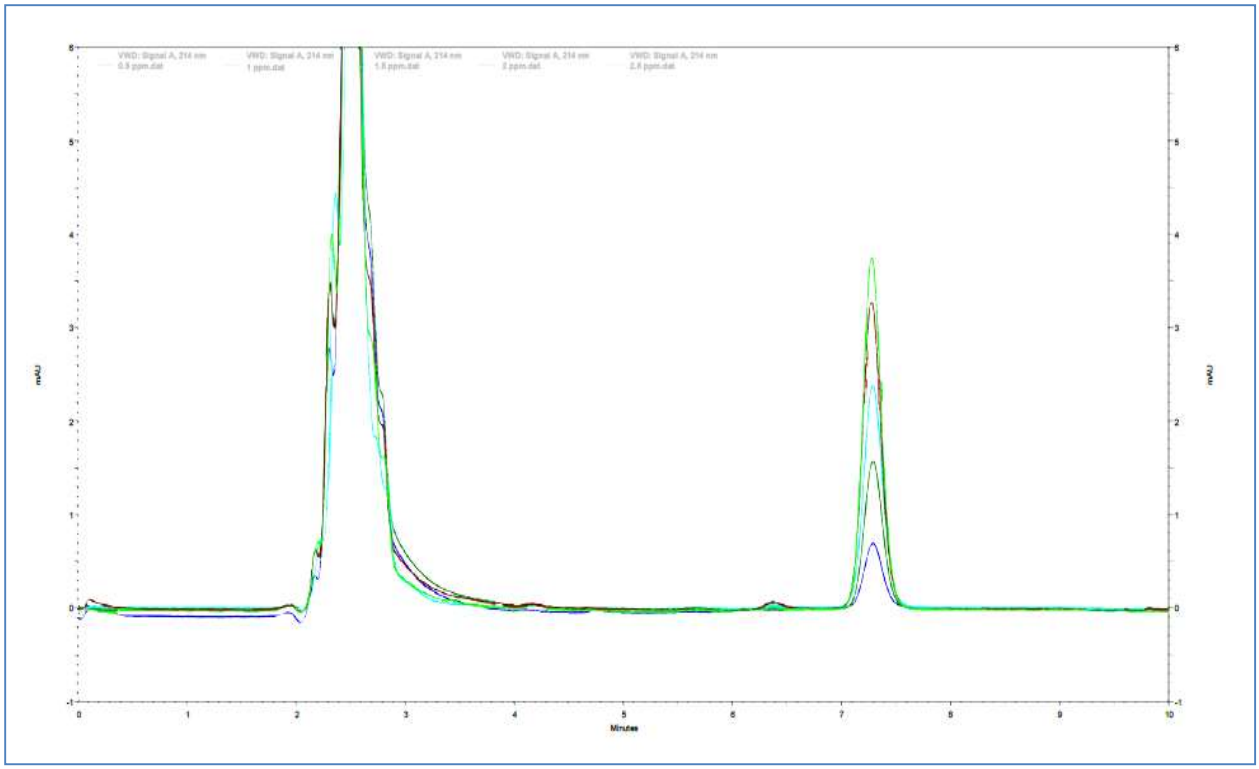

Figure 14: Overlay Chromatogram of Tavaborole by HPLC in skin homogenate

4. Preformulation studies:

4.1 Melting point:

Melting point of the Luliconazole and Tavaborole was found to be 152 °C and 120°C respectively which were measured by the capillary method using melting point apparatus.

4.2 Infrared Spectroscopy (IR Spectroscopy):

The IR spectrum was recorded in the wavelength region of 4000 cm-1 to 400 cm-1 using FT-IR spectrophotometer (BrukerOptik GmbH, Germany). The sample of pure drug was dispersed uniformly in potassium bromide (KBr) by triturating it in mortar and compressed at a pressure of 5 tons for 5 min in a hydraulic press to form a pellet. The pellet was placed in the path-length of IR spectrophotometer and the spectrum was obtained which was compared with the standard spectra. FTIR results indicate the excipients compatibility between Luliconazole and the physical mixture of Luliconazole and excipients as well as Tavaborole and physical mixture of Tavaborole and excipients.[34]

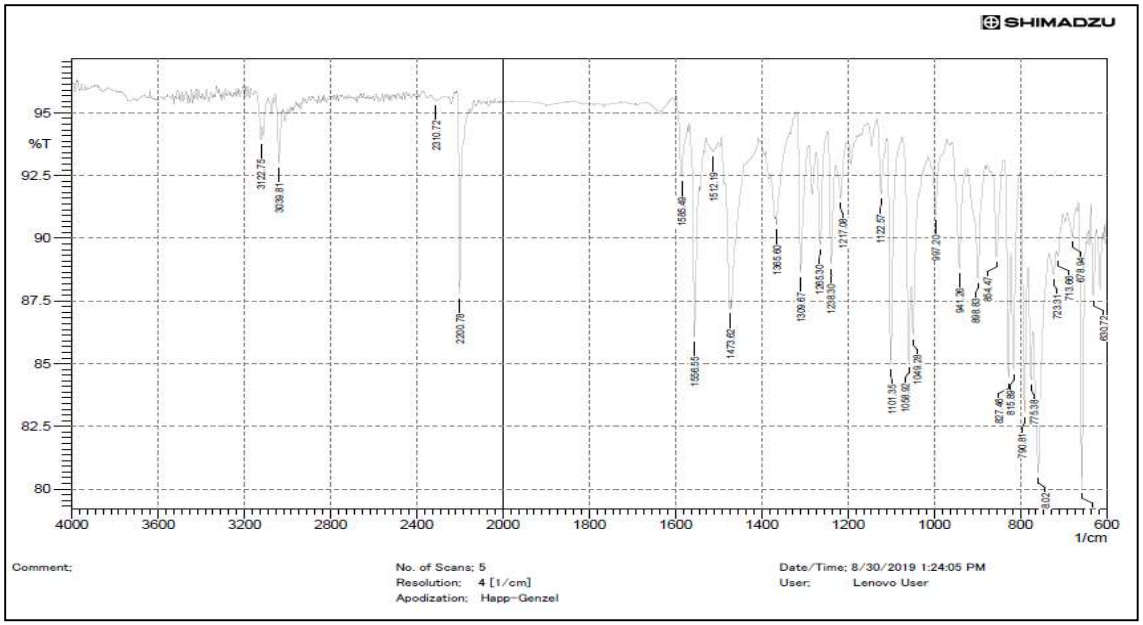

Figure 15: FTIR spectra of Luliconazole

Table 9: Interpretation of FTIR of Luliconazole

Type of Vibration	Standard	Observed	Inference
C-H Aromatic stretch	3000-3100	3122.75 3039.81	Present
C-H aliphatic stretch	2840-3000	2941	Present
S-H stretching	2550-2600	2527 2614	Present
C- N Stretch	2222-2260	2310 2200	Present
C=C aromatic stretch	1450-1650	1512 1556	Present
Aromatic C=C For Chlorobenzene	1446,1478,1584	1473 1585	Chlorobenzene present
C-Cl stretch	550-850 1089-1096	678	Chloro group present

In the table 9, the comparison between observed and standard peaks showed authenticity of Luliconazole.

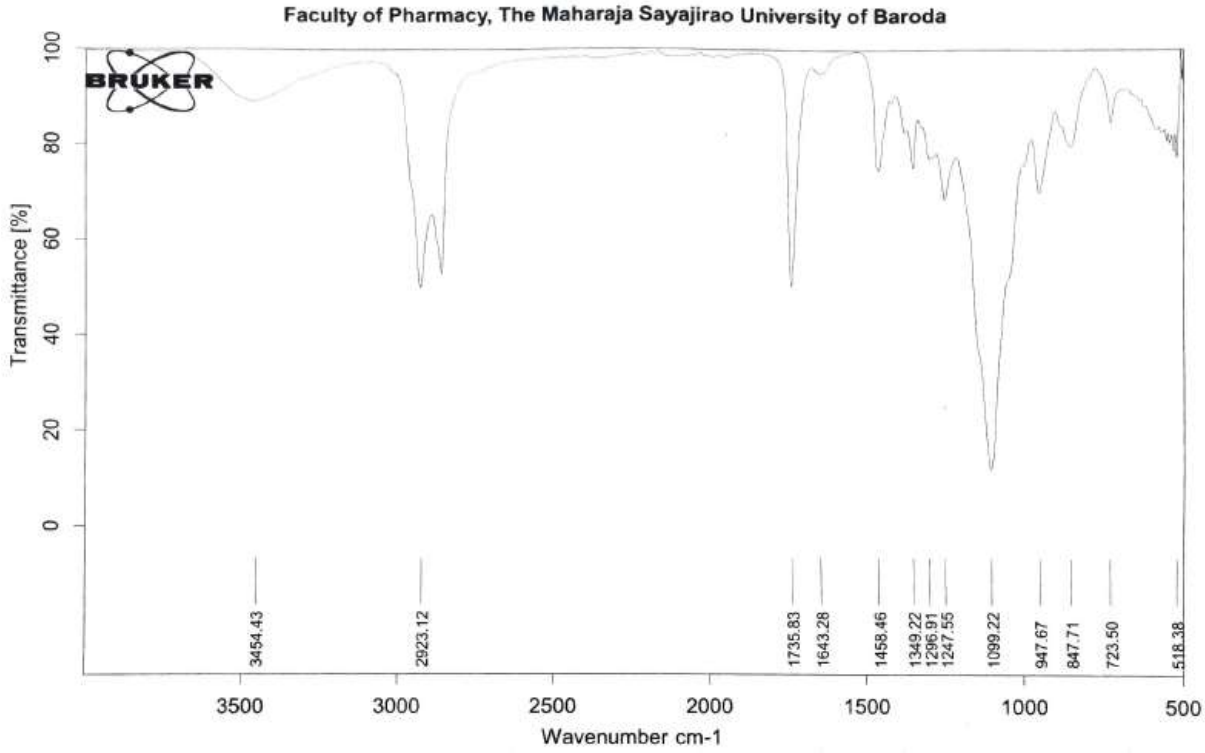

Figure 16: FTIR spectra of Luliconazole + Physical mixture of Capmul MCM C8, Coconut oil, Cremophore EL, Pluronic F127 (For Nanoemulsion)

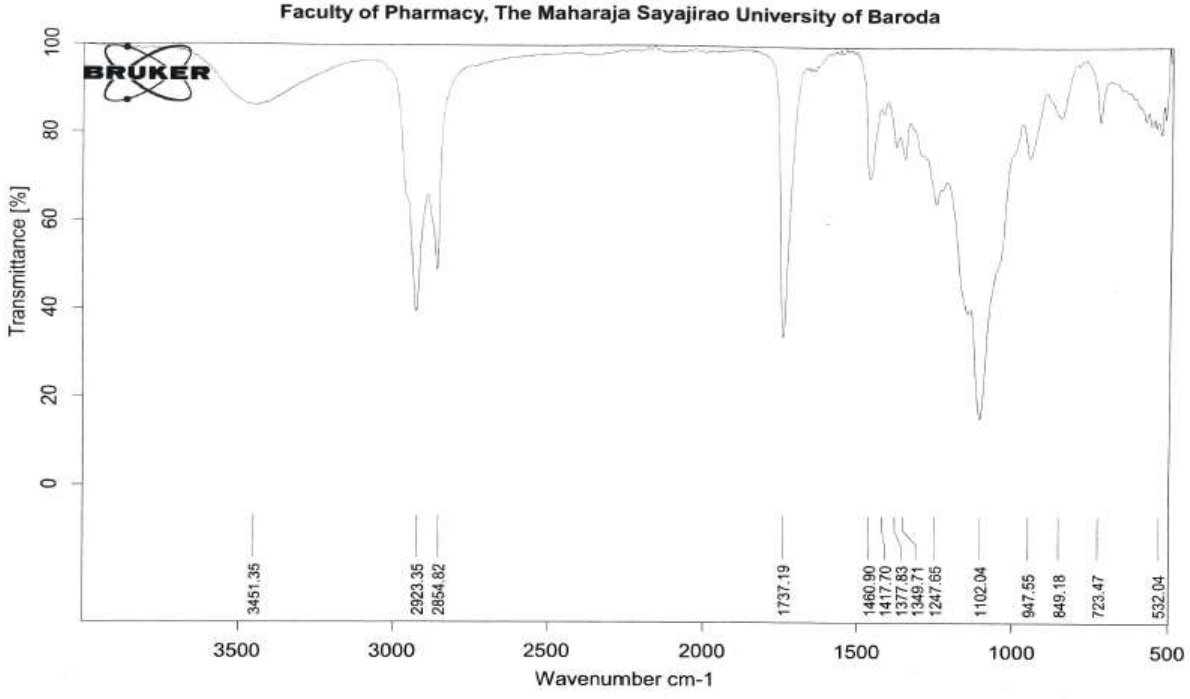

Figure 17: FTIR spectra of Luliconazole + Physical mixture of Capmul MCM C8, Softemul AS, Cremophore EL, Pluronic F127 (For NLCs)

Presence of all characteristic peaks in FTIR spectra indicates the excipients compatibility between Luliconazole and the physical mixtures of Luliconazole.

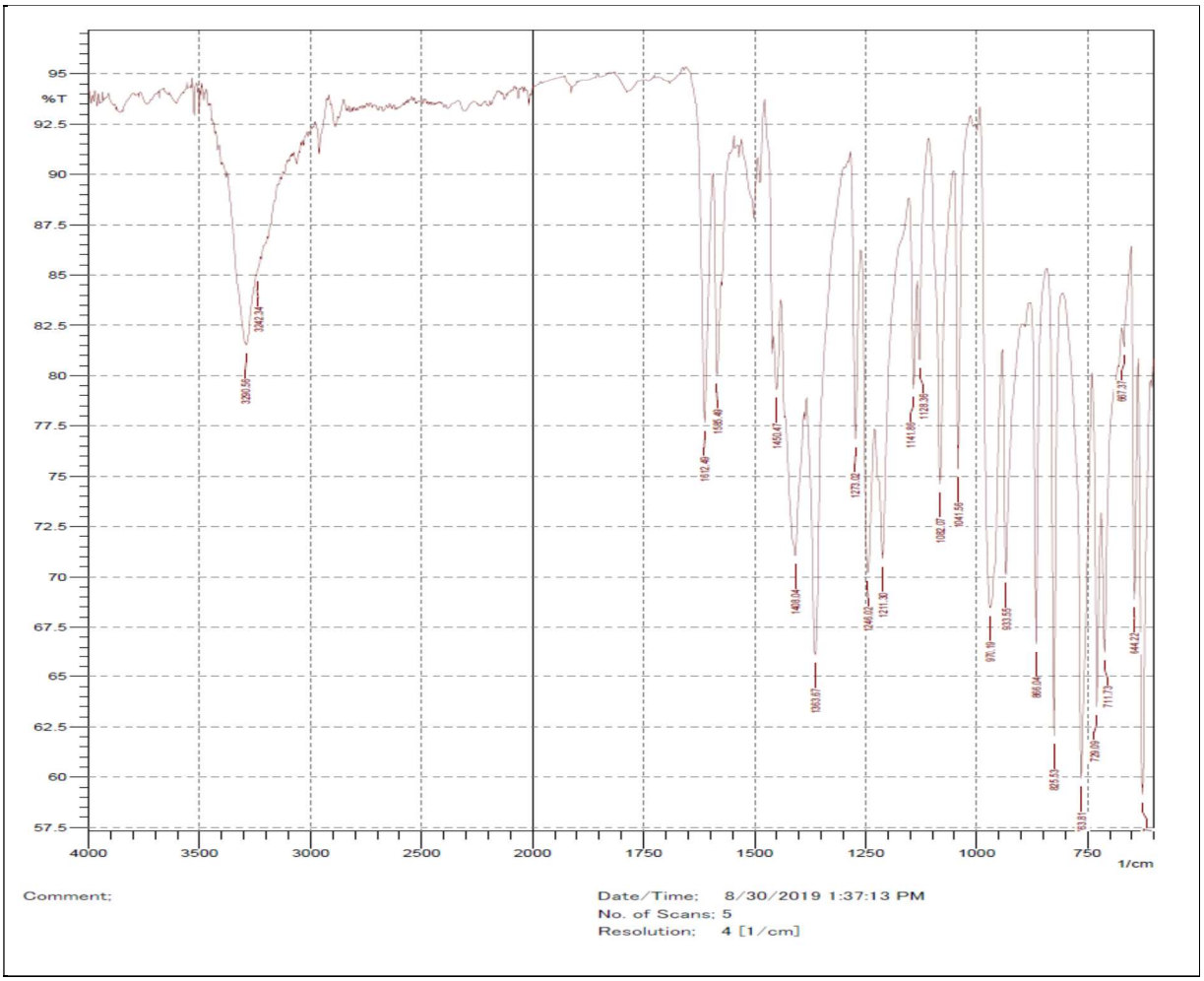

Figure 18: FTIR of Tavaborole

Table 10: Interpretation of FTIR of Tavaborole

Type of Vibration	Standard	Observed	Inference
O-H stretching	3550-3200	3242.34	Present
C=C Stretching	1650-1600	1612.49	Present
B-O stretching	1340-1370	1363.67	Present
C-F stretching	1400-1000	1246.02	Present
C- O Stretching (Aliphatic ether)	1150-1085	1082.07	Present

In the table 10, the comparison between observed and standard peaks showed authenticity of Tavaborole.

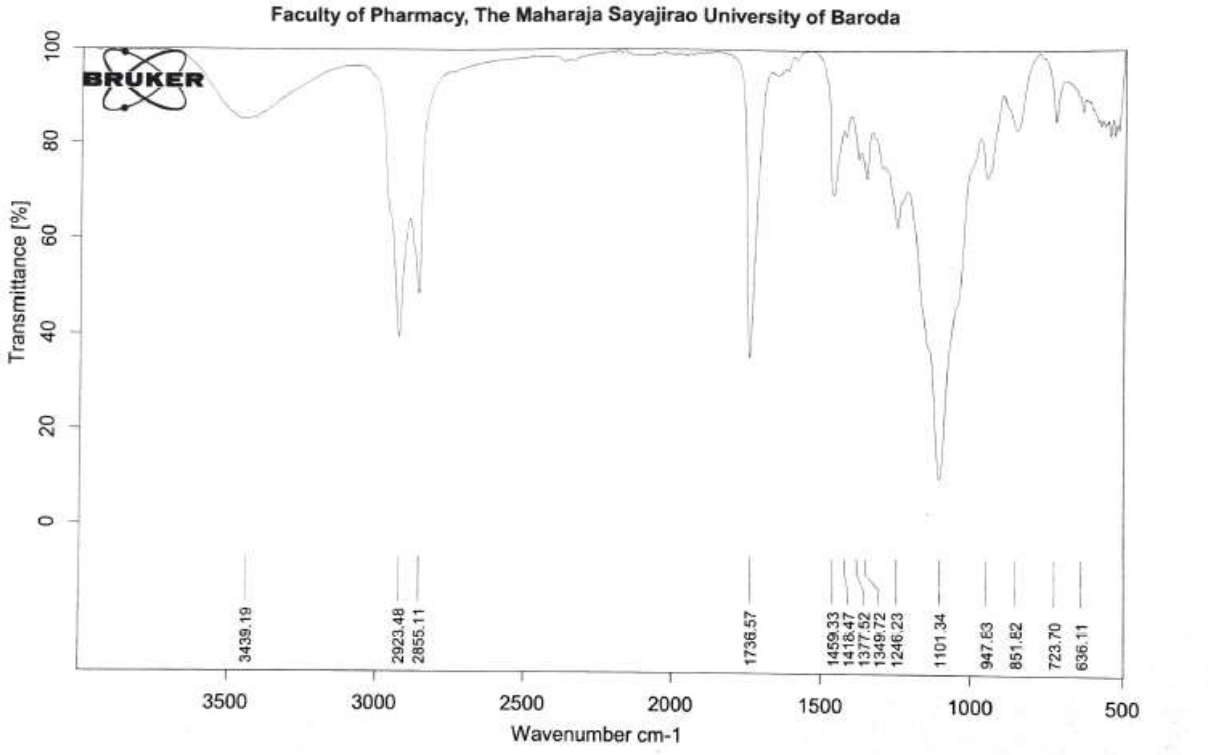

Figure 19: FTIR spectra of Tavaborole + Physical mixture of Capmul MCM C8, Coconut oil, Cremophore EL, Pluronic F127 (For Nanoemulsion)

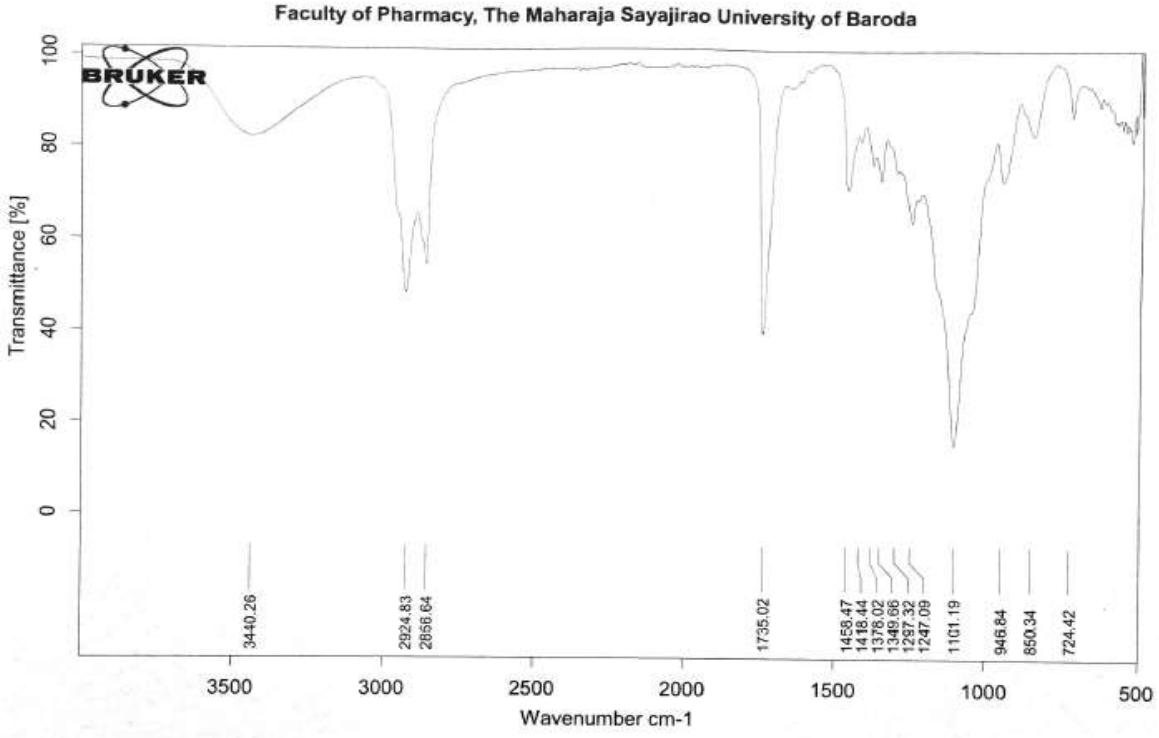

Figure 20: FTIR spectra of Tavaborole + Physical mixture of Capmul MCM C8, Softemul SE, Cremophore EL, Pluronic F127 (For NLCs)

Presence of all characteristic peaks in FTIR spectra indicates the excipients compatibility between Tavaborole and the physical mixtures of Tavaborole.

4.3 Differential scanning Calorimetry (DSC):

Differential scanning calorimetry (DSC) was performed using DSC-41 (Shimadzu, Japan) to study the thermal behavior of drug. Drug sample (2-3 mg) was taken and sealed in aluminum pan by applying external pressure. This aluminum pan was heated from 25°C to 300°C at a scanning rate of 10°C/min under nitrogen flow rate of 40 ml/min to create inert environment and to avoid oxidation due to oxygen.[35]

For Luliconazole:

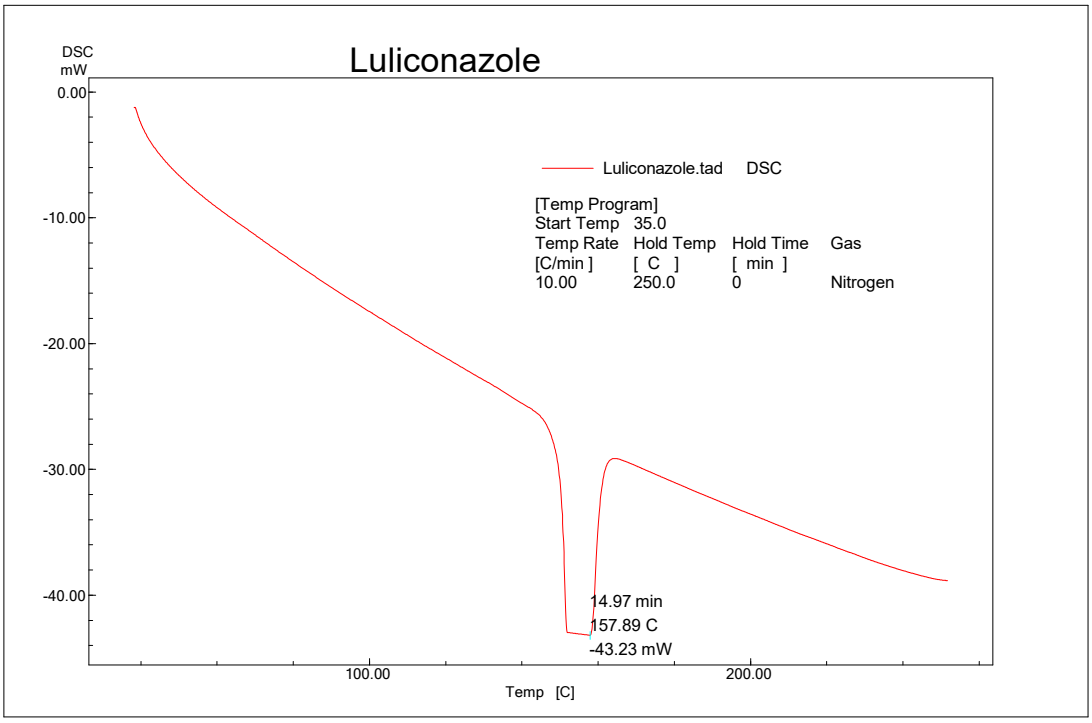

Figure 21: DSC graph of Luliconazole

The reported melting point of Luliconazole is 152 °C and from the DSC graph, a sharp phase transition (melting point) was obtained at 157.89 °C which was almost same as the reported melting point of Luliconazole.

For Tavaborole:

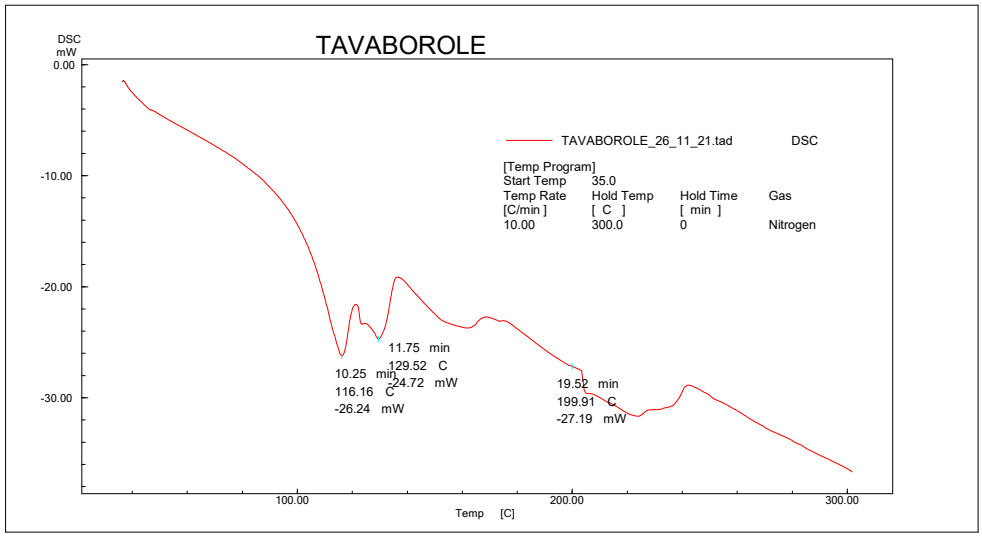

Figure 22: DSC graph of Tavaborole

The reported melting point of Tavaborole is 120 °C and from the DSC graph, a sharp phase transition (melting point) was obtained at 116.16 °C which was almost same as the reported melting point of Tavaborole.

5. Formulation Development and Optimization

5.1 Formulation development of Nanoemulsion of Luliconazole and Tavaborole

5.1.1 Screening of formulation parameters:

Solubility study:

Solubility of Luliconazole and Tavaborole was found out in various oils and surfactants on the basis of saturation solubility. 1 ml of the solvent was taken and incremental amounts of drug were added until the solvent was saturated and no more drug dissolve. This mixture was centrifuged at 3000 rpm to separate the undissolved drug and the supernatant was collected and analyzed using suitable analytical method as described above after suitable dilution as required.[36]

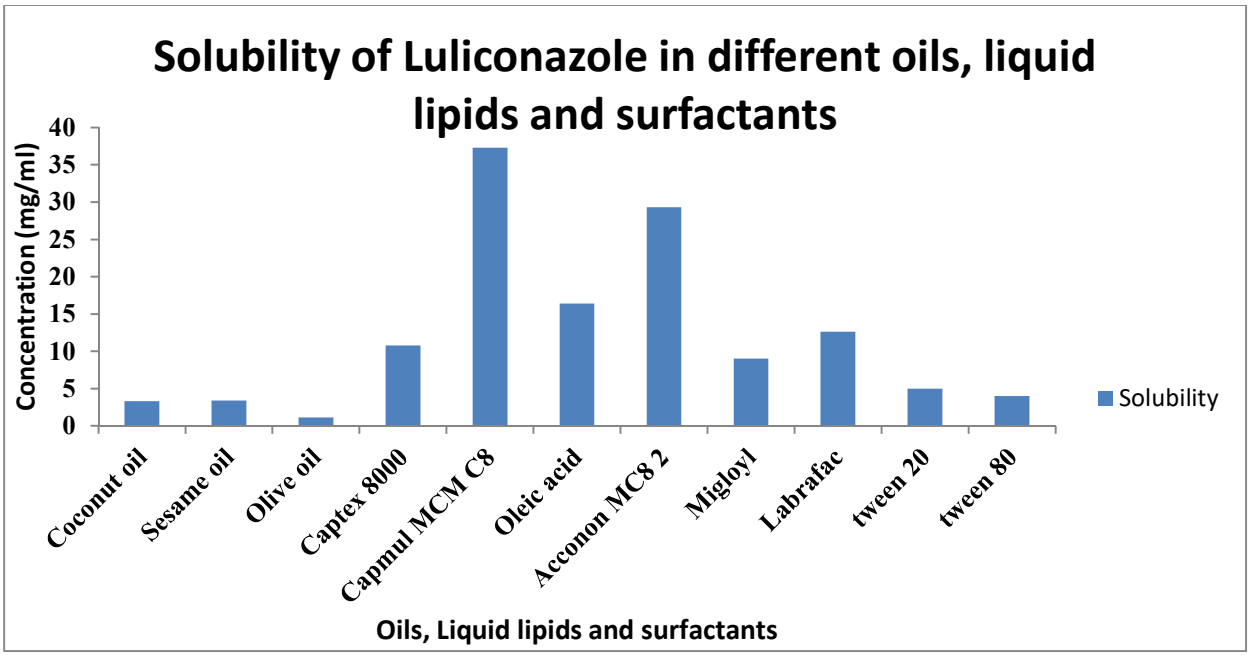

Figure 23: Solubility of Luliconazole in different oils, liquid lipids and surfactants

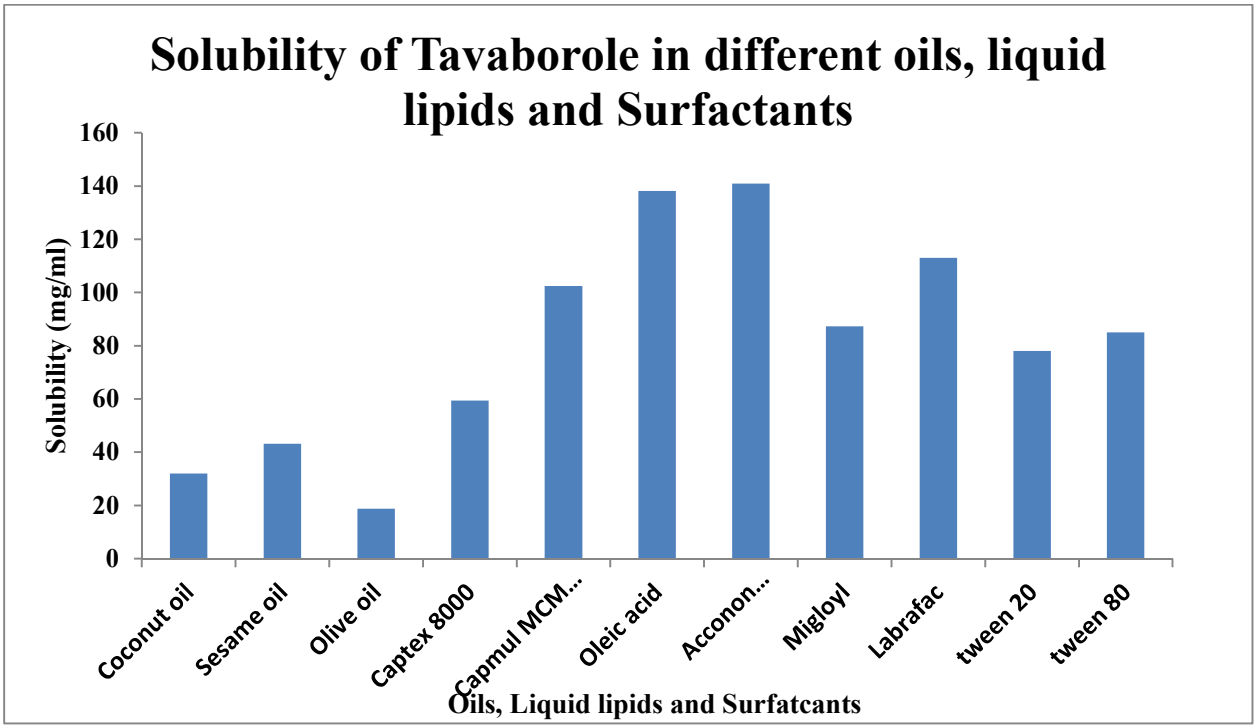

Figure 24: Solubility of Tavaborole in different oils, liquid lipids and surfactants

The highest solubility was found in Capmul MCM C8 so it was selected oil phase. It was reported that coconut oil shows the antifungal activity[37] therefore; to enhance the antifungal activity of formulations, combination of Capmul MCM C8 and coconut oil was selected as oil

phase for Luliconazole nanoemulsion. For Tavaborole, Acconon MC8-2 showed highest solubility. However, nanoemulsions prepared by using Acconon MC8-2 were not stable. Therefore, Tavaborole nanoemulsions were formulated by using Capmul MCM C8 and coconut oil as oil phase. Nanoemulsions were prepared by using different surfactants and co-surfactants. Among them cremophore EL and Pluronic F127 were selected as surfactant and co-surfactant respectively. Different process parameters and formulation parameters were optimized by Box Behnken Design using Design Expert®, (version 12; State-Ease Inc., USA) software.

5.1.2 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 219.4±1.70 nm and 226.8±0.64 respectively. Zeta potential of the optimized formulation of Luliconazole loaded nanoemulsion and Tavaborole loaded nanoemulsion were found to be -28.54±0.94 and -26.12±1.32 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 94.28±1.14% and 96.32±0.72 respectively. Drug loading of the optimized formulation of Luliconazole loaded nanoemulsion and Tavaborole loaded nanoemulsion were found to be 13.24±0.54% and 11.65±1.87% respectively.

Centrifugal stability test: The physical stability of nanoemulsion was determined by the effect of centrifugation on its properties. Centrifugation at various speeds starting from 4000 to 10 000 rpm each for 20 min was carried out and the formulation was visually observed for phase separation. Nanoemulsion showed minute change in globule size upon centrifugation from 4000 to 8000 rpm each for 20 min after 15 days. The freshly prepared nanoemulsion was found to be stable with no sign of instability up to 8000 rpm.[38]

Transmittance: Transmittance was observed by using UV spectrophotometer (UV 1900, Shimadzu, Japan) at 630 nm.[39] 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.

Differential scanning Calorimetry (DSC):

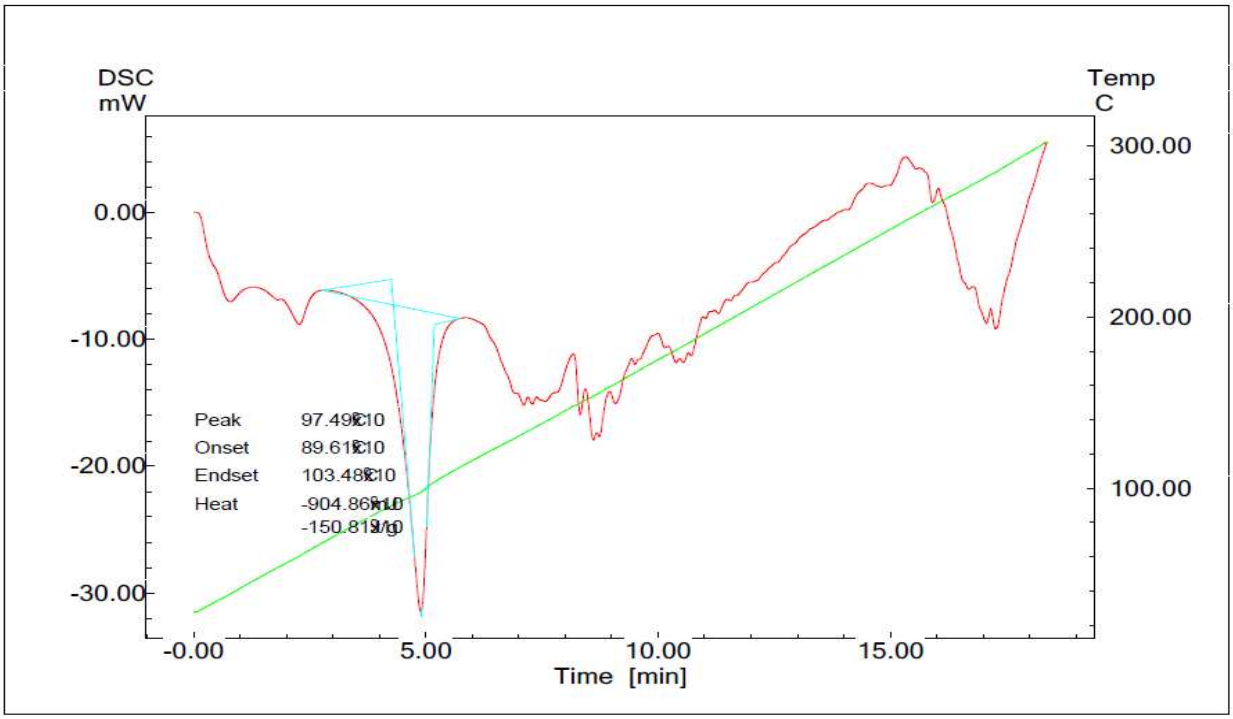

Figure 25: DSC graph of Luliconazole loaded Nanoemulsion

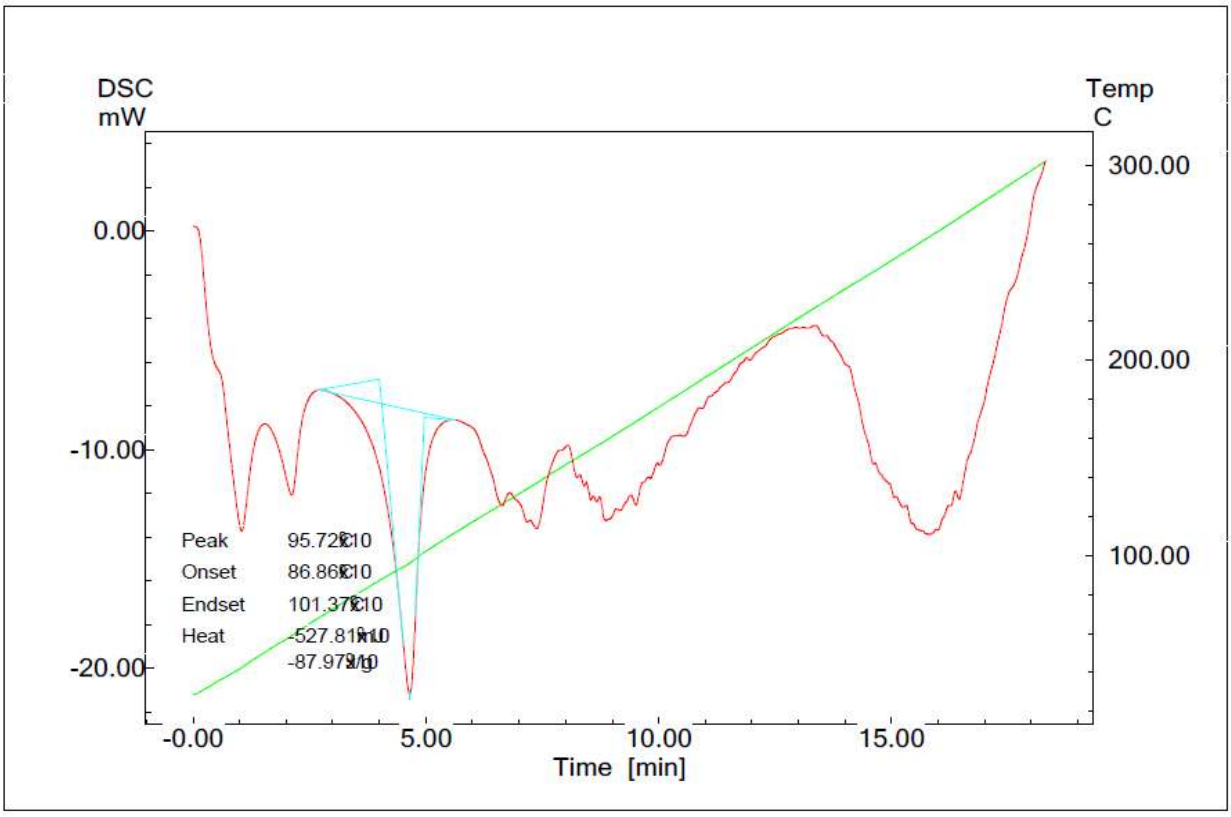

Figure 26: DSC graph of Tavaborole loaded Nanoemulsion

Figure 25 and figure 26 show the DSC graph of Luliconazole and Tavaborole loaded Nanoemulsion respectively.

5.1.3 Incorporation of Nanoemulsion into gel:

Nanoemulsion was 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.

6. Formulation development of NLCs of Luliconazole and Tavaborole

6.1 Primary screening of solid lipid

Solubility study of Luliconazole in different solid lipids and surfactants:

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. For solid lipid screening, Luliconazole and Tavaborole were added separately to 100 mg solid lipid at 70°C in a test tube. Solubility of the drug in molten lipid was determined visually by confirming absence of drug crystals. If the added amount of drug was soluble, then more amount of drug was added until saturation. Solid lipids which can solublize higher amount of Luliconazole and Tavaborole were selected respectively.

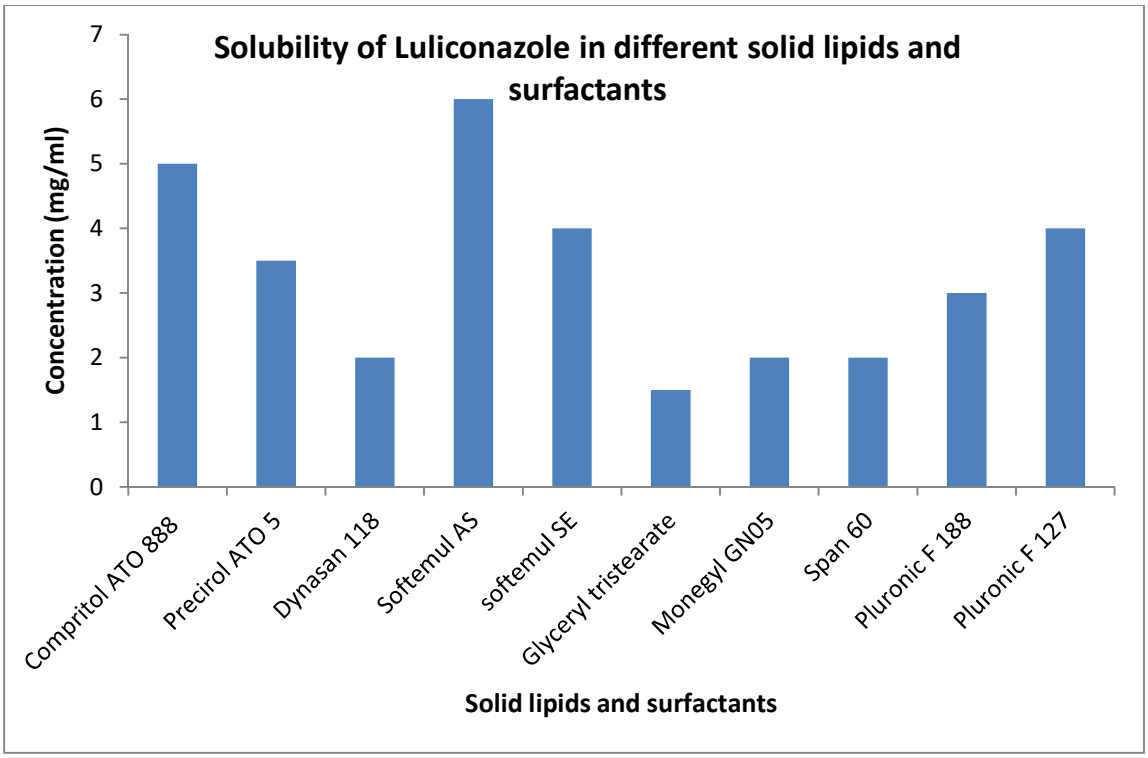

Figure 27: Solubility of Luliconazole in different solid lipids and surfactants

From the graph it was found that Softemul AS and Pluronic F 127 showed highest solubility for Luliconazole so selected as liquid lipid and surfactant respectively. Capmul MCM C8 was selected as liquid lipid based on solubility.

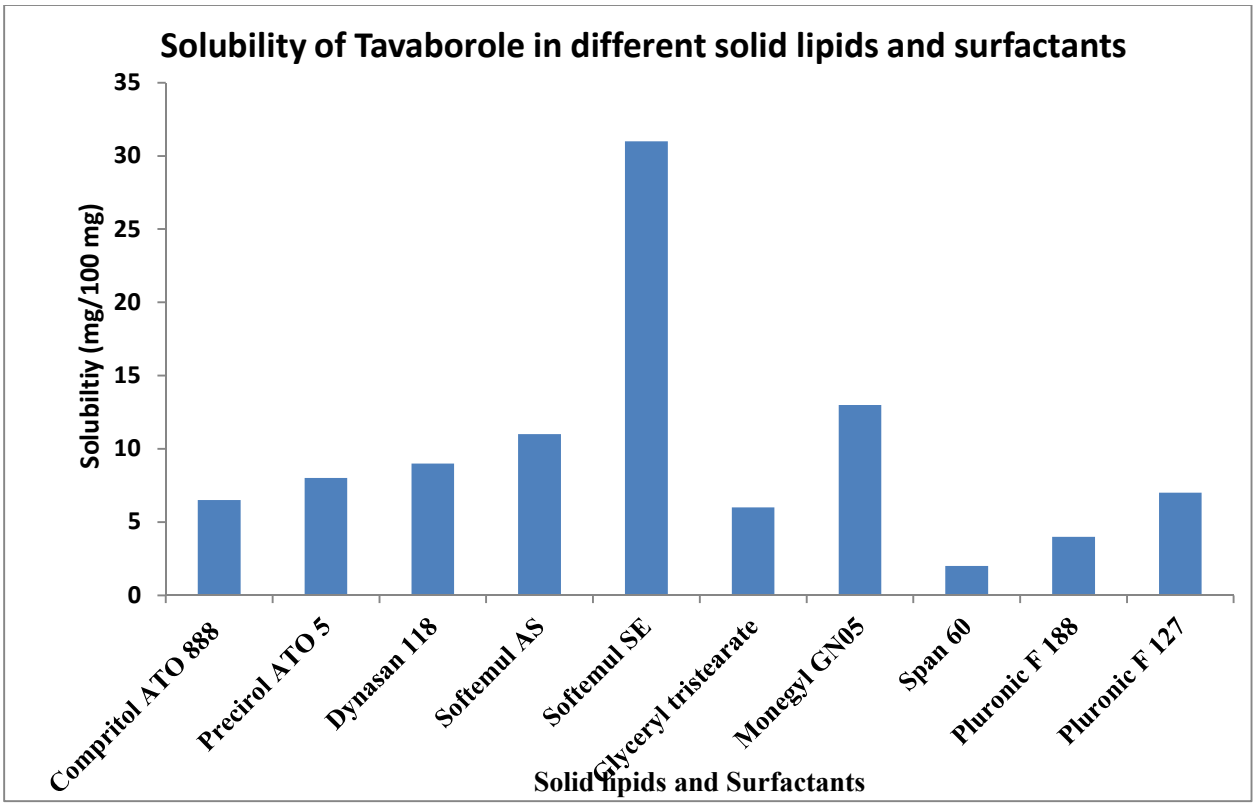

Figure 28: Solubility of Tavaborole in different solid lipids and surfactants

From the graph 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.

6.2 Selection of the ratio of solid lipid to liquid lipid:

The selected solid and liquid lipid having maximum solubilizing capacity for Luliconazole and Tavaborole were mixed together in different proportions %w/w (50:50, 60:40,70:30 and 80:20) respectively to test the miscibility. The mixtures were agitated at 100rpm for 30 minutes at 70ºC The lipid mixtures were cooled to room temperature (25±1°C). After solidification, the miscibility of lipids was assessed by smearing a sample of the mixture onto filter paper, followed by visual observation. The presence of oil droplets on the filter paper revealed immiscibility between the lipids. A binary mixture showing a melting point above 40°C which did not reveal the presence of oil droplets on the filter paper was selected for the preparation of NLCs. Visual observation of samples smeared on the filter paper indicated that binary lipid in the ratio 70:30 w/w (solid: liquid lipid ratio) was optimum for formulation of the NLCs.[40]

6.3 Preparation of NLCs:

NLCs were formulated by hot melt emulsification technique followed by probe sonication.[40] In the lipid phase, 50 mg solid lipid, 50 mg liquid lipid and 25 mg surfactant were taken and dissolved in small quantity of acetone in a beaker to obtain a lipid-drug matrix. Acetone was left for evaporation at 50°C under continuous stirring. Aqueous phase was prepared by taking 25 mg of surfactant in 10 ml distilled water and heated to 50°C under continuous stirring. Aqueous phase was poured dropwise to lipid phase using a magnetic stirrer at 1000 rpm for 15 minutes. The resulting dispersion was probe sonicated for 3 min at 40% amplitude under ice cold conditions.[41] NLCs were optimized by using Box-Behnken design. A randomized design matrix was generated, experimental data was statistically evaluated for achieving an optimized solution and the design space was created with the help of Design Expert® 7.0.0.

6.4 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 215±0.87 nm and 234±1.54 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 95.24±1.87% and 97.21±0.98% respectively. Drug loading of the optimized formulation of Luliconazole loaded NLCs and Tavaborole loaded NLCs were found to be 15.32±1.43% and 13.76±0.65% respectively.

6.5 Head Space Gas Chromatography (HS-GC) Testing for residual solvent:

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.

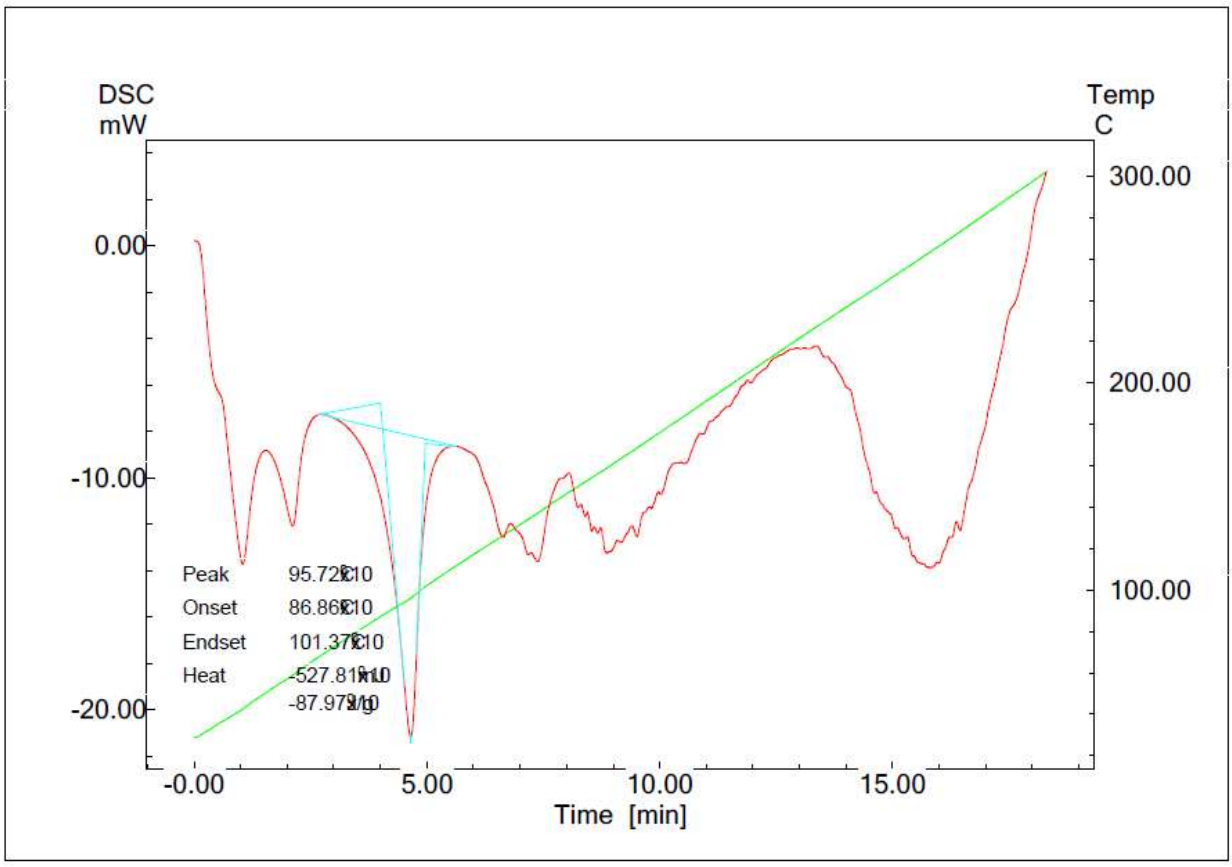

Figure 29: DSC graph of Luliconazole loaded NLCs

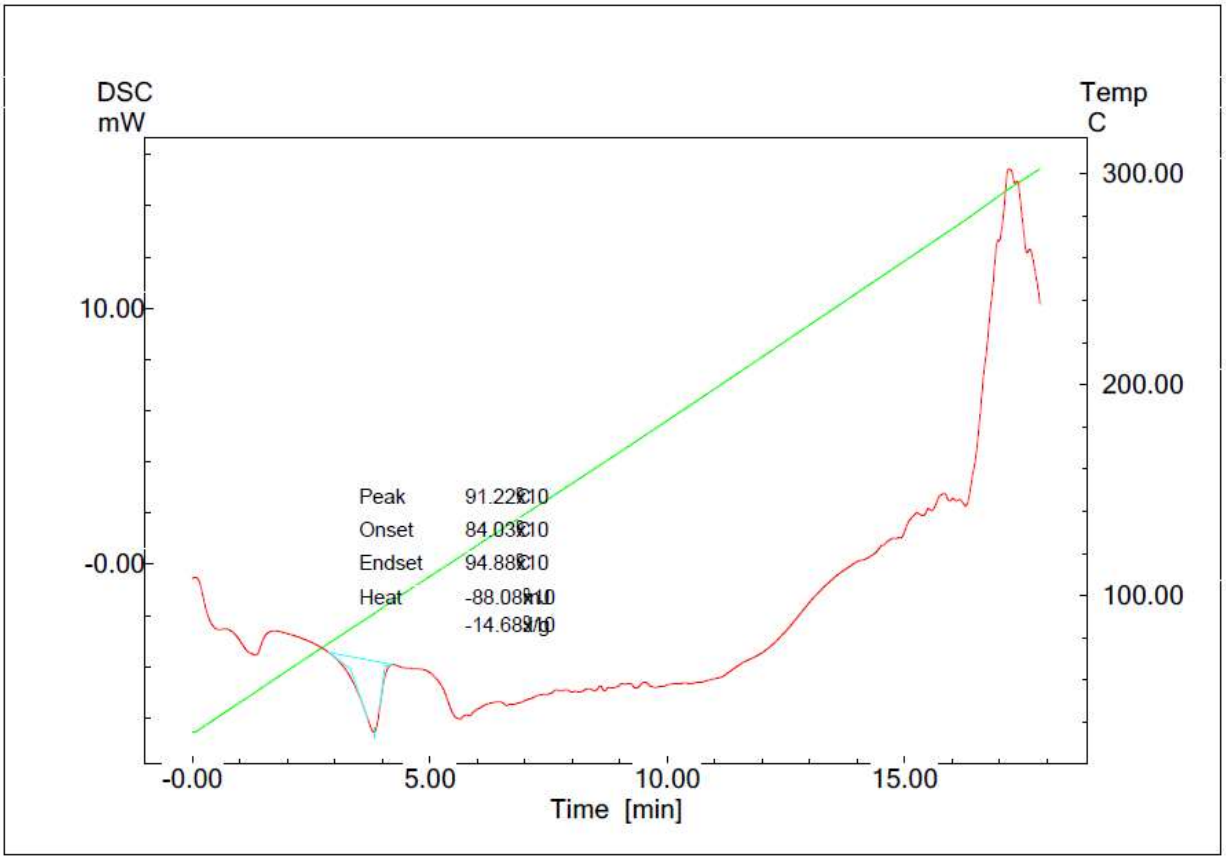

Figure 30: DSC graph of Tavaborole loaded NLCs

Figure 29 and figure 30 show the DSC graph of Luliconazole and Tavaborole loaded NLCs respectively.

6.6 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.

7. Powder X-ray diffraction studies:

The crystalline structure of the Luliconazole, Lyophilized powder of nanoemulsion and NLCs of Luliconazole were analyzed using powder X-ray Diffractometer (Rigaku- Minifex). The samples were analyzed using Cu tube as anode with K radiation on the solid sample. The sample analysis was performed at difractograms a scattering angle of (2θ) over a range of 10–40º using accelerating voltage of 30 kV at 25ºC.[42]

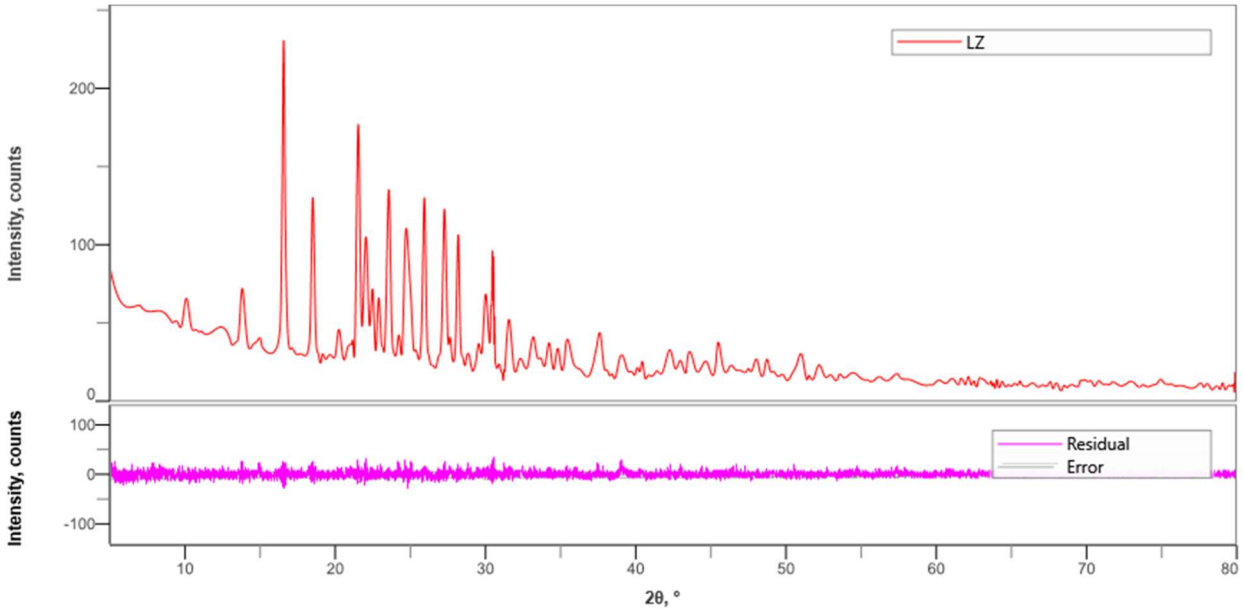

Figure 31: XRD of Luliconazole

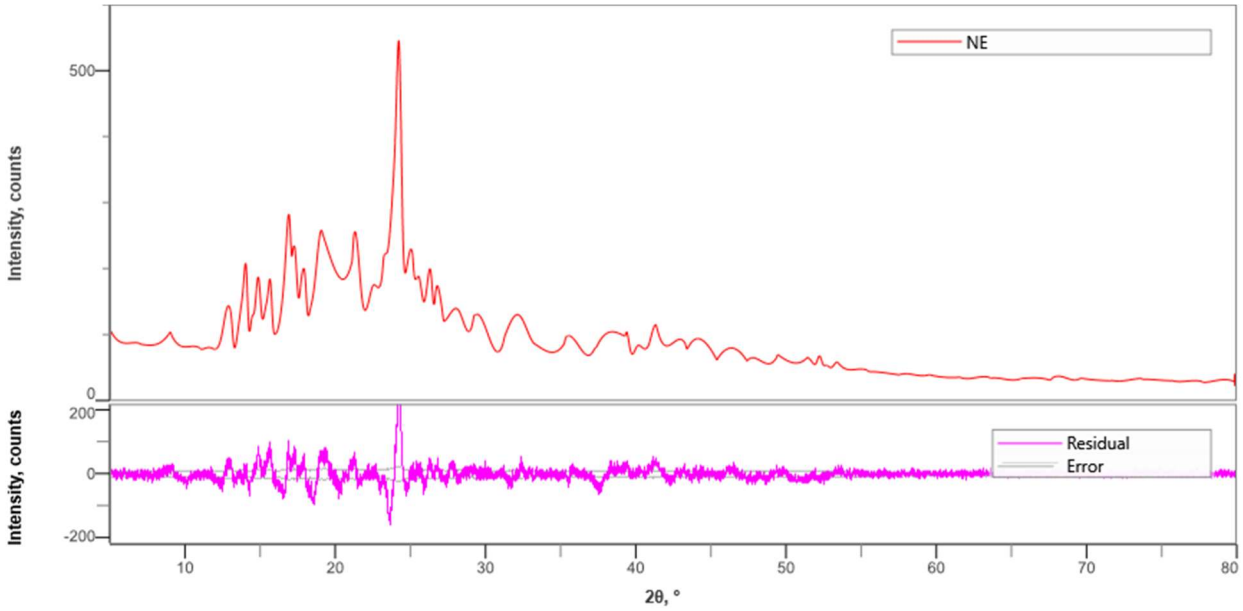

Figure 32: XRD graph of Luliconazole loaded Nanoemulsion

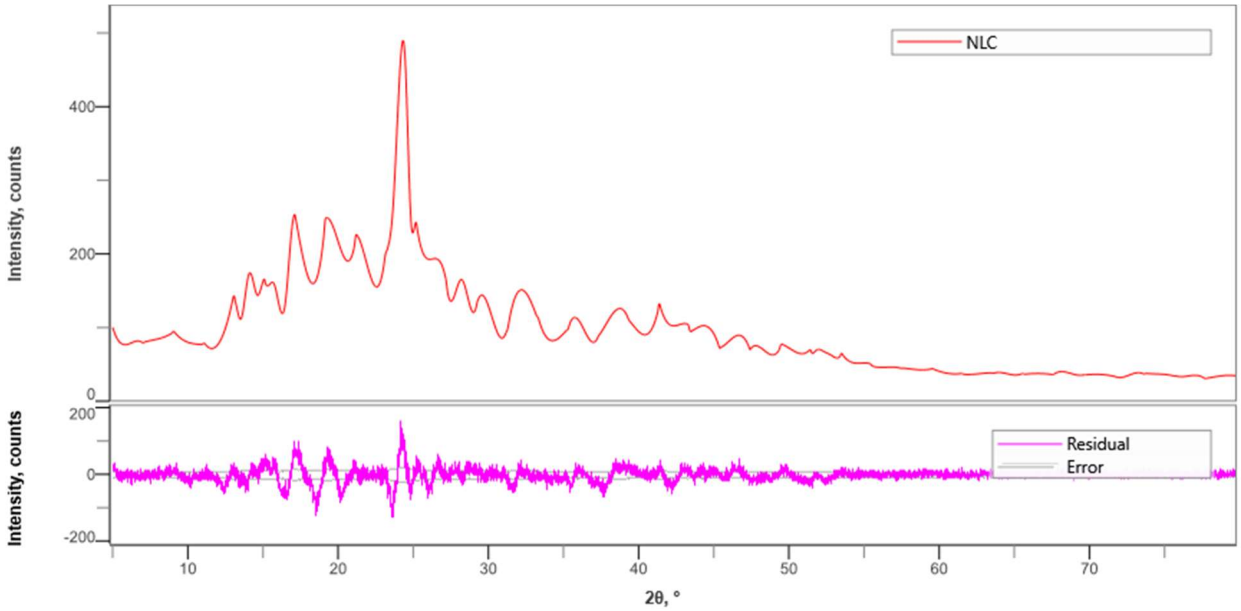

Figure 33: XRD graph of NLCs of Luliconazole

As shown in figure 31 Luliconazole showed intense peaks because of its crystalline structure. Figure 32 and figure 33 show diffraction patterns of Nanoemulsion and NLCs respectively, confirmed the transformation of drug from crystalline state to amorphous state in Nanoemulsion and NLCs.

8. In-vitro drug release:

In-vitro drug release study was performed using dialysis membrane (molecular weight cut-off 12K Dalton) with the help of modified Franz-diffusion cell. Luliconazole containing gel, Luliconazole loaded nanoemulgel and Luliconazole loaded NLCs based gel was placed in donor compartment. Phosphate buffer saline pH 7.4 was filled in a receptor compartment. Samples (0.5 ml) were withdrawn at regular time intervals (1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 hour) from the receptor compartment and the same volume (0.5 ml) was replaced by a fresh diffusion medium. Samples were analyzed using developed HPLC method as described above. All the experiments were performed in triplicate and the average values were taken.[43] Same procedure was followed for Tavaborole containing gel, Tavaborole loaded nanoemulgel and Tavaborole loaded NLCs based gel.

Results:

In-vitro drug release study showed that drug release from Luliconazole loaded gel, Luliconazole loaded Nanoemulgel and Luliconazole loaded NLCs based gel was found to be 55.45±2.28%,

87.97±1.96 and 96.61±2.22 respectively. Permeation of drug from Tavaborole loaded gel, Tavaborole loaded Nanoemulgel and Tavaborole loaded NLCs based gel was found to be 76.17±1.72%, 98.67±2.34 and 96.43± 1.44 respectively.

8. Ex-vivo characterization:

8.1 Skin collection and preservation:

Isolated rat skins were thoroughly cleaned with PBS pH 7.4, immediately rinsed and soaked in Glycerol solution in PBS 7.4 and preserved in dry ice for transportation to laboratory. PBS 7.4 was used for thawing rat skins at room temperature. Care was taken while separating full thickness of rat skin and was done with the help of forceps and scalpel. Fat present in the skin was cleaned and then the skin was thoroughly inspected for surface and thickness. After establishing that the skin is suitable, it was cut into pieces of circular shape having uniform thickness and a diameter suitable for fastening in Franz diffusion cell. These skin pieces were soaked in 15% glycerol solution prepared in PBS 7.4, transferred to zip lock polybags and preserved in deep refrigerator at -70°C for not more than two months.

8.2 Ex-vivo permeation and deposition study:

Developed formulations were tested for deposition profile and permeation with the help of full thickness of rat skin. The evaluation was conducted by employing a Franz-type diffusion cell having a 7 ml receptor chamber. For performing this experiment, phosphate buffer pH 7.4 was used for filling the receptor compartment and circular water bath was employed for maintaining its temperature at 37°C. Further the skin sections were affixed between the receptor and donor compartment. Care was taken that the stratum corneum faces the donor compartment. Diffusion media used in the Franz diffusion cell was stirred lightly at a speed of 100 rpm. Developed formulation was added in donor compartment. From the sampling arm of the diffusion cell, samples having a volume of 1 ml were taken at various time points i.e. 1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 hours. Further, fresh diffusion media of the same volume was replaced in order to maintain the total volume. The skin section was removed from the Franz diffusion cell after 24 hours and the skin was washed with 5 ml diffusion media three times. For calculation of the drug adhered to the skin, washings of the skin were saved. Scalpel was used for cutting the washed skin into small pieces. Then, these pieces were suspended in methanol, homogenized in cold

condition for a period of 5 minutes and then it was sonicated using bath sonicator for a period of 15 minutes. For quantification of the drug accumulated in skin, the drug was removed by centrifuging it at an RPM of 5000 for a period of 10 minutes. All the samples were filtered with the help of 0.2 μm syringe filter and the quantification of the drug was performed by employing developed HPLC method in skin homogenate. Similar method was employed for the ex-vivo study of all prepared formulations.[44]

Table 11: Ex-vivo Drug release from various prepared formulation

Formulations	Drug permeated across skin (%)	Drug deposited within skin (%)	Drug retained on skin surface (%)
Luliconazole loaded gel	14.21	18.16	64.98
Luliconazole loaded nanoemulgel	24.32	64.76	10.21
Luliconazole loaded NLCs based gel	19.91	68.66	10.43
Tavaborole loaded gel	28.68	14.39	56.32
Tavaborole loaded nanoemulgel	35.62	55.98	7.41
Tavaborole loaded NLCs based gel	38.12	52.54	9.16

8.3 Ex-vivo fluorescence microscopy study:

With the help of fluorescence microscopy, permeation behavior of the formulations which were developed was illustrated. FITC suspension, FITC loaded nanoemulsion and NLCs were formulated and utilized for the study. The rat skin was thawed at room temperature, equilibrated and fastened on franz diffusion cell in the same way as explained in earlier section. FITC loaded formulations were smeared onto the stratum corneum layer of the skin in a similar way as explained in earlier sections. After a period of 12 hours, skin sectioning was performed in dark environment using cryo-microtome, sections were fixed on a glass slide. Confocal laser scanning microscope was utilized for examining the fluorescence on the slide.[45]

8.4 Transungual Permeation Study:

Hooves from freshly slaughtered goat, free of connective and cartilaginous tissues, were taken from the local slaughterhouse and soaked in distilled water for 24 h. From the lower part of the hooves, a section of about 1 mm thickness was cut. The hoof membrane was placed carefully on the Franz diffusion cell of 7-mL capacity. Luliconazole loaded gel, Luliconazole loaded Nanoemulgel and Luliconazole loaded NLCs based gel were applied evenly on the surface of the membrane. The receptor compartment was filled with phosphate buffer, pH 7.4 and the whole assembly was maintained at 37 ± 1°C with constant stirring (100 rpm) for 24 h. From the sampling arm of the diffusion cell, samples having a volume of 1 ml were taken at various time points i.e. 1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 hours. Further, fresh diffusion media of the same volume was replaced in order to maintain the total volume. The samples were analysed by the HPLC. Same methods were applied for the Tavaborole loaded gel, Tavaborole loaded Nanoemulgel and Tavaborole loaded NLC based gel.[46]

Results:

Permeation of drug from Luliconazole loaded gel, Luliconazole loaded Nanoemulgel and Luliconazole loaded NLCs based gel was found to be 40.50±1.28%, 80.77±1.57 and 76.61±1.22 respectively. Permeation of drug from Tavaborole loaded gel, Tavaborole loaded Nanoemulgel and Tavaborole loaded NLCs based gel was found to be 60.37±1.47%, 91.38±1.96 and 95.85± 2.08 respectively.

9. Antifungal Activity:

9.1 In-vitro determination of minimum inhibitory concentration (MIC):

In-vitro antimicrobial study was executed to determine the MIC of the developed formulations of Luliconazole and Tavaborole in microbial strains of Candida albicans. Broth micro-dilution technique was used to determine the MIC of developed formulations. Briefly, C. albicans were seeded in 96-well plates having cell density of 5 x 10^3 CFU/well. Different concentrations of developed formulations in Mueller Hinton Broth media were added to each well. The microbes were incubated under standard conditions upto 24 hr and the optical density was measured using microplate reader at 600 nm.[47]

Results:

Luliconazole loaded formulations exhibited a MIC in the range of 0.05-0.2 µg/ml against Candida albicans. Tavaborole loaded formulations exhibited a MIC in the range of 1-3 µg/ml against Candida albicans. The results indicate that Luliconazole and Tavaborole loaded formulations have strong antifungal activity against Candida albicans.

10. Cell line Study:

10.1 In-vitro MTT Assay:

MTT assay using 3T3-fibroblast cells: A study was performed in the fibroblast cells to assess the cytotoxicity of developed formulations. Cells were seeded with 5×103 cells/well cell density in a 96 well microtiter plate using DMEM supplemented with 10% Fetal Bovine Serum. The cell culture was grown for 24 hours in a CO2 incubator maintained at 5% concentration and humidified with saturated Copper sulfate solution. After 24 hours, cells were exposed at different w/w ratio and evaluated for 6 hours. After the exposure time, cell media was replaced with a complete medium containing 1% solution of antibiotic and 10% Fetal Bovine Serum. After 24 hours, cells were washed with PBS pH 7.4, and MTT dye (5mg/mL) solution (20 µl) was added to each well plate. The MTT dye was allowed to react for 4 hours under incubator condition and after that, cell medium in each plate was replaced with 100 µl of DMSO (Himedia, Mumbai) and the microtiter plate was shaken gently to dissolve the crystals of formazan. The color of formazan was determined using a Biorad microtiter plate reader (Biorad, California) at 570 nm. PBS treated Cells were used as control cells. The absorbance values of cells treated with PBS were taken as 100% cell viability and all other treatments were expressed relative to it. Luliconazole suspension, Luliconazole loaded Nanoemulsion, Luliconazole loaded NLCs, Tavaborole suspension, Tavaborole loaded Nanoemulsion and Tavaborole loaded NLCs in the concentration range of 0.001 – 500 µg/mL were evaluated for the MTT assay with Triton X 100 and PBS pH 7.4 as positive and negative control respectively.[48]

Results:

The developed Luliconazole loaded Nanoemulsion, Luliconazole loaded NLCs, Tavaborole loaded Nanoemulsion and Tavaborole loaded NLCs have no potential toxic effects on the 3T3-fibroblast cells and are found to be safe for dermal delivery.

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