

**7. *In-vitro* and Ex-vivo studies****7.1 Introduction**

The optimized formulations of Luliconazole and Tavaborole were evaluated by *in-vitro* and ex-vivo studies like *in-vitro* drug release, *in-vitro* cytotoxicity, cellular uptake, *in-vitro* antifungal efficacy, ex-vivo drug permeation and skin retention studies.

**7.2 Materials and Instruments****Table 7.1 List of Materials and Reagents**

<b>Chemical/Reagent</b>	<b>Manufacturer/Supplier</b>
Luliconazole	Sun Pharmaceutical Industries Ltd., Vadodara
Tavaborole	Symed labs limited, Hyderabad
Methanol (HPLC grade)	Spectrochem Pvt. Ltd., Mumbai
Dimethyl Sulphoxide	Himedia Pvt. Ltd., Mumbai
Dialysis membrane (12-14 KDa cut-off)	Himedia Pvt.Ltd., Mumbai
Sodium Chloride(AR)	S.D Fine Chemicals, Mumbai
Sodium Hydroxide (AR)	Spectrochem Labs Ltd, Vadodara
Potassium Chloride (AR)	Spectrochem Labs Ltd, Vadodara
Potassium dihydrogen phosphate (AR)	Spectrochem Labs Ltd, Vadodara
Sodium dihydrogen phosphate (AR)	Spectrochem Labs Ltd, Vadodara
Hydrochloric acid (AR)	Rankem chemical, Vadodara
3T3-fibroblast cells	NCCS, Pune
6 and 96 well cell culture plates	Sigma Aldrich, USA
Dulbecco's Modified Eagle Medium (DMEM-high glucose)	Sigma Aldrich, USA
Fluorescein-5-isothiocynate (FITC)	Sigma Aldrich, USA
Fetal Bovine Serum (FBS)	Sigma Aldrich, USA
Trypsin-EDTA solution	Sigma Aldrich, USA
Antibiotic antimycotic solution	Sigma Aldrich, USA
Thiazolyl blue tetrazolium bromide (MTT)	Sigma Aldrich, USA

Distilled water	Prepared in house
Sabouraud dextrose agar –SDA plates	SRL Chem, India
Fungal Culture ( <i>Trichophyton rubrum</i> , MTCC 3272)	NCIM, India
Dimethyl Sulfoxide- DMSO	SRL Chem, India

**Table 7.2 List of Equipments**

<b>Equipment/Instrument</b>	<b>Manufacturer/Supplier</b>
Digital Analytical Balance	Shimadzu, Japan
RP-HPLC with UV detector (gradient)	Agilent, Germany
pH meter	Lab India Pvt. Ltd., Mumbai
Magnetic Stirrer	Remi equipment Pvt. Ltd., India
CO <sub>2</sub> Incubator	Jouan, Thermo Fisher Scientific, USA
Microtiter plate reader	Biorad, India
Confocal Microscope	LSA 710, Carl-zeiss Inc., USA
Fluorescence microscope	Nikon, India
Vortex mixer	Spinix, Japan
Incubator thermo shaker	Bombay Lab services, Mumbai
Centrifuge	Remi Instrument, India
Scanning Electron Microscope (SEM)	JEOL, JSM-6490, Japan

### **7.3 Methodology**

#### **7.3.1 *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. 0.2 g (equivalent to 2 mg Luliconazole) Luliconazole containing gel, Luliconazole loaded nanoemulsion based gel and Luliconazole loaded NLCs based gel were placed in the donor compartments of Franz diffusion cells. 7 ml phosphate buffer saline pH 7.4 was filled in a receptor compartment. Samples (1 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 (1 ml) was replaced by a fresh diffusion medium to maintain sink condition. Samples were analyzed using developed HPLC method as described in section of

3.3.2 of chapter 3. All the experiments were performed in triplicate and the average values were taken (1,2). Same procedure was followed by using 0.2 g (equivalent to 10 mg Tavaborole) Tavaborole containing gel, Tavaborole loaded nanoemulsion based gel and Tavaborole loaded NLCs based gel.

### **7.3.2 *In-vitro* cytotoxicity and cellular uptake studies**

*In-vitro* cell line studies are essential as they offer the methods for primary evaluation of direct effects of drugs and formulations on cells and tissues so as to form a basis for in vivo animal studies and clinical studies. These studies are important as they give an idea on clinical applicability in pathological conditions and understand molecular mechanisms as well as can screen the test samples for their efficacy and toxicity (3, 4). The objective of the *in-vitro* cytotoxicity study was to evaluate the toxic potential of the developed formulations, if any on the 3T3-fibroblast cells. Additionally, the cellular uptake examination was also carried out to assess the intracellular uptake of the developed formulations in 3T3-fibroblast cell line using confocal microscopy.

#### **7.3.2.1 Cell culture handling and sub-culturing protocol**

The 3T3 fibroblast cell culture was purchased from NCCS Pune. Without removing the media, the receiving flask was maintained in an anaerobic incubator for 24 hours at 37 °C and 5% CO<sub>2</sub>. Upon completion of incubation, culture media was removed from the flask. The adhering cells in the flask were cleaned using PBS pH 7.4. 2 ml of freshly made Trypsin-EDTA solution was added to thoroughly cover the cell monolayer in the flask. The cell culture was subjected to centrifugation for 5 min duration at 1200 rpm. The resultant cells were again suspended in a new growth medium after the supernatant was discarded. The cells were counted with a Neubauer counting chamber, the cells were transferred at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> in separate flask. To promote cell proliferation, these flasks were maintained in an incubator with a temperature of 37°C and 5% CO<sub>2</sub>. Every third day, the growth media were changed, and once the culture reached 80–90% confluency, passaging was carried out (5-7).

#### **7.3.2.2 MTT Assay**

3T3-fibroblast cells were used in the MTT assay to determine the cytotoxicity of the developed formulations. Using DMEM supplemented with 10% Foetal Bovine Serum,  $5 \times 10^3$  cells/well were added to a 96-well microtiter plate. The cell culture was incubated for a 24-hour period in a CO<sub>2</sub> incubator with a 5% concentration and a saturated copper sulphate solution to provide

humidity. Cells were exposed to various concentrations of the developed formulations and they were incubated for 6 hours. Following the exposure period, a full medium containing 10% foetal bovine serum and 1%v/v antibiotic solution (Penicillin/streptomycin) was added to the cell media. Following a 24-hour period, each well plate received 20 µl of MTT dye (5 mg/mL) solution after the cells had been cleaned with PBS pH 7.4. After allowing the MTT dye to react for 4 hours in an incubator, each plate's cell media was replaced with 100 µl of DMSO, and the microtiter plate was gently shaken to dissolve the formazan crystals. At 570 nm, the colour of formazan was measured with a Biorad microtiter plate reader. All other treatments were expressed in relation to the absorbance values of PBS-treated cells, which were assumed to represent 100% cell viability (8). Luliconazole suspension, Luliconazole loaded Nanoemulsion, Luliconazole loaded NLCs, Tavaborole suspension, Tavaborole loaded Nanoemulsion, and Tavaborole loaded NLCs were assessed for the MTT test using Triton X 100 (1%v/v) and PBS pH 7.4 as positive and negative control respectively.

### **7.3.2.3 Cellular uptake studies:**

The 3T3-fibroblast cells were used in the *in-vitro* cellular uptake studies of the developed formulations. In short, cells were seeded using DMEM treated with 10% FBS at a density of  $10^4$  cells/well in a 6-well plate. The cell culture was incubated in DMEM at 37°C with 5% CO<sub>2</sub> for 24 hours. A saturated solution of copper sulphate was used to humidify the culture. The media was removed and the cells were washed with sterile PBS after a 24-hour period. Subsequently, 100 nM concentrations of FITC containing developed formulations were added to the cells. Using transfection reagent, transfection medium, and dilution buffer, the transfection stage was carried out in accordance with the manufacturer's instructions (Santacruz Biotechnology, USA). To guarantee that any remaining formulation was removed, cells were twice washed with PBS after being exposed for six hours. Following that, the cells were fixed with 1 mL of 4% paraformaldehyde solution per well and incubated for 3–5 minutes at room temperature. Following the exposure period, the paraformaldehyde was removed immediately and the cells were thoroughly washed three times using PBS while being shaken intermittently throughout each wash to get rid of any remaining paraformaldehyde. The cells' nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 1 µg/mL in a volume sufficient to cover the cells. The cells were then left at room temperature for 15 minutes to allow the dye to permeate while being shielded from the light by aluminium foil. After that, cells undergo another

PBS 7.4 wash. The coverslips were placed on a glass slide using a 50:50 PBS:glycerin solution, and a confocal laser scanning microscope (LSA 710, Carl-zeiss Inc., USA) was used for confocal microscopy. At 1 and 4 hours, the cellular uptake was assessed (9, 10).

### **7.3.3 *In-vitro* determination of minimum inhibitory concentration (MIC)**

The MIC of the produced formulations of Luliconazole and Tavaborole in microbiological strains of *Candida albicans* and *Trichophyton rubrum* was determined using an *in-vitro* antimicrobial investigation. The MIC of the developed formulations was ascertained using the broth microdilution technique. In short,  $5 \times 10^3$  CFU/well of *Candida albicans* and *Trichophyton rubrum* were seeded in 96-well plates. Developed formulations were added in each well which was supplemented with Mueller Hinton broth medium. The microorganisms were cultured for an entire day under standard conditions, and a microplate reader was used to detect the optical density at 600 nm (n=3) (11).

### **7.3.4 *Ex-vivo* characterization**

#### **7.3.4.1 Skin collection and preservation**

Rat skin was obtained from the Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Gujarat, India under the IAEC (institutional animal ethics committee) protocol number MSU/IAEC/2021-22/2108. Rat skins that were previously isolated were properly washed using PBS pH 7.4, rinsed, and then immersed in a glycerol solution in PBS pH 7.4. They were then preserved in dry ice and brought to the laboratory. Rat skins were thawed at room temperature using PBS 7.4. Using a scalpel and forceps, the whole thickness of the rat's skin was carefully separated. The skin's fat content was removed, and its thickness and surface were carefully examined. Once the suitability of the skin was established, it was cut into circular pieces with a diameter appropriate for fastening in a Franz diffusion cell and a homogeneous thickness. The skin pieces were placed in zip-lock polybags and kept in a deep refrigerator at  $-70^{\circ}\text{C}$  for no more than of two months after being soaked in a 15% glycerol solution made in PBS 7.4 (12, 13).

#### **7.3.4.2 *Ex-vivo* permeation and deposition study**

Developed formulations were evaluated for penetration and deposition profile in skin by using rat skin. Using a Franz-type diffusion cell with a 7 ml receptor chamber, the evaluation was carried out. In order to conduct this experiment, the receptor compartment was filled with phosphate buffer pH 7.4, and a circular water bath was used to maintain the temperature at  $37^{\circ}\text{C}$ . Additionally, the skin segments were attached between the donor and receptor compartments.

The stratum corneum was carefully positioned to face the donor chamber. The Franz diffusion cell's diffusion media were gently agitated at a speed of 100 revolutions per minute. 0.2 g (equivalent to 2 mg LZ and 10 mg TB) of NE based gel and NLCs based gel of LZ and TB was introduced to the donor compartment, respectively. Samples with a volume of 1 ml were taken from the diffusion cell's sampling arm at several intervals, including 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours. In addition, new diffusion media of the same volume were added to keep the overall volume (sink condition) constant. After a day in the Franz diffusion cell, the skin portion was taken out and three washes with 5 millilitres of diffusion media were performed. Washings of the skin were saved for the computation of the medication adhered to the skin. The cleaned skin was chopped into little pieces using a scalpel. After that, these fragments were suspended in methanol and homogenized for five minutes. The drug was extracted from the skin by centrifuging it for 10 minutes at 5000 rpm in order to quantify the amount of drug that is deposited within the skin. Using a 0.2 µm syringe filter, all the samples were filtered, and an HPLC method for LZ and TB (section 3.4.2 and 3.5 of chapter 3) that was established for skin homogenate was used to quantify the drug (14, 15).

#### **7.3.4.3 Ex-vivo fluorescence microscopy study**

The developed formulations' penetration behavior was demonstrated by the use of fluorescence microscopy. For the investigation, FITC suspension, FITC loaded nanoemulsion, and NLCs were prepared and used. The rat skin was defrosted to room temperature, allowed to thaw, and then secured to the Franz diffusion cell and developed formulation were applied using the same procedure as previously described in section 7.3.3.2. After a 12-hour interval, pieces of the skin were mounted on a glass slide and skin sectioning was done using a cryo-microtome in a dark atmosphere. The fluorescence on the slide was examined using a confocal laser scanning microscope (LSA 710, Carl-zeiss Inc., USA) (16, 17).

#### **7.3.4.4 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. 0.2 g Luliconazole loaded gel, Luliconazole loaded Nanoemulsion based gel and Luliconazole loaded NLCs based gel (equivalent to 2 mg LZ) was

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  $37 \pm 1^\circ\text{C}$  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 sink condition (18). The samples were analysed by the HPLC method described in section 3.4.2 and 3.5 of chapter 3. Same methods were applied for the Tavaborole loaded gel, Tavaborole loaded Nanoemulsion based gel and Tavaborole loaded NLC based gel by using 0.2 g gel (equivalent to 10 mg TB).

### **7.3.5 Anti-Fungal activity of developed formulations against *T. rubrum* by zone of inhibition method**

This study was performed at Aakaar Biotechnologies Pvt. Ltd. Lucknow. The Antifungal activity of developed formulations was checked by zone Inhibition Method (Kirby-Bauer method) (19). The SDA (Sabouraud dextrose agar) plates were inoculated by spreading with 100  $\mu\text{l}$  of Fungal culture, *T. rubrum* (adjusted to 0.5 McFarland Unit - Approx cell density ( $1.5 \times 10^8$  CFU/mL) and followed by placing the discs containing 10  $\mu\text{l}$  of different formulations. One disc in each plate was loaded with solvent alone which served as vehicle control and Amphotericin B disc (25 $\mu\text{g}$ ) were taken as positive control. The plates of *T. rubrum* were incubated (Basil Scientific Corp. India- Incubator) at  $37^\circ\text{C}$  for 24 hrs. The clear zones created around the disc were measured and recorded (20).

### **7.3.6 Development of Ex-vivo onychomycosis model**

This study was performed at Aakaar Biotechnologies Pvt. Ltd. Lucknow. The effect of developed formulations of Luliconazole and Tavaborole on the onychomycosis was investigated by using onychomycosis model. Using Ex-vivo onychomycosis model, the effect of developed formulations on the morphology of the growing hyphae of *Trichophyton rubrum* was investigated by scanning electron microscopy (SEM) (21).

Different samples were taken as below table:

**Table 7.3 List of Sample code and sample name**

<b>Sample Code</b>	<b>Sample Name</b>
S1	Luliconazole marketed cream
S2	Luliconazole Nanoemulsion based gel

S3	Luliconazole NLCs based gel
S4	Tavaborole marketed solution
S5	Tavaborole Nanoemulsion based gel
S6	Tavaborole NLCs based gel

**7.3.6.1 Preparation of *T. rubrum* infected Ex- vivo Onychomycosis Model**

To prepare Ex-vivo onychomycosis model, the toenail samples from the healthy volunteer were cut into approximately 1 x 2 mm in size. The ventral surface of the nail was placed face down on the agar plate seeded with  $1 \times 10^6$  *T. rubrum* culture, then incubated at 27°C for 3 days. After that, an aliquot of 5 µL of Luliconazole was applied to the surface of the nail plate with a micropipette and then cultured for an additional 24 h (22).

**7.3.6.2 Scanning Electron Microscopy**

The agar plate on which the infected nails were placed was punched out with a cork borer, and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 18 hr. After being washed with the buffer, specimens were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 18 hr at 4°C. The samples were dehydrated in graded acetone and freeze-dried in t-butyl alcohol. The dried samples of nails were mounted on SEM stubs, coated with osmium with an osmium plasma coater (OPC 60A; Filgen, Japan), and examined in a JSM-7500F field emission scanning electron microscope (JEOL Ltd, Tokyo, Japan), operated at 1 kV (23, 24).

**7.4 Results and Discussion**

**7.4.1 *In-vitro* drug release study**

The % cumulative drug release of Luliconazole and Tavaborole from Plain drug gel and developed formulations at each time point was calculated and shown in tables 7.4 and 7.5, as illustrated in fig. 7.1, 7.2 respectively.

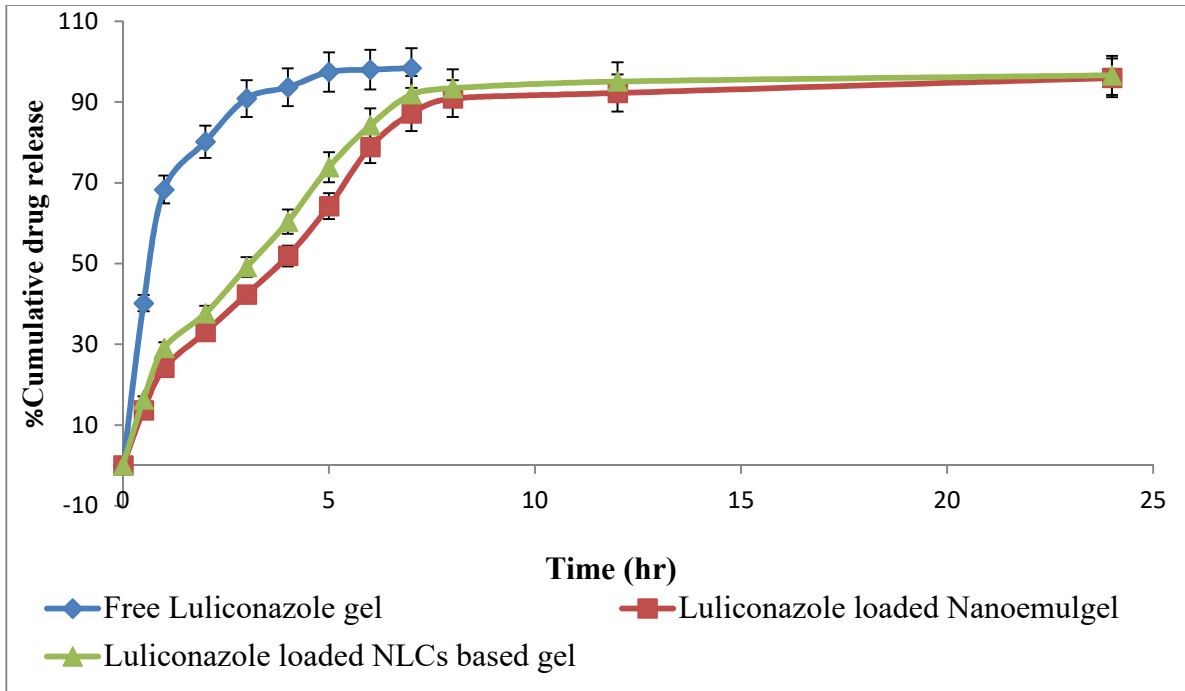
**Table 7.4 *In-vitro* drug release study of Luliconazole loaded formulations**

Time (hr)	%Cumulative drug release		
	Plain Luliconazole gel	Luliconazole loaded Nanoemulsion based	Luliconazole loaded NLCs based gel

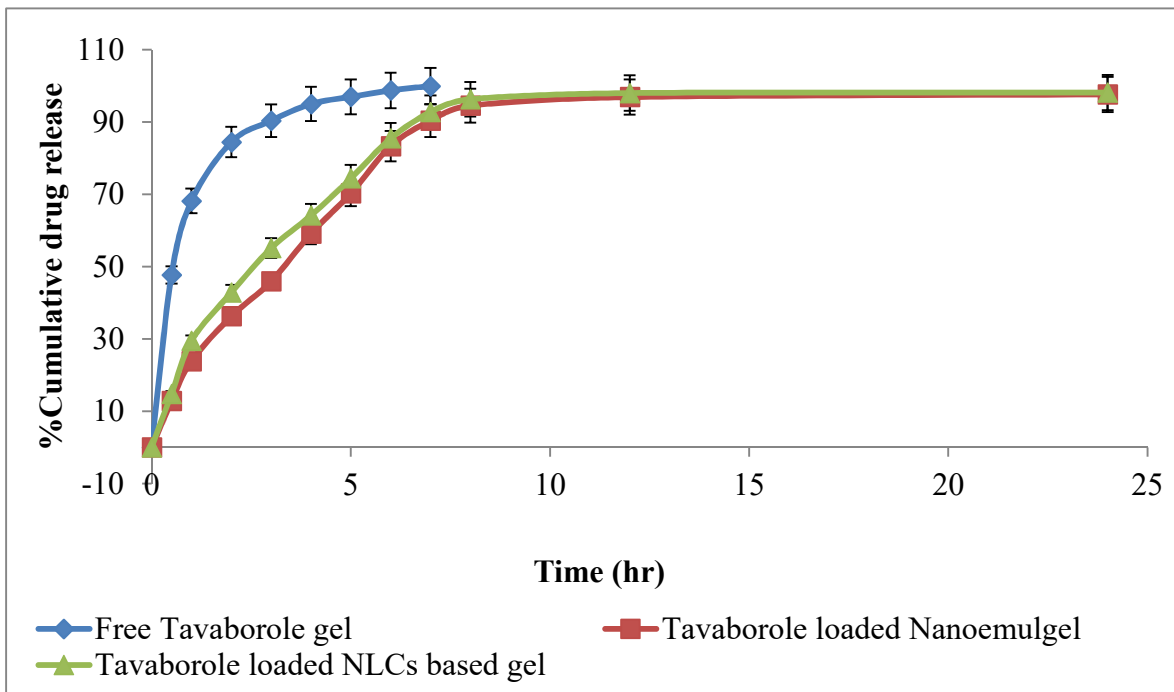
		<b>Gel</b>	
0.5	40.20±0.68	13.65±0.95	16.32±1.25
1	68.34±1.28	24.12±0.28	29.05±2.14
2	80.18±2.07	33.02±1.27	37.66±1.68
3	90.87±1.75	42.32±0.91	49.09±0.21
4	93.66±1.38	51.87±0.06	60.37±0.45
5	97.44±0.88	64.21±0.93	73.85±1.20
6	98.01±2.04	78.84±0.65	84.22±1.05
7	98.39±1.12	87.18±1.04	91.88±0.98
8	-	90.87±0.43	93.43±0.77
12	-	92.23±1.92	95.07±1.25
24	-	95.97±1.96	96.61±1.73

**Table 7.5 *In-vitro* drug release study of Tavaborole loaded formulations**

<b>Time (hr)</b>	<b>%Cumulative drug release</b>		
	<b>Plain Tavaborole gel</b>	<b>Tavaborole loaded Nanoemulsion based gel</b>	<b>Tavaborole loaded NLCs based gel</b>
0.5	47.65±1.28	12.77±0.19	14.77±0.87
1	68.14±0.08	23.82±1.43	29.44±1.65
2	84.43±2.19	36.22±0.97	42.81±0.88
3	90.32±0.21	45.88±1.29	55.09±0.21
4	94.98±1.61	59.12±0.77	64.12±0.33
5	96.94±1.64	70.21±0.93	75.38±0.99
6	98.72±0.81	83.30±1.14	85.43±0.51
7	99.93±0.74	90.37±0.61	92.71±1.26
8	-	94.49±0.17	96.27±0.38
12	-	96.88±1.77	97.98±1.87
24	-	97.61±2.34	98.13±1.44



**Figure 7.1** *In-vitro* drug release study of Luliconazole loaded formulations



**Figure 7.2** *In-vitro* drug release study of Tavaborole loaded formulations

*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  $98.39 \pm 1.12\%$ ,  $95.97 \pm 1.96\%$  and  $96.61 \pm 1.73\%$  respectively. Permeation of drug from Tavaborole loaded gel, Tavaborole loaded Nanoemulsion based gel and Tavaborole loaded NLCs based gel was found to be  $99.93 \pm 0.74\%$ ,  $97.61 \pm 2.34\%$  and  $98.13 \pm 1.44\%$  respectively. Release data of all formulations showed that compare to plain gel Nanoemulgel and NLCs based gel showed sustained release.

The result of different mathematical models, i.e., Zero order, First order, Higuchi model, Hixon Crowell, and Korsmeyer- Peppas model were applied to understand the Luliconazole and Tavaborole release kinetics from nanoemulgel and NLC gel respectively, are presented in table 7.3.

**Table 7.6 Various mathematical models and their correlation coefficient ( $R^2$ ) values**

Mathematical models	Luliconazole loaded Nanoemulgel	Luliconazole loaded NLCs based gel	Tavaborole loaded Nanoemulgel	Tavaborole loaded NLCs based gel
Zero-order	0.599	0.548	0.569	0.537
First-order	0.816	0.756	0.789	0.761
Hixon Crowell	0.739	0.684	0.715	0.693
Higuchi	0.852	0.827	0.836	0.825
Korsmeyer- Peppas	0.926 (n=0.559)	0.910 (n=0.506)	0.913 (n=0.557)	0.898 (n=0.518)

The regression coefficient of the plot of Korsmeyer- Peppas model for Luliconazole loaded Nanoemulsion based gel and Luliconazole loaded NLCs based gel was found to be 0.926 and 0.910 respectively, which are higher than other models. Additionally, the n (release exponent) value was found to be 0.559 and 0.506 for Luliconazole loaded Nanoemulsion based gel and Luliconazole loaded NLCs based gel, respectively. The regression coefficient of the plot of Korsmeyer- Peppas model for Tavaborole loaded Nanoemulsion based gel and Tavaborole loaded NLCs based gel was found to be 0.913 and 0.898 respectively, which are higher than other models. Additionally, the n (release exponent) value was found to be 0.557 and 0.518 for Tavaborole loaded Nanoemulsion based gel and Tavaborole loaded NLCs based gel,

respectively. Moreover, If (n) value is within the range of (0.43– 0.50), then release follows Fickian diffusion, while (n) value greater than (0.5) corresponding to non-fickian transport mechanism. It is further divided into three ranges as follows:  $0.50 < n < 0.89$  for anomalous release,  $0.89 < n < 1.00$  for case II transport and  $n > 1.00$  for super case II transport. Here all the developed formulation followed non-fickian transport mechanism (anomalous release). One interpretation of this diffusion-based mechanism is that the oil phase will create a (filter) or a (membrane-like barrier) against the hydrophilic sheath of NE. As a result, the CC molecules leakage from the NE followed a linear release behavior as long as this oily barrier is intact (25).

#### 7.4.2 *In-vitro* cytotoxicity and cellular uptake studies

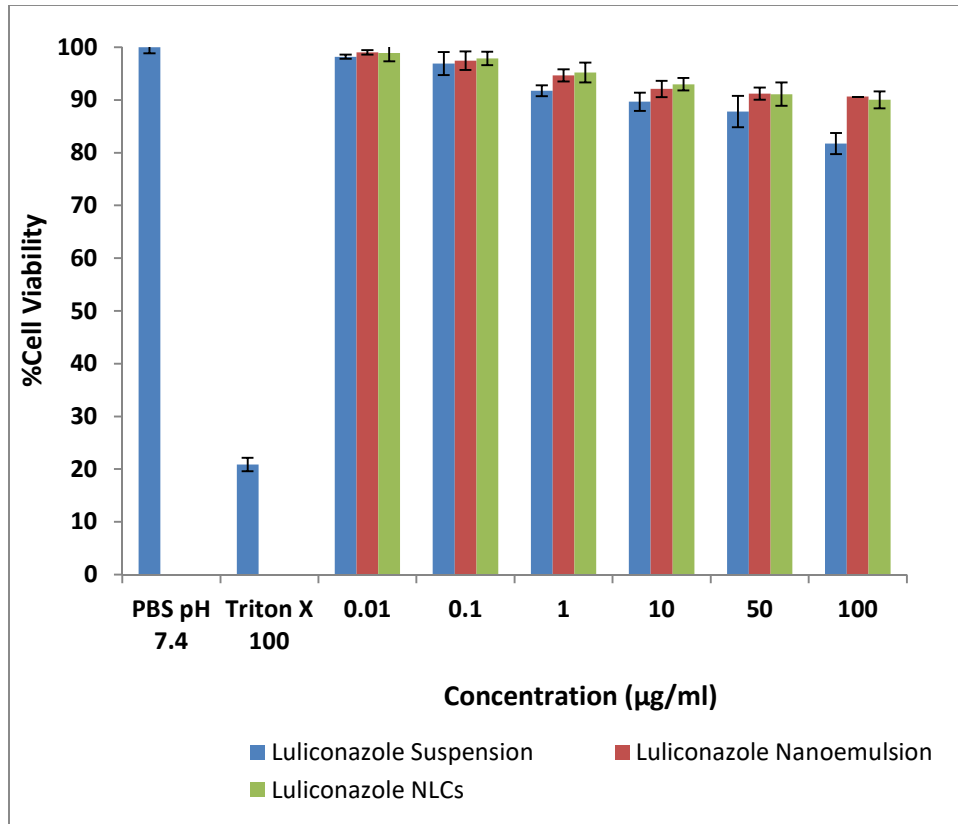
##### 7.4.2.1 MTT assay

The cell viability data for Luliconazole and Tavaborole loaded formulations are summarized in table 7.3 and illustrated in fig. 7.3 and 7.4 respectively,

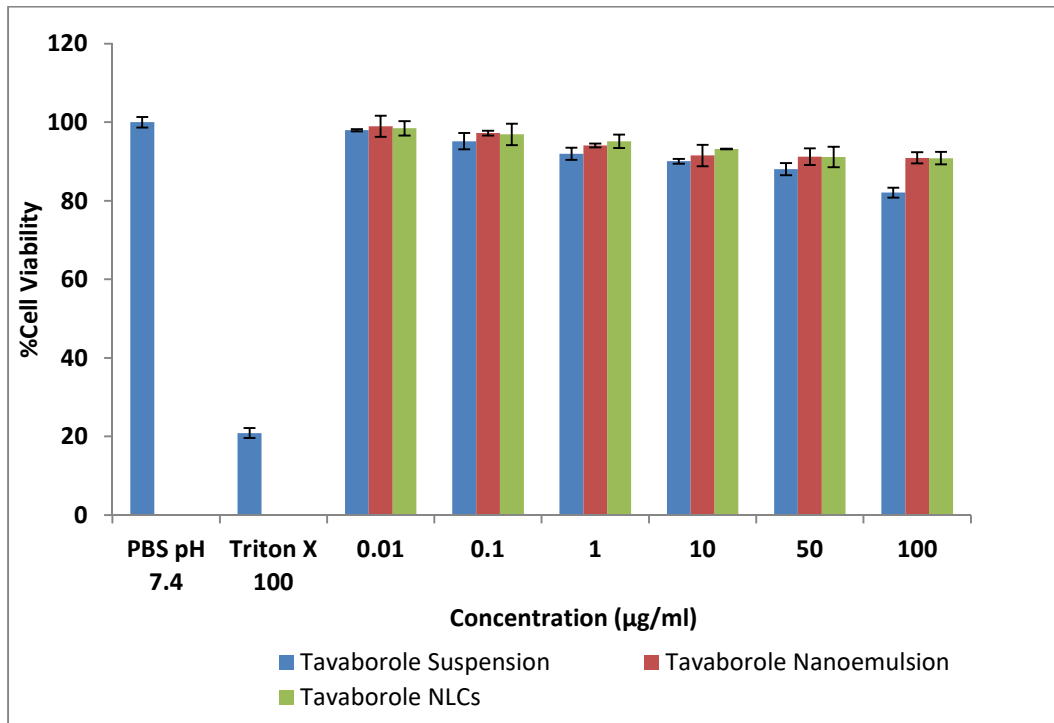
**Table 7.7 *In-vitro* cell viability data for Luliconazole and Tavaborole loaded formulations in 3T3 fibroblast cells**

Conc. ( $\mu\text{g/ml}$ )	%Cell viability					
	Luliconazole			Tavaborole		
	Suspension	Nanoemuls ion	NLCs	Suspension	Nanoemulsi on	NLCs
0.01	98.21 $\pm$ 0.37	99.03 $\pm$ 0.07	98.91 $\pm$ 0.68	97.97 $\pm$ 0.31	98.97 $\pm$ 2.66	98.45 $\pm$ 1.82
0.1	96.89 $\pm$ 2.18	97.45 $\pm$ 0.65	97.88 $\pm$ 0.29	95.19 $\pm$ 2.08	97.25 $\pm$ 0.62	96.91 $\pm$ 2.74
1	91.76 $\pm$ 0.05	94.65 $\pm$ 1.98	95.21 $\pm$ 1.59	91.96 $\pm$ 1.55	94.09 $\pm$ 0.48	95.13 $\pm$ 1.73
10	89.66 $\pm$ 1.72	92.10 $\pm$ 0.77	92.98 $\pm$ 1.25	90.07 $\pm$ 0.62	91.55 $\pm$ 2.74	93.22 $\pm$ 0.08
50	87.82 $\pm$ 2.97	91.21 $\pm$ 2.11	91.09 $\pm$ 0.88	88.05 $\pm$ 1.57	91.21 $\pm$ 2.11	91.19 $\pm$ 2.61
100	81.74 $\pm$ 1.98	90.67 $\pm$ 0.54	90.03 $\pm$ 0.60	82.08 $\pm$ 1.29	90.92 $\pm$ 1.42	90.87 $\pm$ 1.59

Cells treated with PBS pH 7.4 were taken as 100% cell viability. %Cell viability of positive control (Triton X 100) was found to be 20.89 $\pm$ 1.98%.



**Figure 7.3** *In-vitro* cell viability data for Luliconazole formulations in 3T3 fibroblast cells

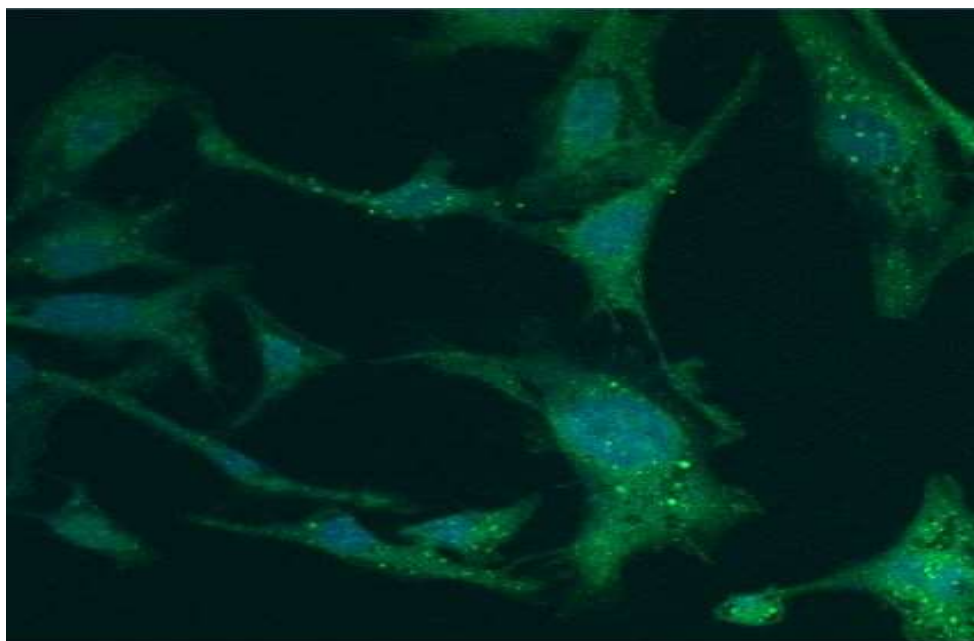


**Figure 7.4** *In-vitro* cell viability data for Tavaborole formulations in 3T3 fibroblast cells

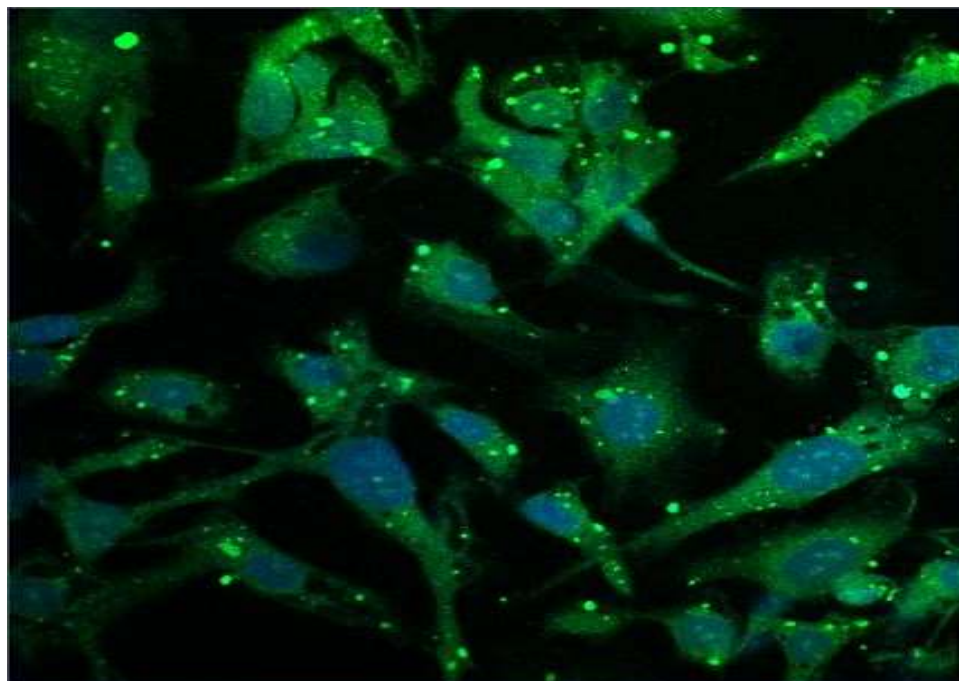
The developed Luliconazole loaded Nanoemulsion, Luliconazole loaded NLCs, Tavaborole loaded Nanoemulsion and Tavaborole loaded NLCs have no potential toxic effects on the 3T3-fibroblast cells and are found to be safe for dermal delivery.

#### **7.4.2.2 Cellular uptake studies**

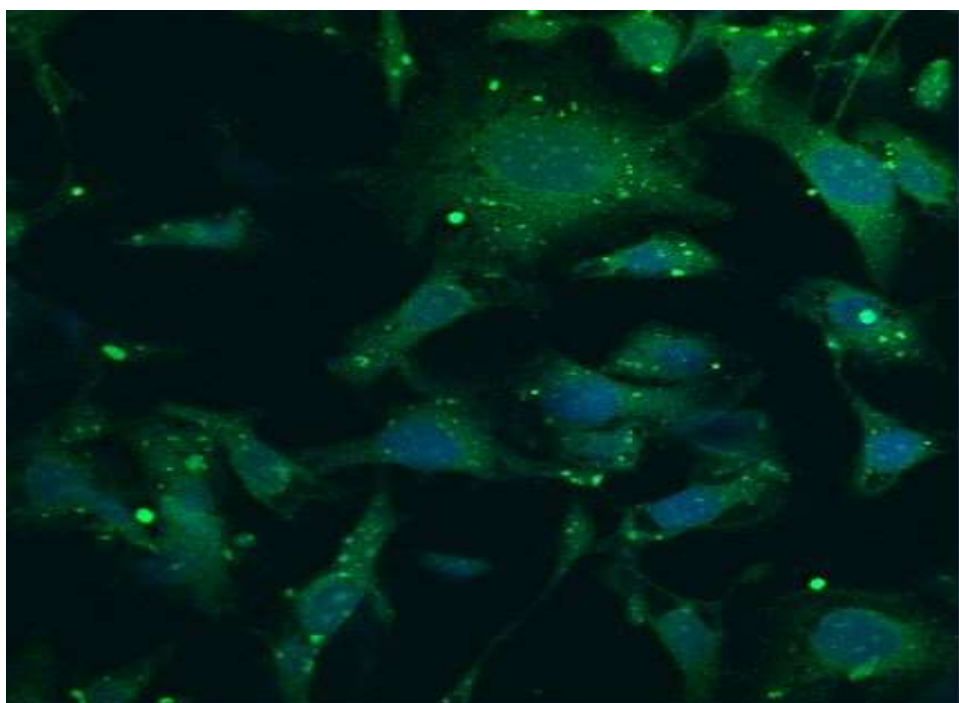
The cellular uptake studies of Luliconazole and Tavaborole formulations are demonstrated in figure 7.5 and 7.6, respectively. Result showed the higher cellular uptake of the Luliconazole loaded NE and NLCs gel compared to Plain Luliconazole gel and the higher cellular uptake of the Tavaborole loaded NE and NLCs gel compared to Plain Tavaborole gel after 4 hr. Higher cellular uptake can correlate with the enhancement of therapeutic efficacy of Luliconazole and Tavaborole.



**(A) Luliconazole gel**

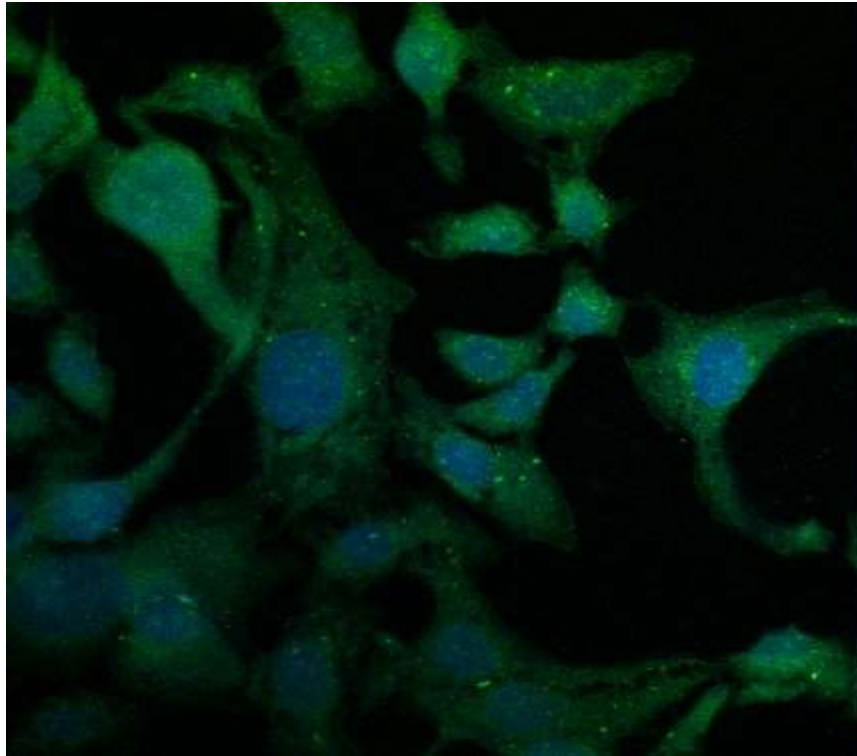


**(B)** *Luliconazole* Nanoemulsion based gel

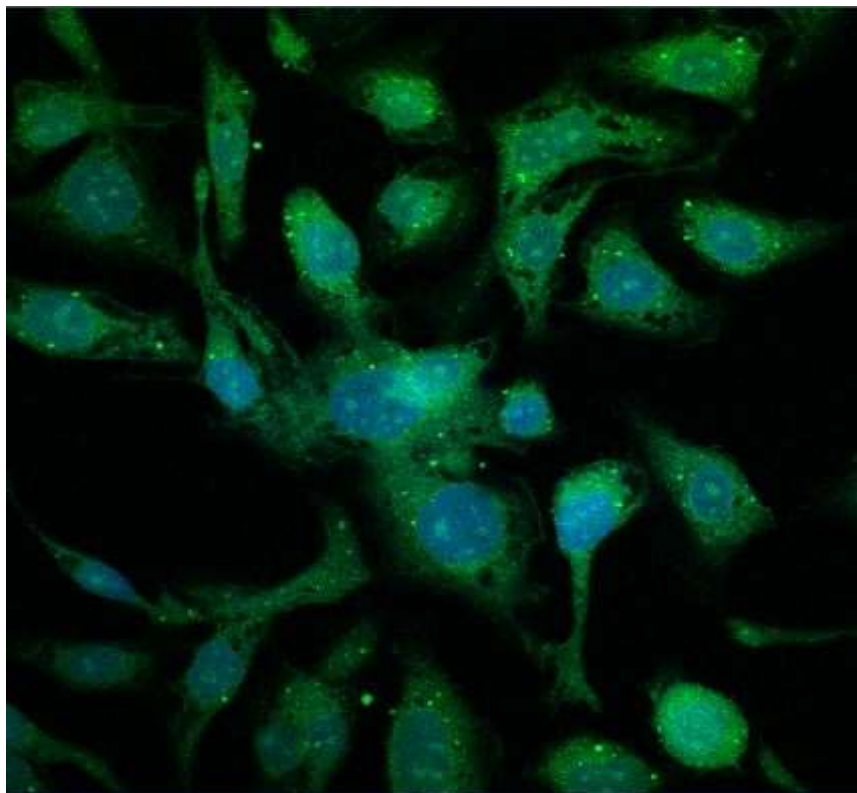


**(C)** *Luliconazole* NLC based gel

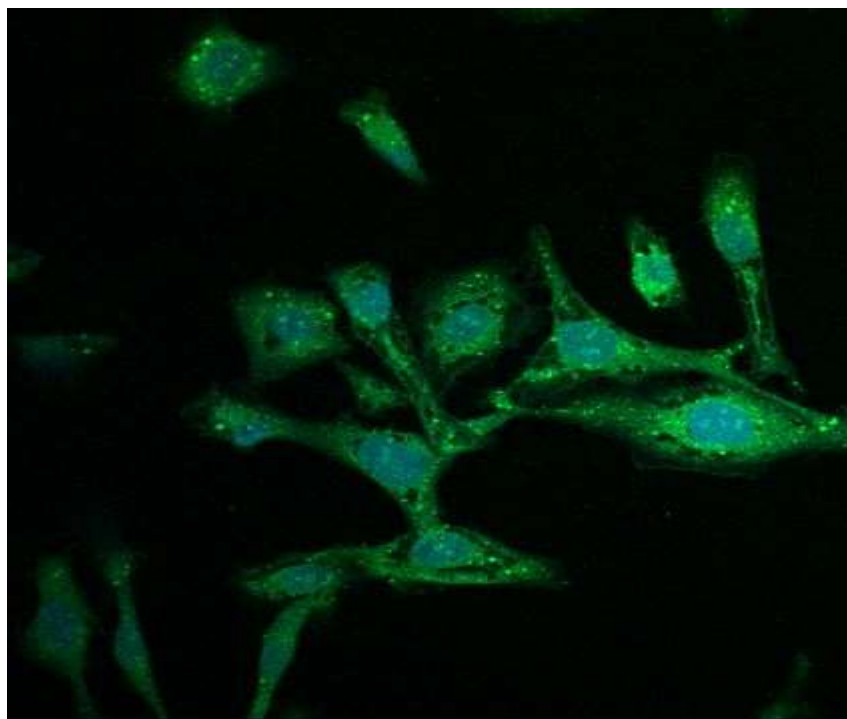
**Figure 7.5** *In-vitro* cell uptake of *Luliconazole* formulations in 3T3-fibroblast cells



**(A) Tavaborole gel**



**(B) Tavaborole Nanoemulsion based gel**



(C)Tavaborole NLCs based gel

**Figure 7.6 *In-vitro* cellular uptake of Tavaborole formulations in 3T3-fibroblast cells**

**7.4.3 *In-vitro* determination of minimum inhibitory concentration (MIC)**

Luliconazole loaded formulations exhibited MIC in the range of 0.063-0.07 µg/ml and 0.0018-0.0024 against *Candida albicans* and *Trichophyton rubrum* respectively. Tavaborole loaded formulations exhibited a MIC in the range of 0.8-1 µg/ml and 1-1.3 µg/ml against *Candida albicans* and *Trichophyton rubrum* respectively. The results indicate that Luliconazole and Tavaborole loaded formulations have strong antifungal activity against *Candida albicans* and *Trichophyton rubrum*.

**Table 7.8 MIC values of developed formulations of Luliconazole and Tavaborole against *Candida albicans* and *Trichophyton rubrum***

Formulations	MIC (µg/ml)	
	<i>Candida albicans</i>	<i>Trichophyton rubrum</i>
Plain Luliconazole gel	0.063±0.001	0.0018±0.0006
Luliconazole Nanoemulsion based gel	0.07±0.01	0.002±0.001
Luliconazole NLCs based gel	0.08±0.01	0.0024±0.001

Plain Tavaborole gel	0.8±0.1	1±0.2
Tavaborole Nanoemulsion based gel	1±0.3	1.2±0.4
Tavaborole NLCs based gel	1±0.5	1.3±0.3

From the table 7.8, it can be concluded that there is no significant difference in MIC of plain drug gel and developed formulations of LZ and TB.

#### 7.4.4 Ex-vivo characterization

##### 7.4.4.1 Ex-vivo permeation and deposition study

The results of ex-vivo drug permeation and skin retention of Luliconazole and Tavaborole loaded formulations are summarized in tables 7.9 and 7.10 respectively.

**Table 7.9 Ex-vivo drug release study of Luliconazole loaded formulations**

Time (hr)	%Cumulative drug release		
	Plain Luliconazole gel	Luliconazole loaded Nanoemulsion based gel	Luliconazole loaded NLCs based gel
0.5	1.32±1.08	2.47±1.58	2.01±0.97
1	3.45±1.17	5.48±0.95	4.91±0.82
2	5.22±2.11	10.02±1.66	7.21±0.66
3	6.98±1.56	12.32±0.68	9.01±1.76
4	9.43±0.04	14.76±1.03	10.51±0.21
5	10.62±0.98	15.97±2.31	11.08±1.44
6	11.33±1.88	17.08±0.33	12.77±0.90
7	12.11±2.07	19.22±1.88	14.21±2.33
8	13.42±0.96	21.87±0.39	16.05±0.92
12	14.03±1.05	22.99±1.41	18.58±0.39
24	14.21±0.87	24.32±1.90	19.91±0.54

**Table 7.10 Ex-vivo drug release study of Tavaborole loaded formulations**

Time (hr)	%Cumulative drug release		
	Plain Tavaborole loaded gel	Tavaborole loaded Nanoemulsion based gel	Tavaborole loaded NLCs based gel
0.5	5.74±0.28	6.20±1.60	7.73±0.52
1	8.45±0.75	10.81±0.37	10.61±0.27
2	9.82±0.71	14.66±0.28	14.95±0.81
3	12.98±1.56	16.80±0.03	17.39±0.65
4	14.85±2.36	20.01±0.79	20.44±0.09
5	16.62±1.29	22.77±0.04	24.72±1.93
6	18.10±0.72	25.99±0.84	27.21±0.67
7	21.01±0.97	28.04±0.75	30.01±0.93
8	24.29±1.66	30.27±0.02	33.85±2.03
12	26.76±0.95	33.71±0.94	36.97±1.18
24	28.68±0.08	35.62±2.41	38.12±1.67

**Table 7.11: Ex-vivo Drug release from developed formulations of Luliconazole and Tavaborole**

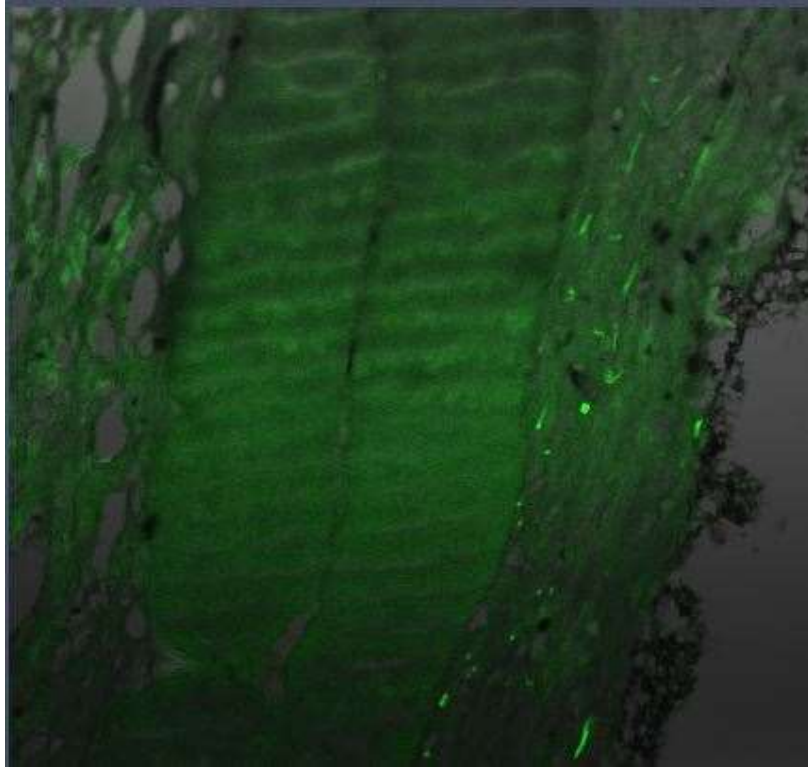
Formulations	Drug permeated across skin after 24 hrs(%)	Drug deposited within skin (%)	Drug retained on skin surface (%)
Plain Luliconazole loaded gel	14.21±0.87	18.16±1.29	64.98±0.87
Luliconazole loaded nanoemulsion based gel	24.32±1.90	64.76±2.32	10.21±1.06
Luliconazole loaded NLCs based gel	19.91±0.54	68.66±1.15	10.43±1.58
Plain Tavaborole loaded gel	28.68±0.08	14.39±1.77	56.32±2.13

Tavaborole loaded nanoemulsion based gel	35.62±2.41	55.98±0.96	7.41±1.60
Tavaborole loaded NLCs based gel	38.12±1.67	52.54±0.41	9.16±1.83

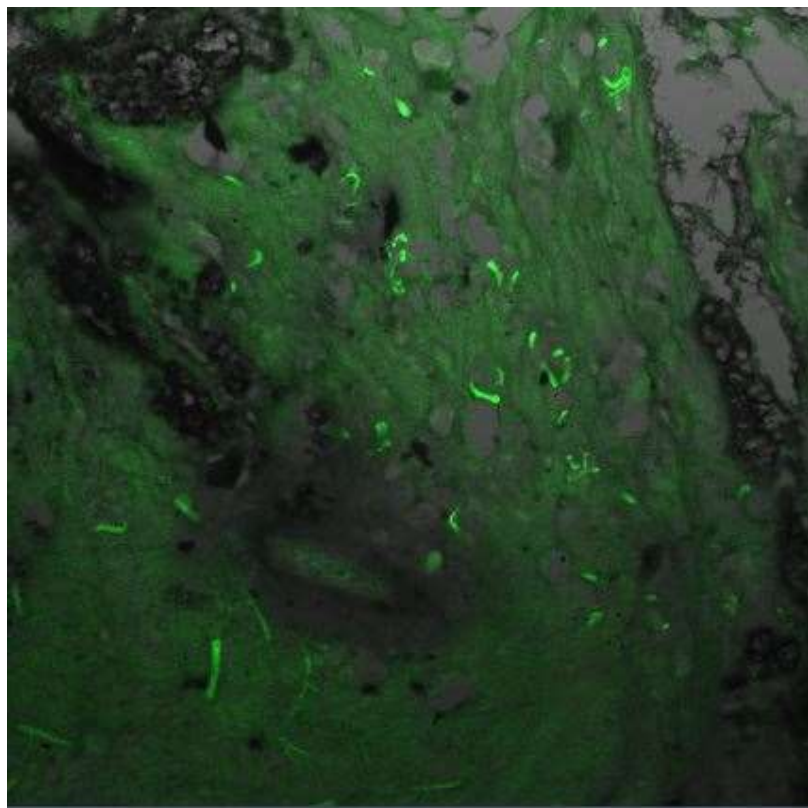
Table 7.11 shows the drug permeation through skin after 24 hours, drug deposited within skin and drug retention on skin surface from different formulations. LZ NE and LZ NLCs showed higher drug deposition within skin compared to LZ plain gel. It may be due to lipophilic nature of formulations which has higher affinity towards lipid presents in skin. TB NE and TB NLCs showed higher drug deposition compared to plain drug. This indicates the higher availability of the drugs from lipidic nanoformulations compared to plain gel. The drug retained on the skin surface is very less in case of the lipid nanoformulations compared to plain gel. This indicates the suitability of the developed formulations to treat deep fungal infections as majority of the drug gets deposited in the skin.

#### **7.4.4.2 Ex-vivo fluorescence microscopy study:**

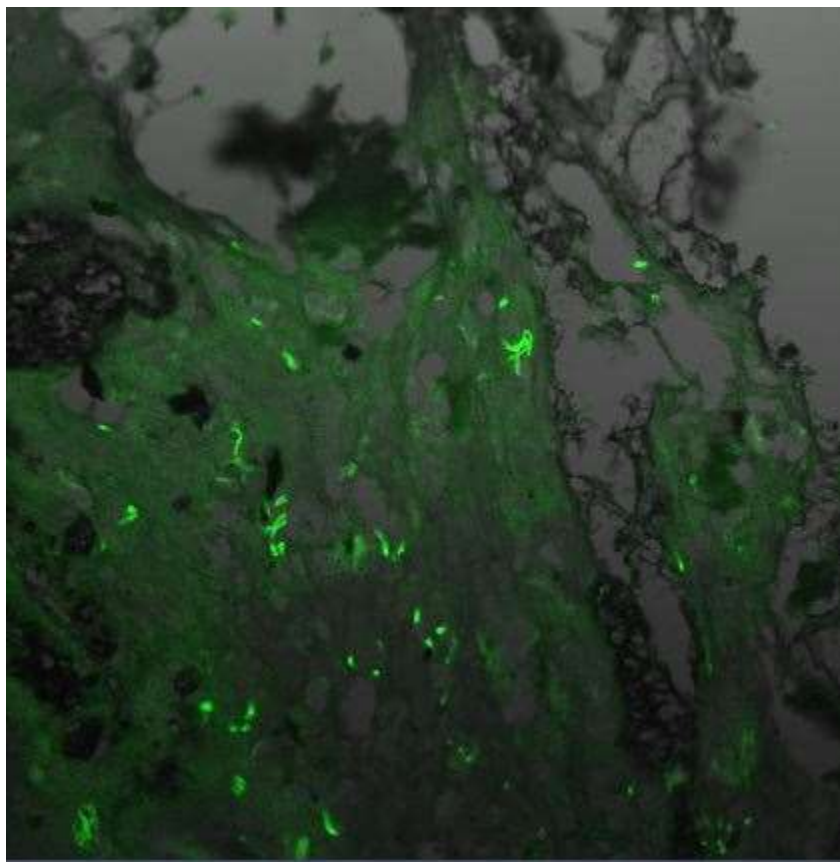
Sections of the rat skin were taken and exposed to FITC loaded formulations for a period of 12 hours. Further, after exposure fluorescence microscopic images of rat skin sections was taken and the images are presented in figure 7.7 and figure 7.8. It is noted that the sections of skin exposed to FITC suspension has least fluorescence.



**A. Luliconazole gel**

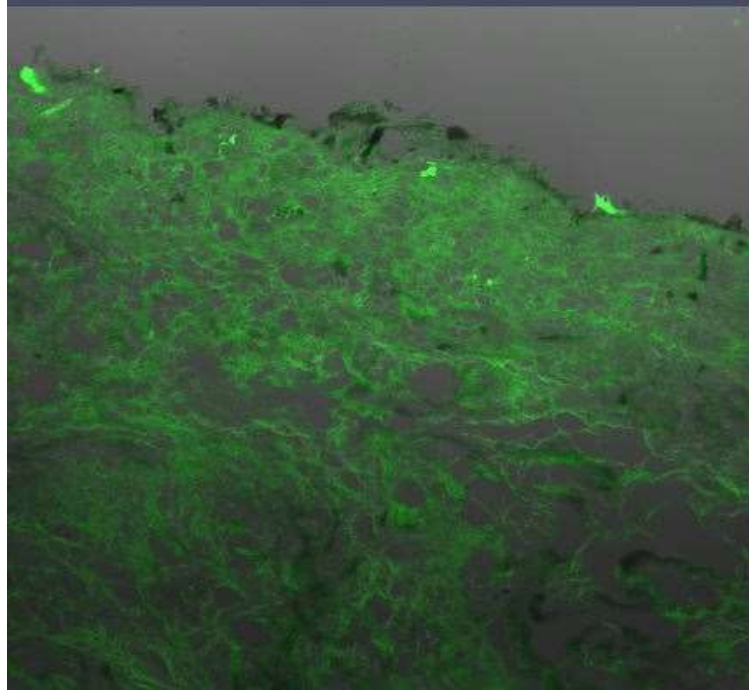


**B. Luliconazole loaded nanoemulsion based gel**

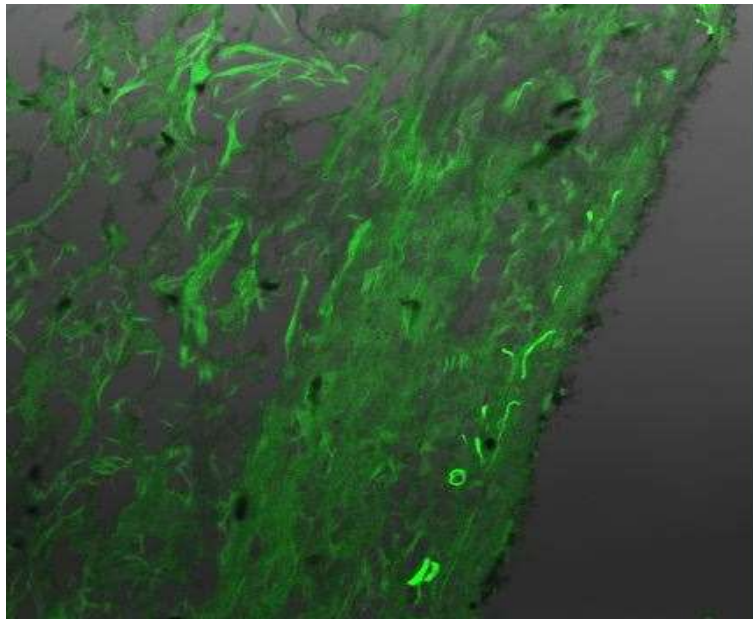


**C. Luliconazole loaded NLCs based gel**

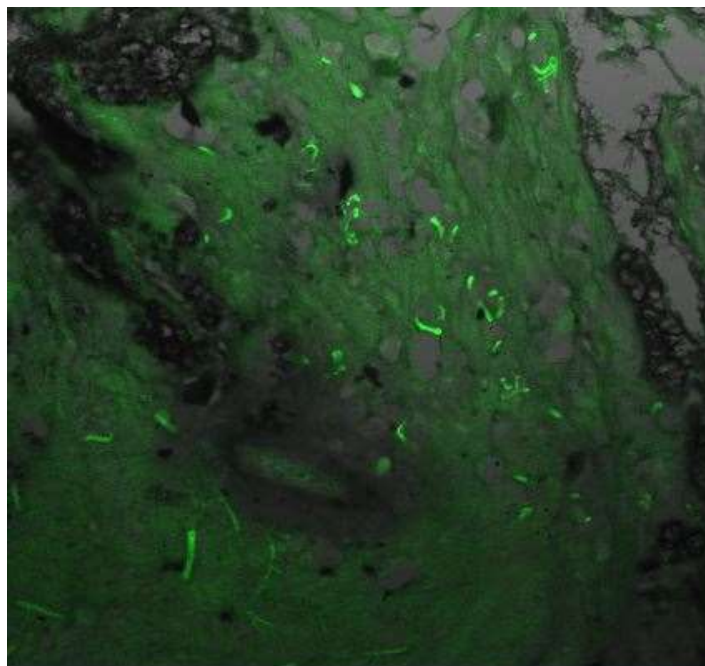
**Figure 7.7 Fluorescence microscopic images of rat skin sections after 12h of treatment with A. Luliconazole gel B. Luliconazole loaded nanoemulsion based gel and C. Luliconazole loaded NLCs based gel**



**A. Tavorole gel**



**B. Tavorole loaded nanoemulsion based gel**



**C. Tavorole loaded NLCs based gel**

**Figure 7.8 Fluorescence microscopic images of rat skin sections after 12h of treatment with A. Tavorole gel B. Tavorole loaded nanoemulsion based gel and C. Tavorole loaded NLCs based gel**

It was noted that the data collected from the fluorescence microscope experiment, complied with the ex vivo permeation and deposition data wherein more fluorescence was reported in the section of skin which were exposed to the Luliconazole and Tavorole loaded lipid based nano formulations. Therefore, it can be concluded that there is enhanced permeation of the drugs through the developed nanocarriers loaded gel.

**7.4.4.3 Transungual Permeation Study:**

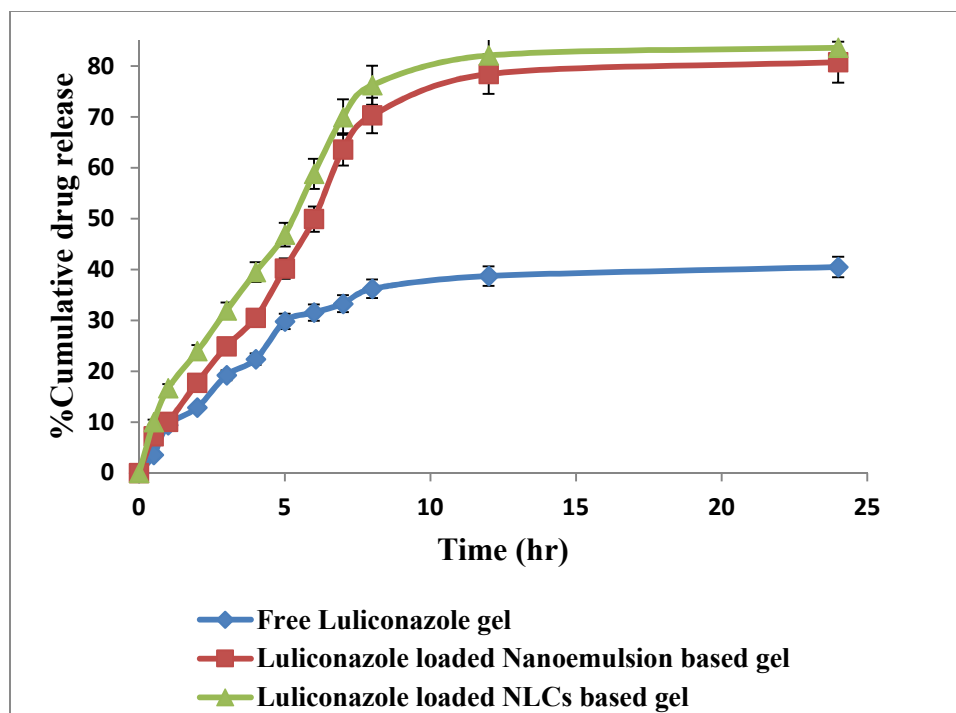
**Table 7.12 Transungual Permeation by Luliconazole loaded formulations**

Time (hr)	%Cumulative drug release		
	Plain Luliconazole gel	Luliconazole loaded Nanoemulsion based gel	Luliconazole loaded NLCs based gel
0.5	3.57±0.98	7.24±0.68	9.98±1.29
1	9.42±0.34	10.08±1.24	16.65±1.02

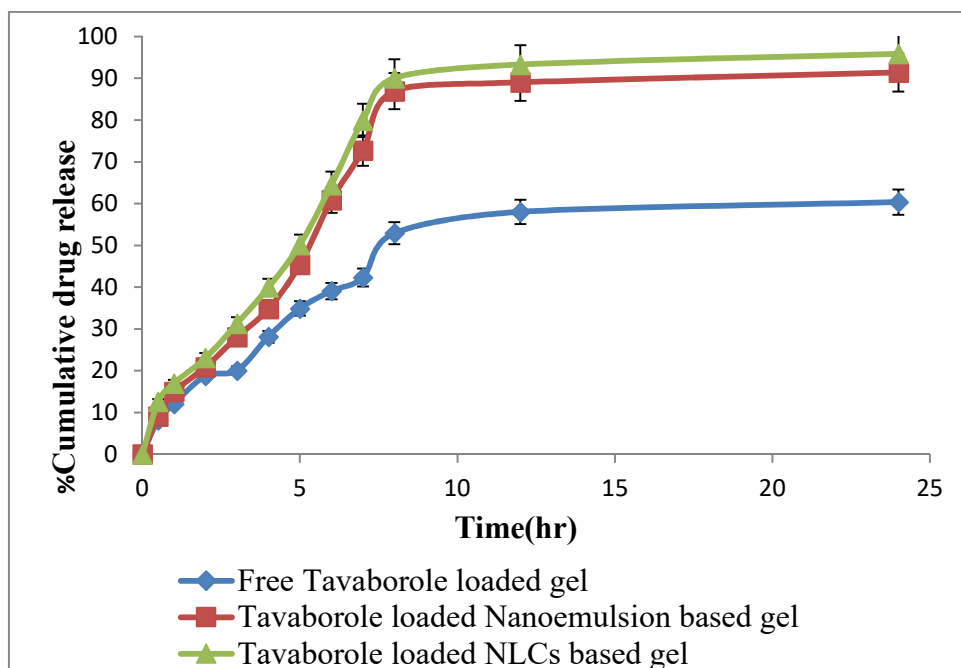
2	12.91±0.54	17.76±0.31	23.94±0.98
3	19.22±1.28	24.92±1.54	31.92±0.64
4	22.37±0.87	30.52±2.05	39.49±1.27
5	29.81±1.17	40.21±0.99	46.84±0.59
6	31.54±2.56	49.92±1.23	58.82±1.61
7	33.28±0.09	63.61±0.51	69.96±0.33
8	36.21±1.73	70.29±1.72	76.23±0.41
12	38.71±0.42	78.42±0.81	82.08±0.76
24	40.50±1.28	80.77±1.57	83.61±1.22

**Table 7.13 Transungual Permeation by Tavaborole loaded formulations**

Time (hr)	%Cumulative drug release		
	Plain Tavaborole loaded gel	Tavaborole loaded Nanoemulsion based gel	Tavaborole loaded NLCs based gel
0.5	8.14±0.61	9.03±0.45	12.56±1.09
1	12.07±0.74	14.92±0.09	16.98±0.85
2	18.75±1.25	20.86±1.54	23.05±2.04
3	20.04±0.81	28.03±0.97	31.28±0.98
4	28.12±0.04	34.81±2.13	39.98±0.52
5	34.91±0.65	45.39±2.06	50.09±2.31
6	39.07±0.94	60.84±1.73	64.43±1.24
7	42.31±1.60	72.69±0.94	79.93±1.62
8	52.92±0.81	86.93±0.72	90.03±0.73
12	58.03±2.28	89.02±2.04	93.29±0.42
24	60.37±1.47	91.38±1.96	95.85± 2.08

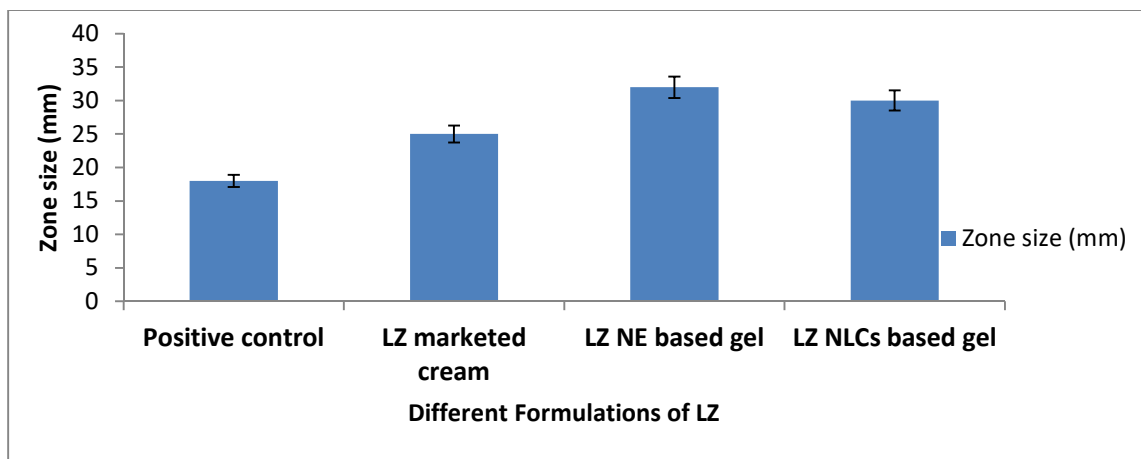


**Figure 7.9** Transungual Permeation study of Luliconazole loaded developed formulations

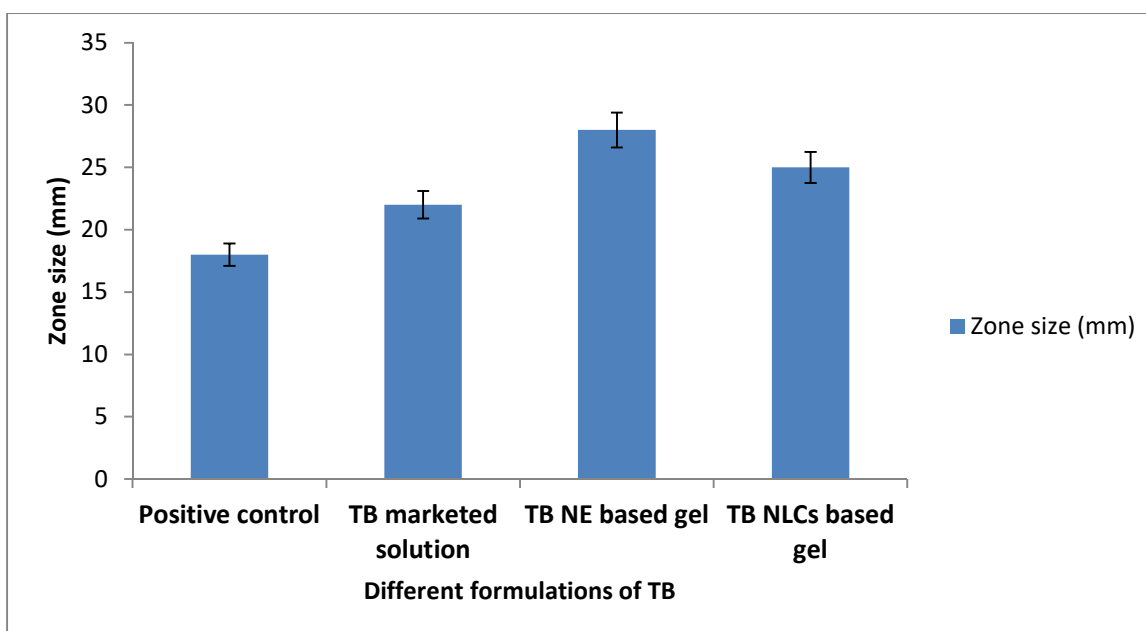


**Figure 7.10** Transungual Permeation study of Tavaborole loaded developed formulations

**7.4.5 Anti-microbial activity assay against *T. rubrum* by Zone of Inhibition method**



**Figure 7.11 Antifungal activity of different formulations of LZ**

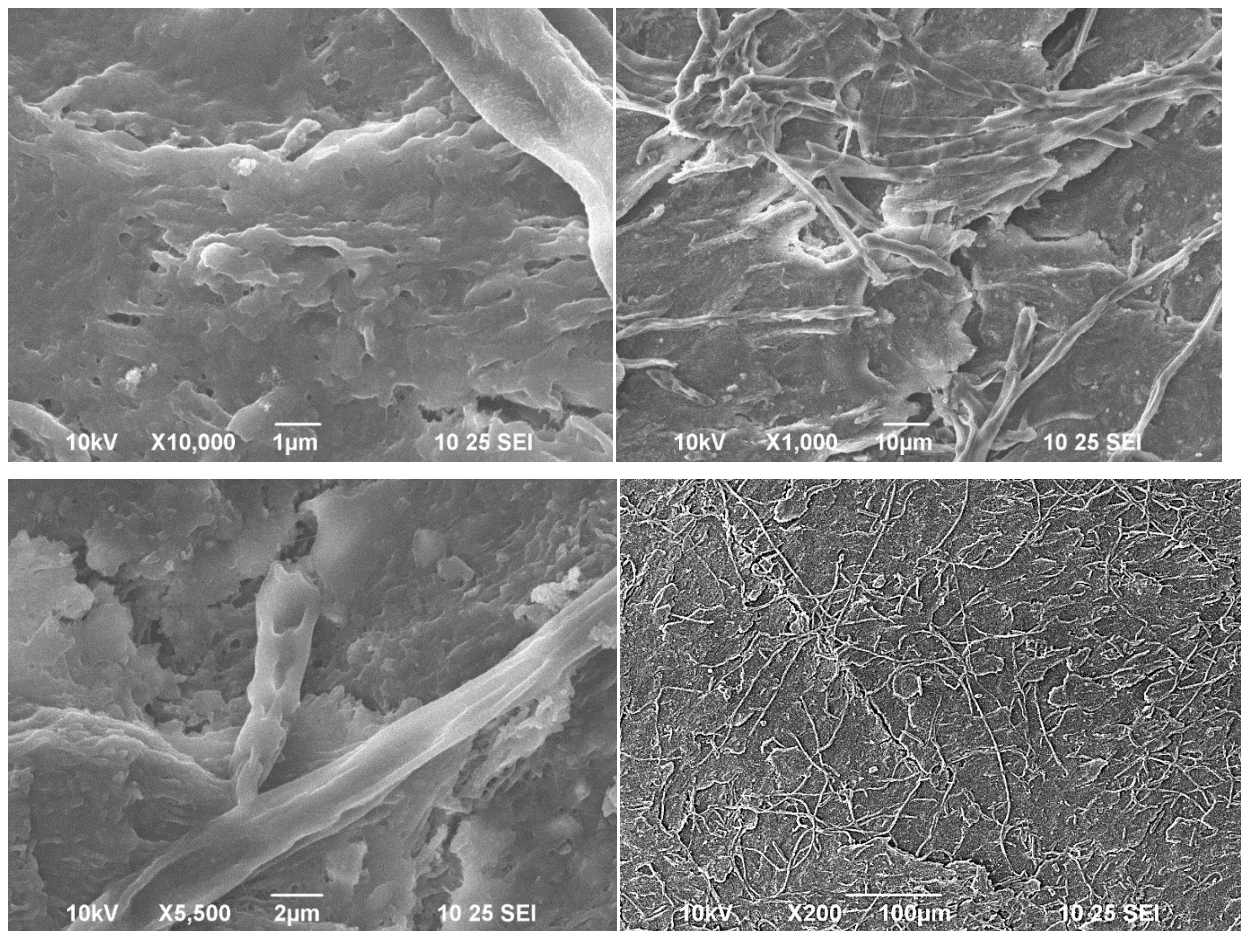


**Figure 7.12 Antifungal activity of different formulations of TB**

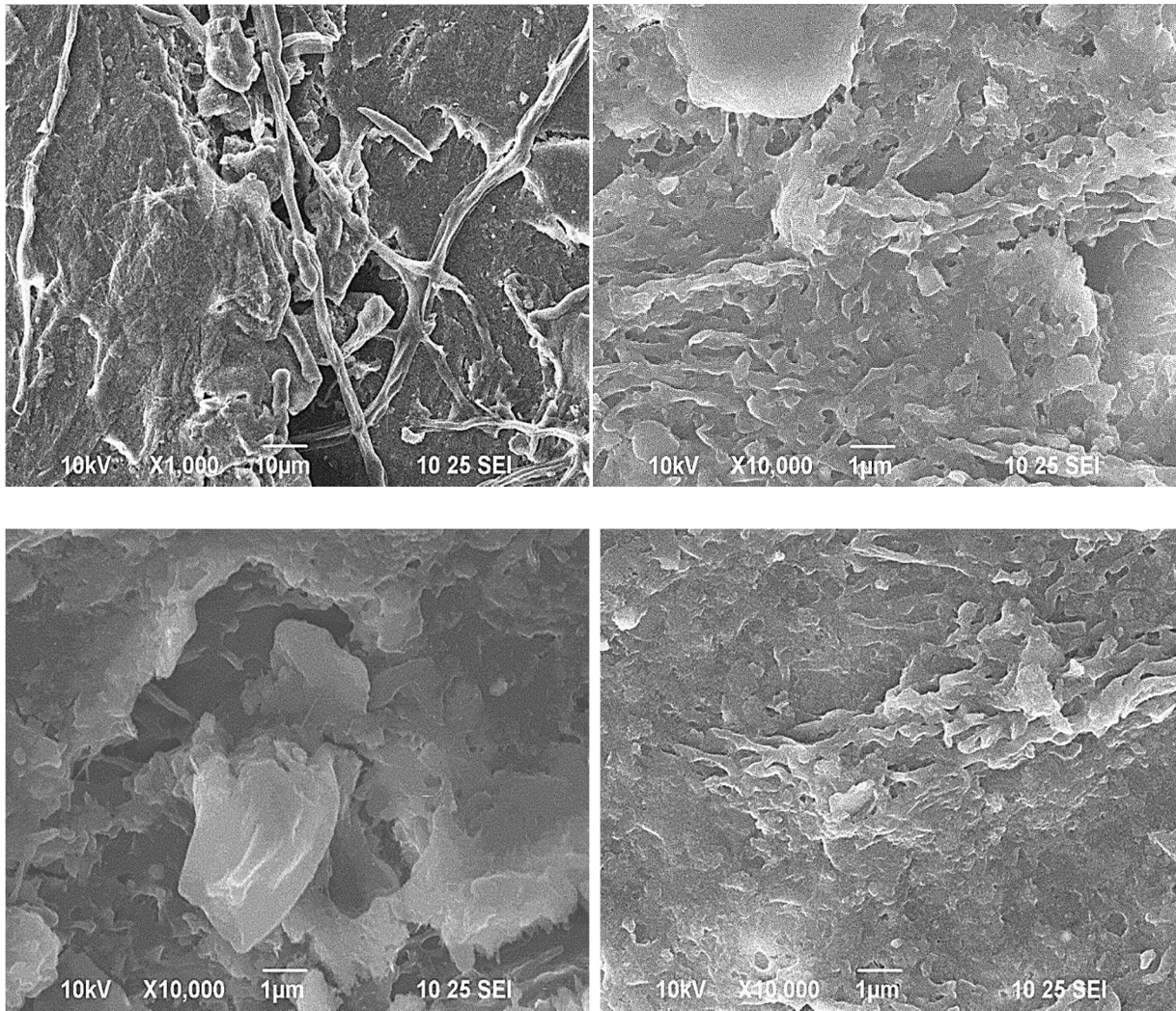
The zone of inhibition is an area around a disk on an agar plate where no fungal growth is observed due to the presence of an antimicrobial agent i.e. Luliconazole and Tavaborole respectively. It is used to determine whether a particular test organism is susceptible to the action of a particular antimicrobial agent or not. Figure 7.11 shows zone of inhibition of marketed product of LZ, LZ NE based gel and LZ NLCs based gel. It can be concluded from the figure that both developed formulations of LZ show higher zone of inhibition compare to marketed

formulation of LZ. LZ NE based gel shows higher zone of inhibition compare to LZ NLCs based gel because LZ NE based gel contains coconut oil which produce synergistic anti fungal activity. Figure 7.12 shows zone of inhibition of marketed product of TB, TB NE based gel and TB NLCs based gel. It can be concluded from the figure that both developed formulations of TB show higher zone of inhibition compare to marketed formulation of TB. TB NE based gel shows higher zone of inhibition compare to TB NLCs based gel because TB NE based gel contains coconut oil which produce synergistic anti fungal activity.

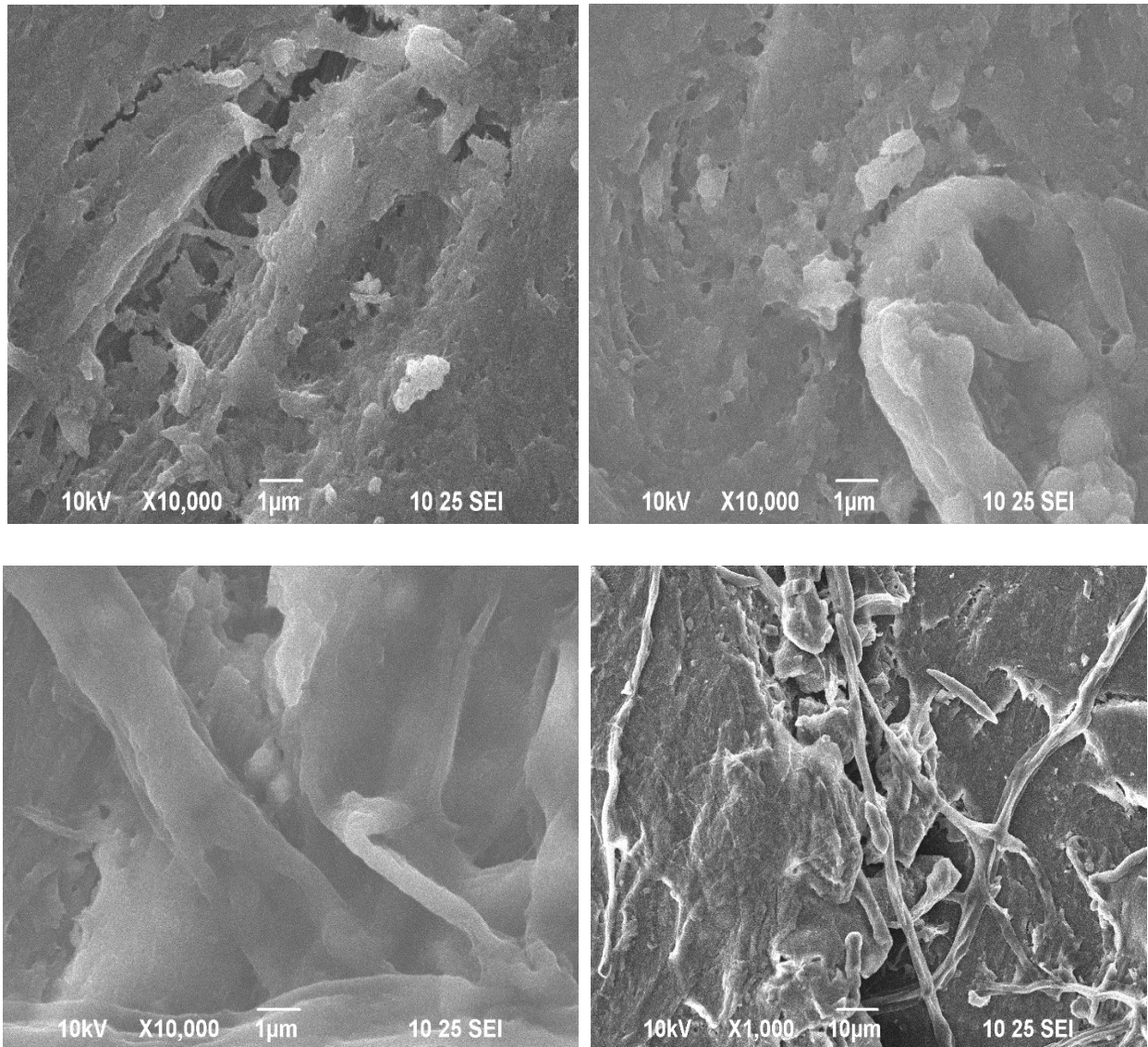
#### 7.4.6 Development of Ex-vivo onychomycosis model



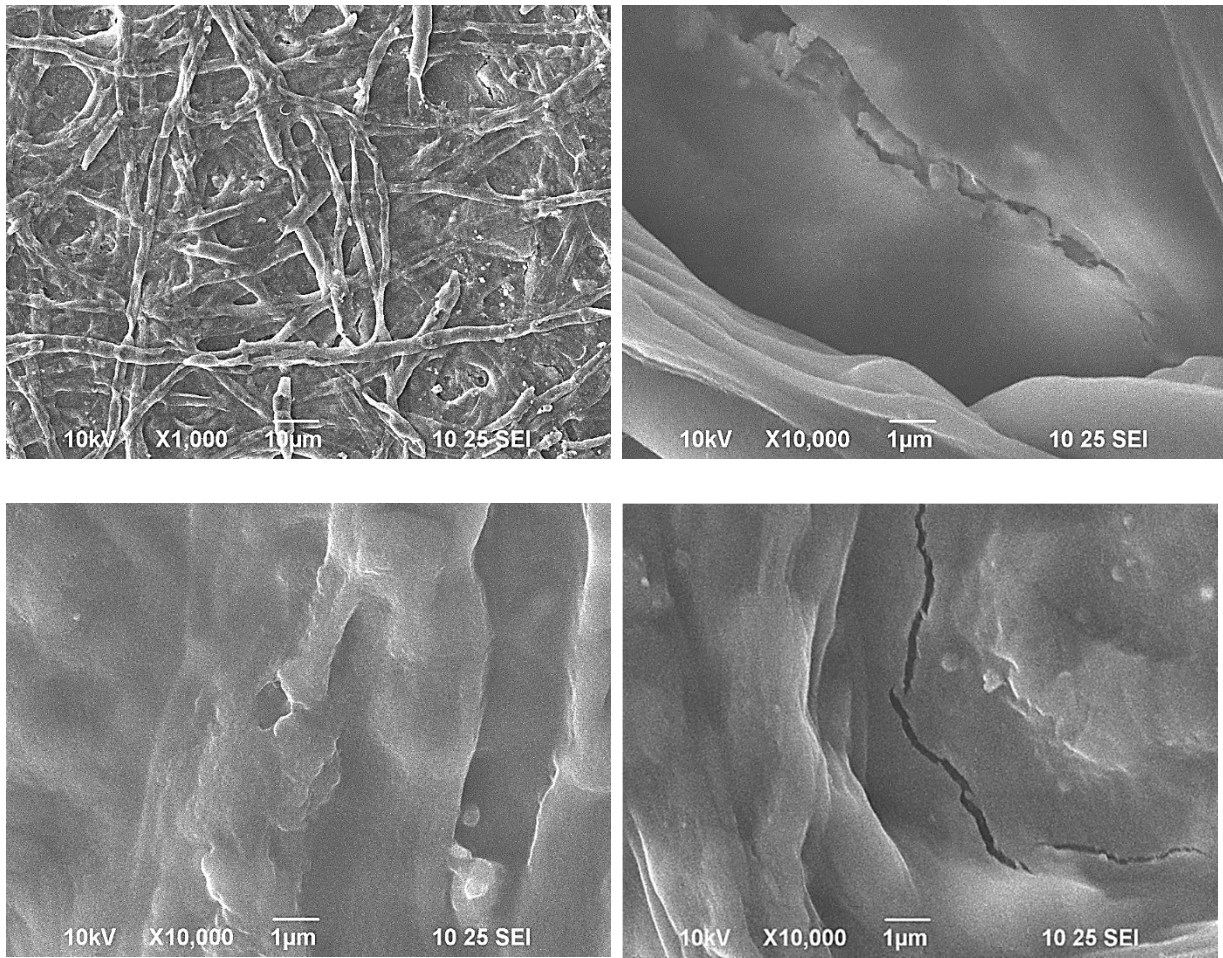
**Figure 7.13 Sample-1 (Culture + Nail Treated with Sample-1)**



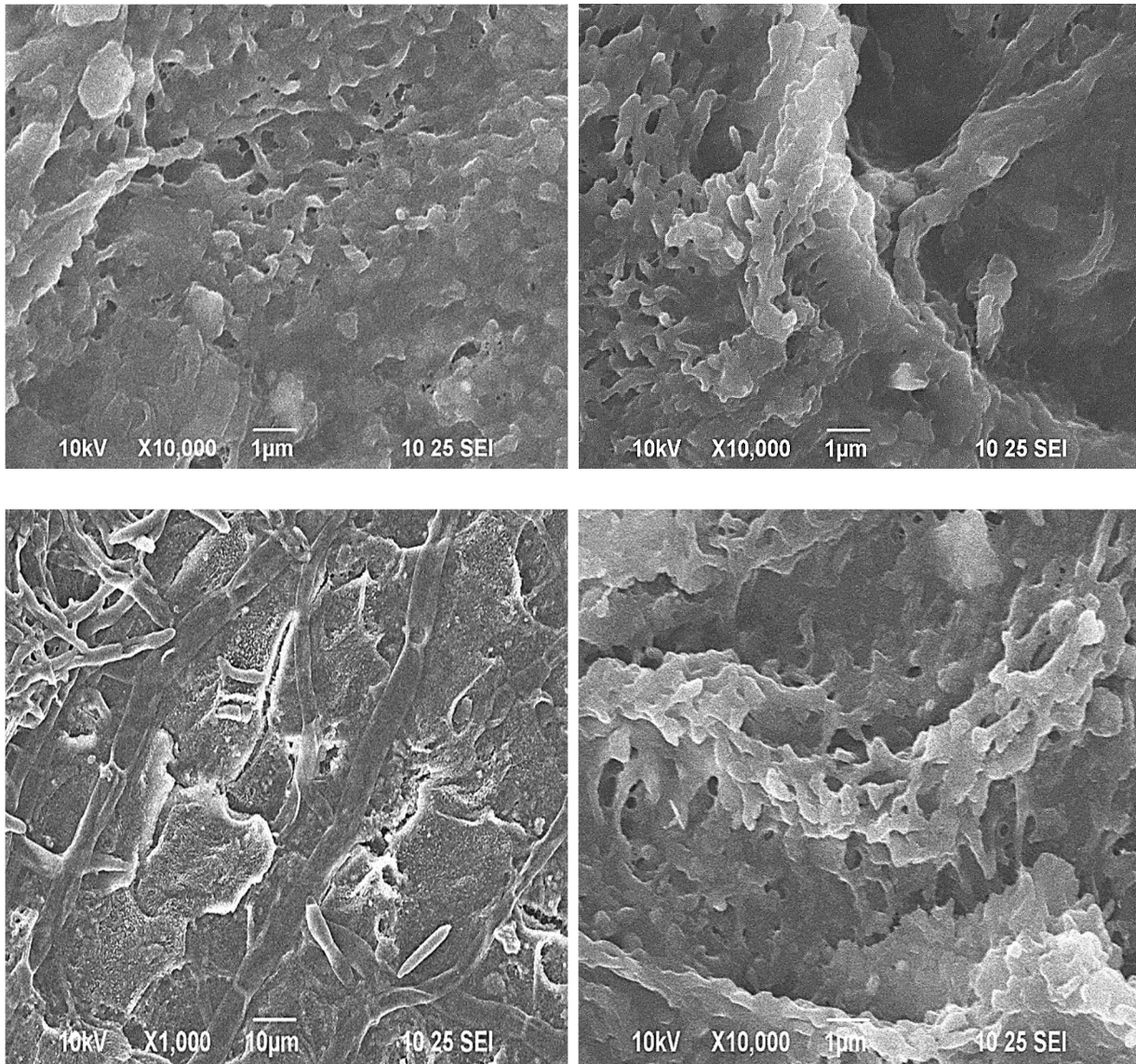
**Figure 7.14 Sample-2 (Culture + Nail Treated with Sample-2)**



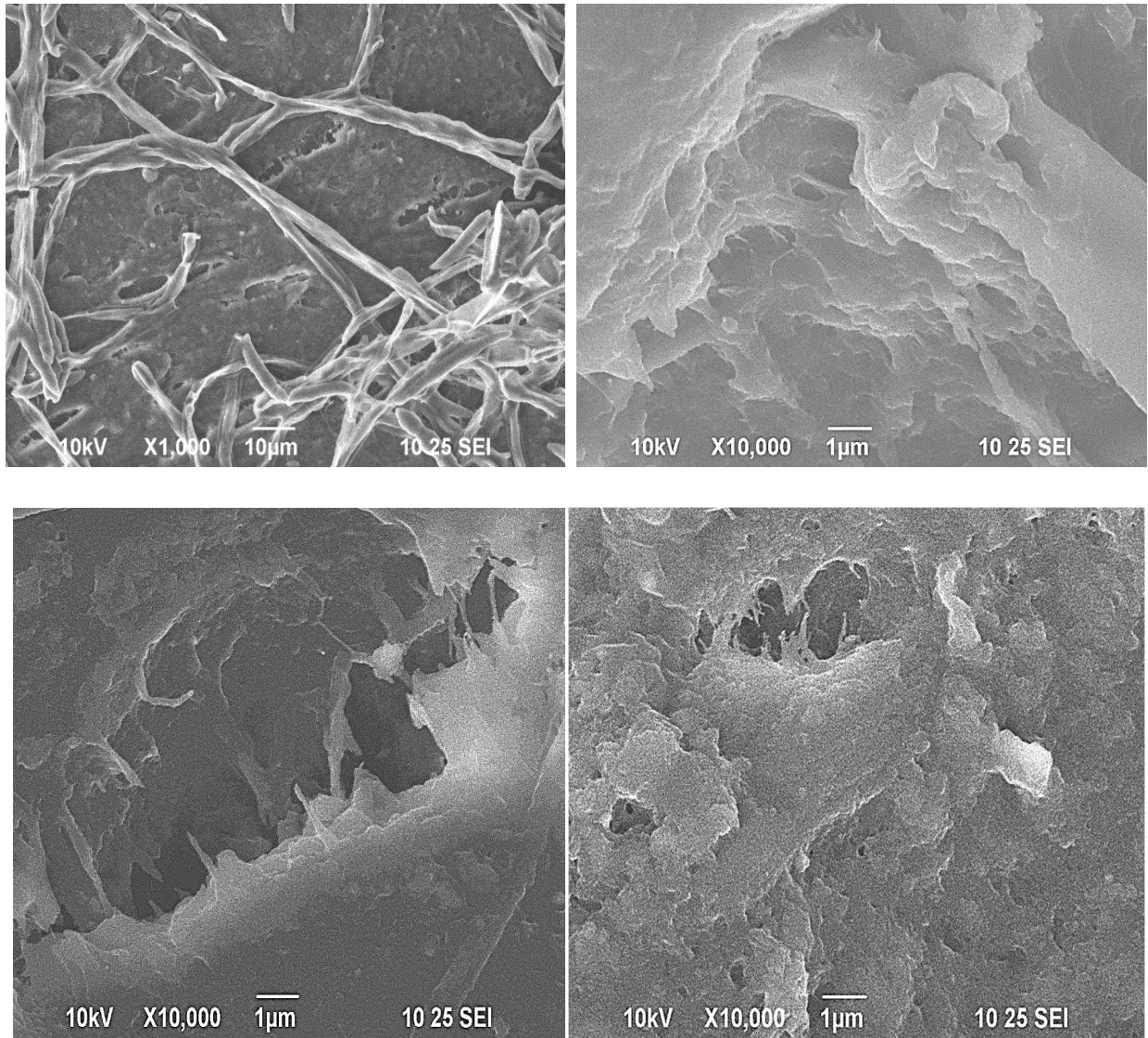
**Figure 7.15 Sample-3 (Culture + Nail Treated with Sample-3)**



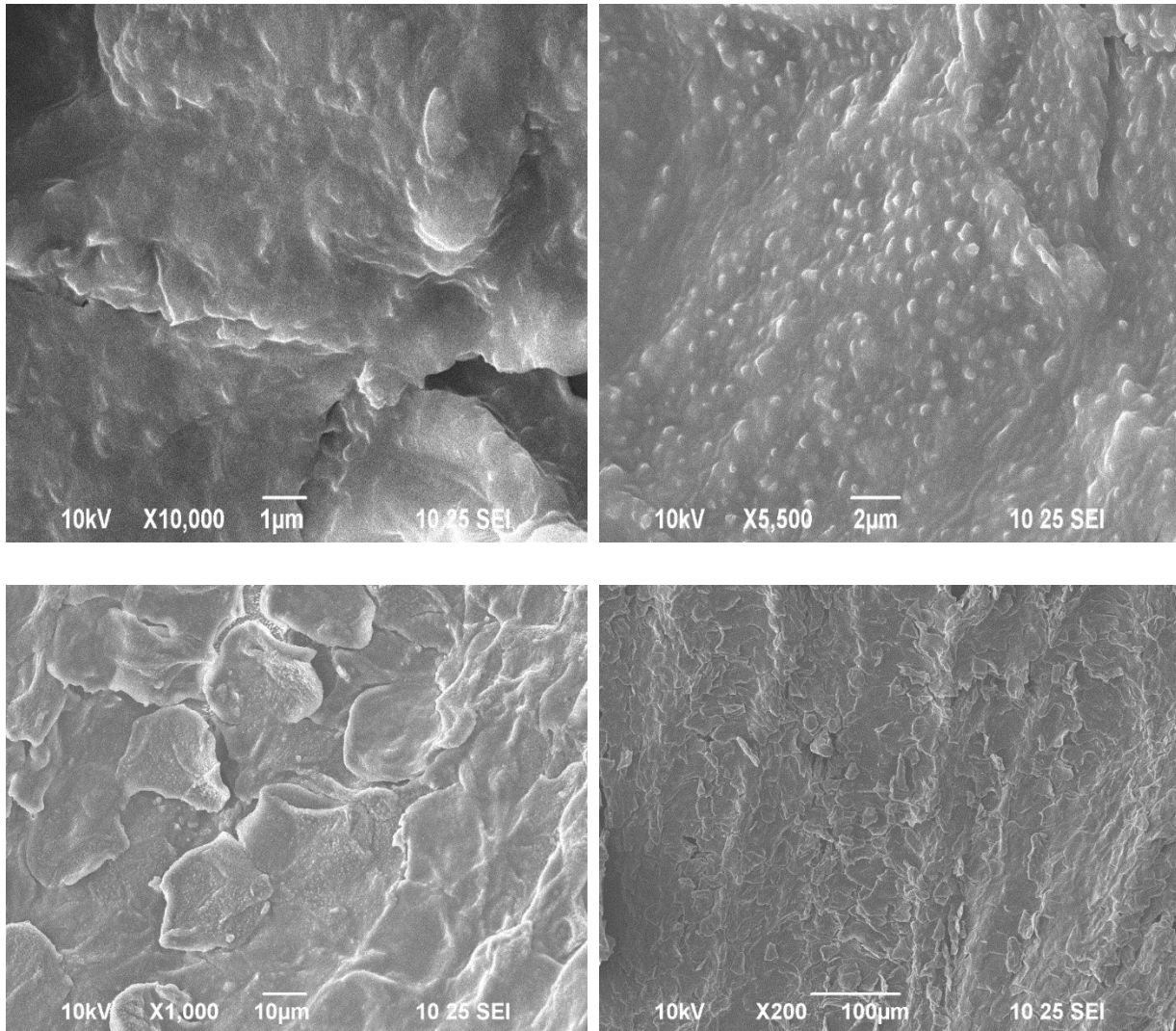
**Figure 7.16 Sample-4 (Culture + Nail Treated with Sample-4)**



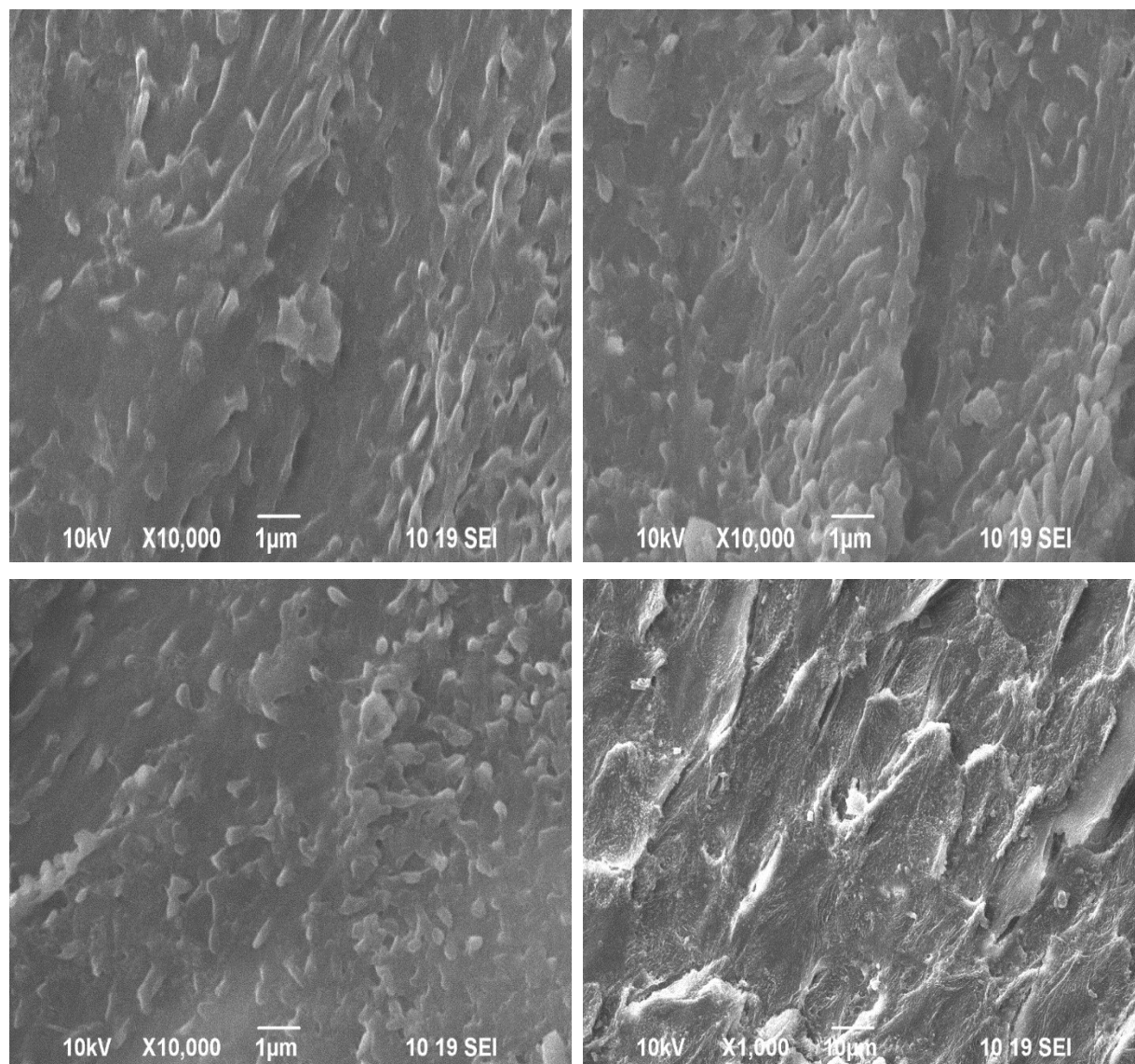
**Figure 7.17 Sample-5 (Culture + Nail Treated with Sample-5)**



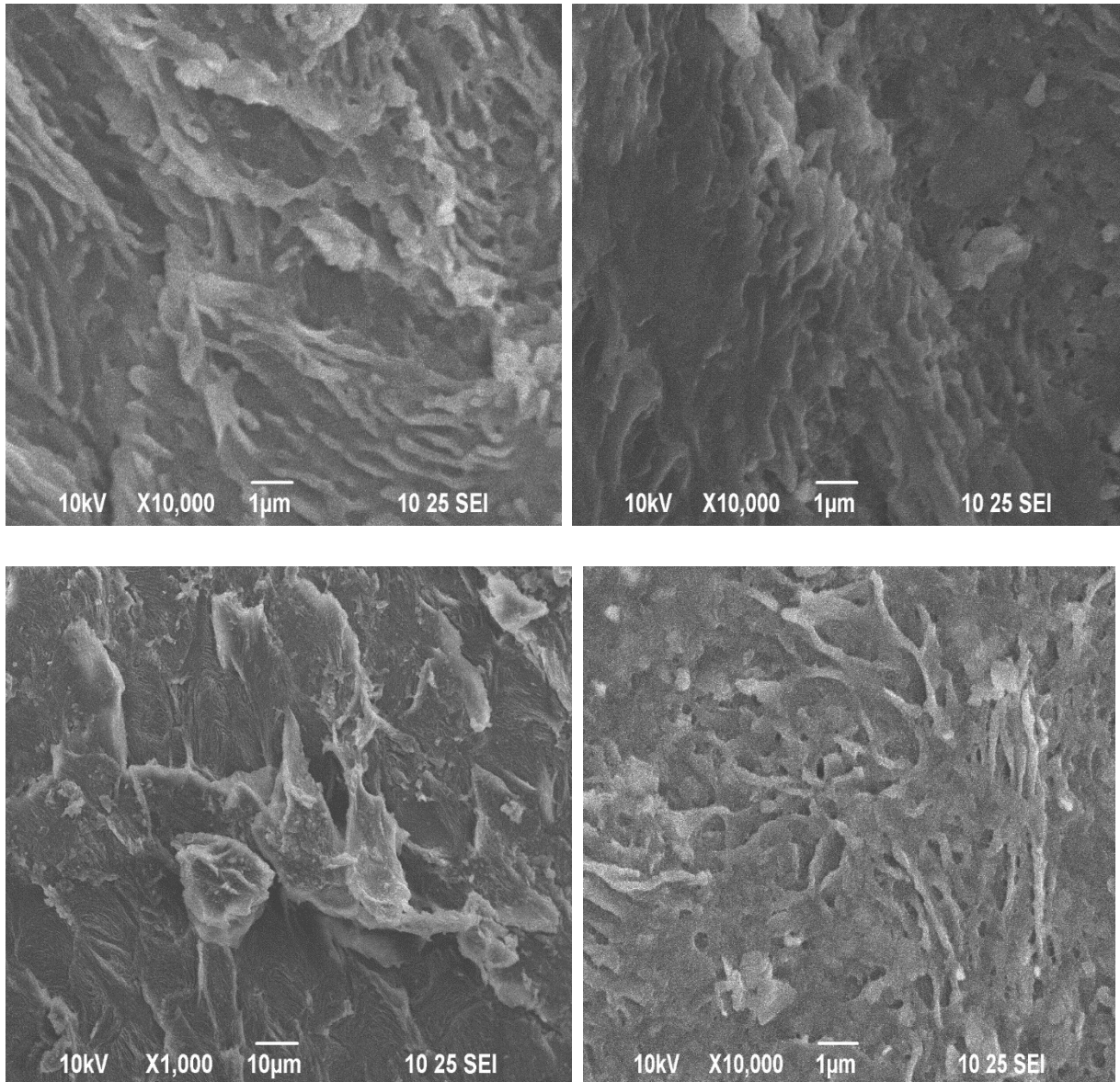
**Figure 7.18 Sample-6 (Culture + Nail Treated with Sample-6)**



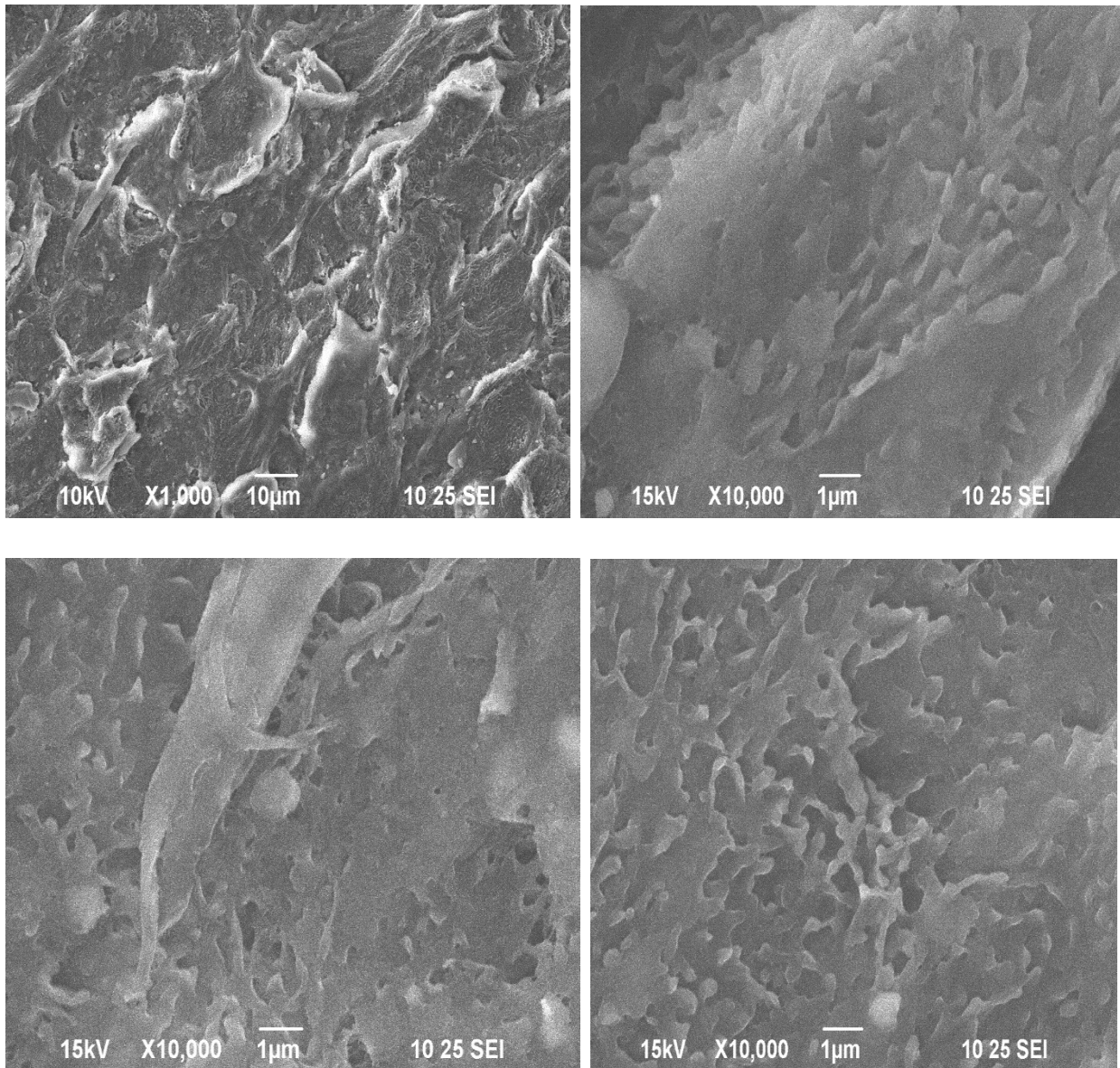
**Figure 7.19 Blank Nail Treated with Sample-1**



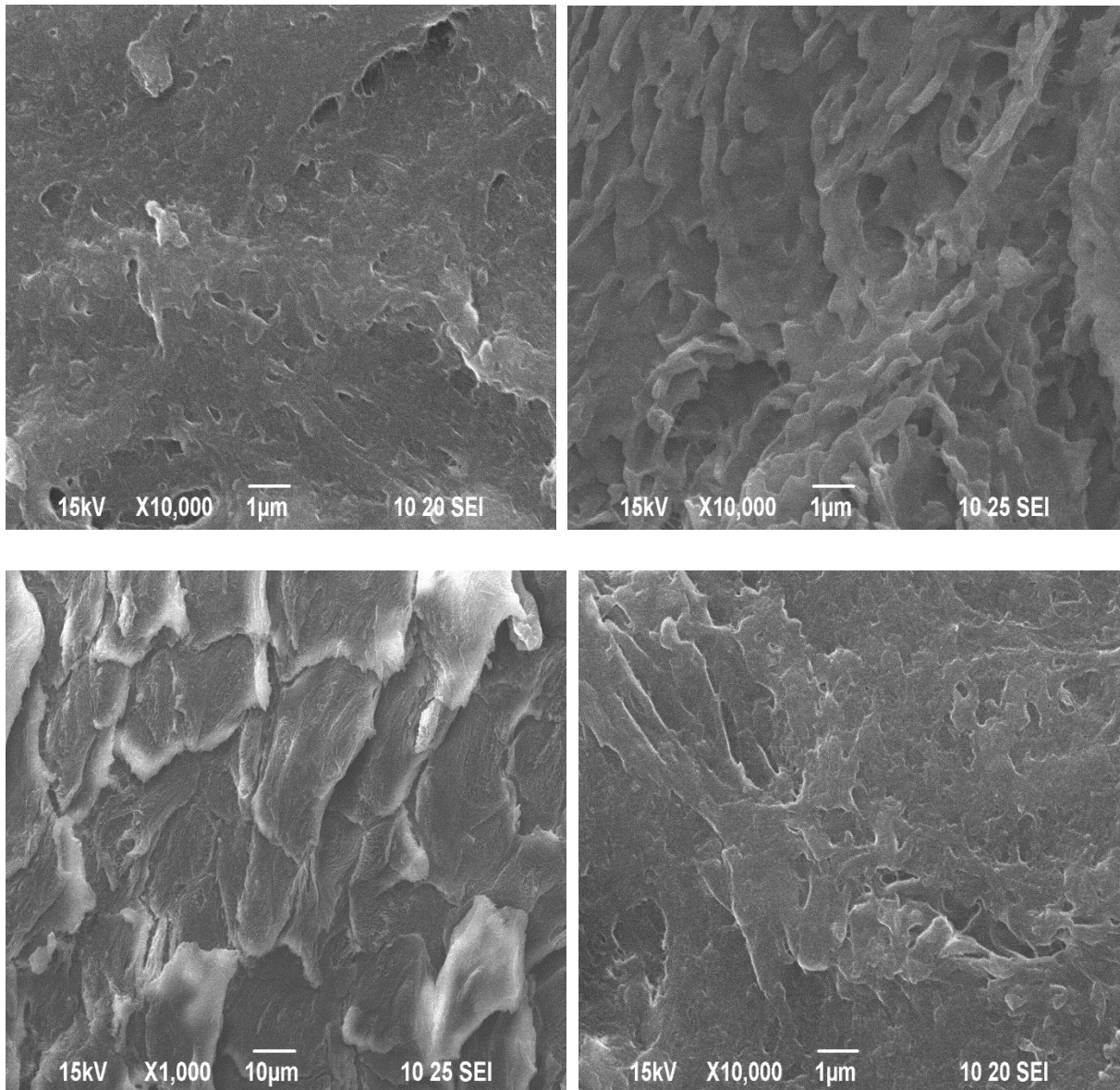
**Figure 7.20** Blank Nail Treated with Sample-2



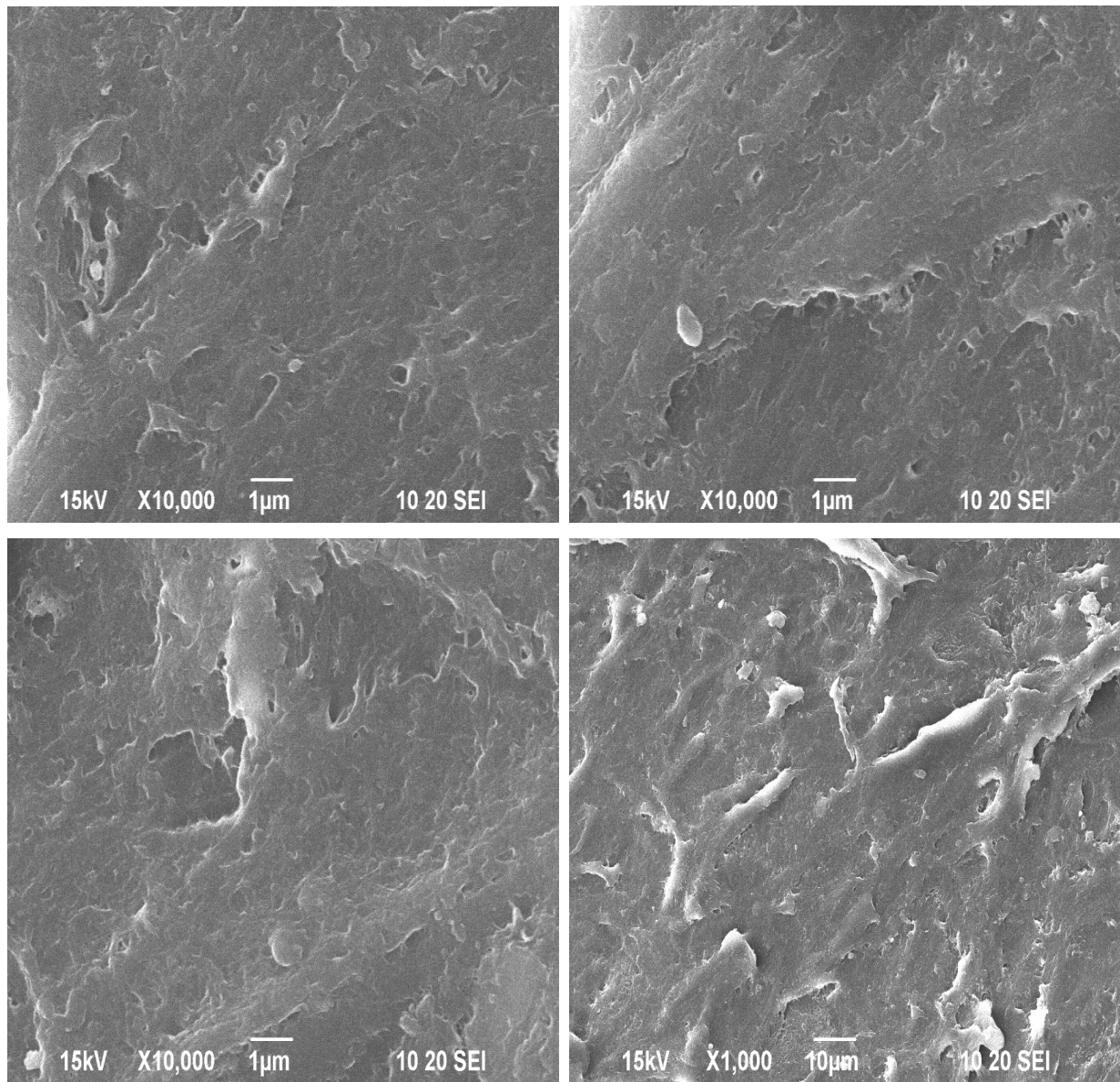
**Figure 7.21** Blank Nail Treated with Sample-3



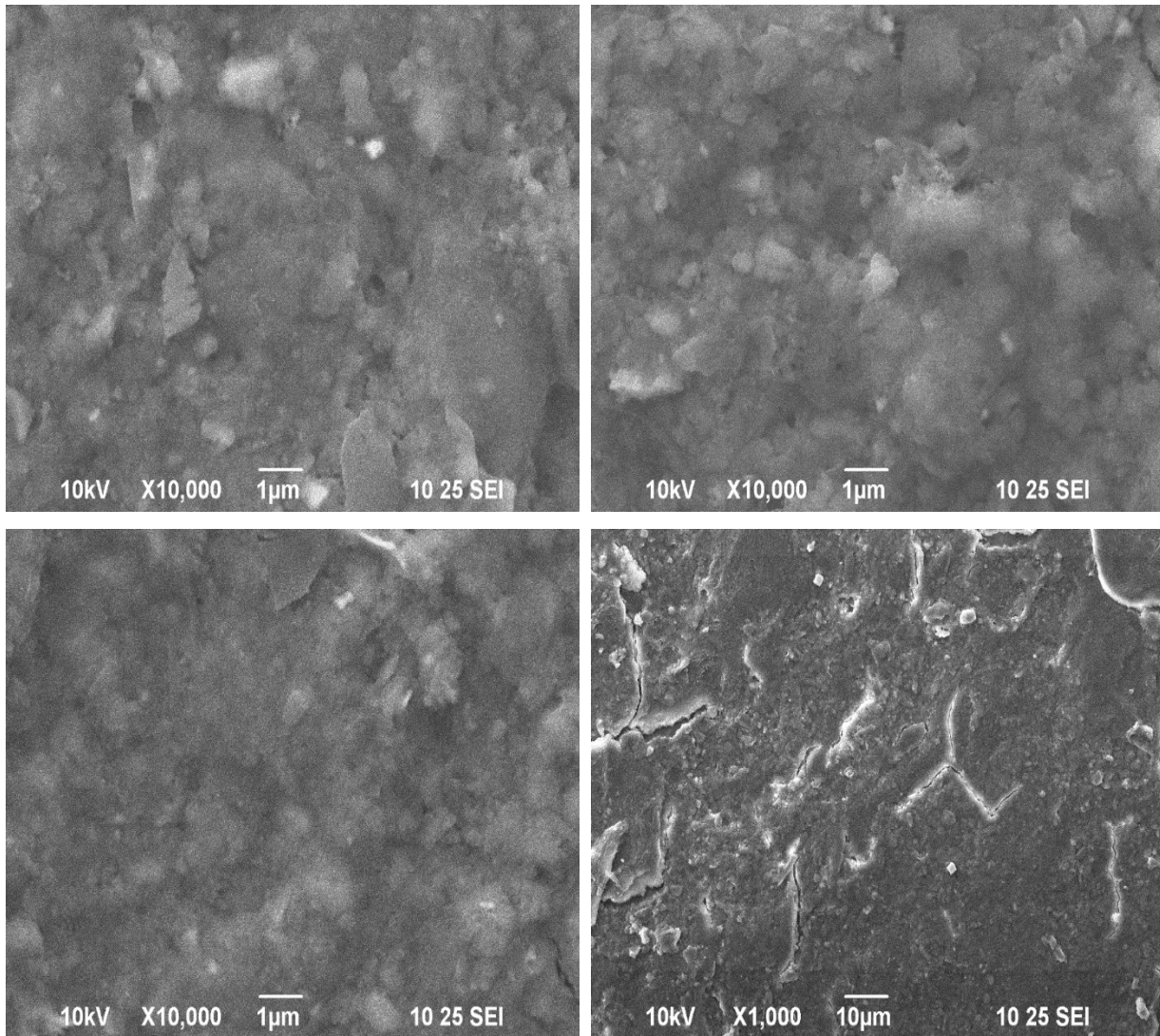
**Figure 7.22** Blank Nail Treated with Sample-4



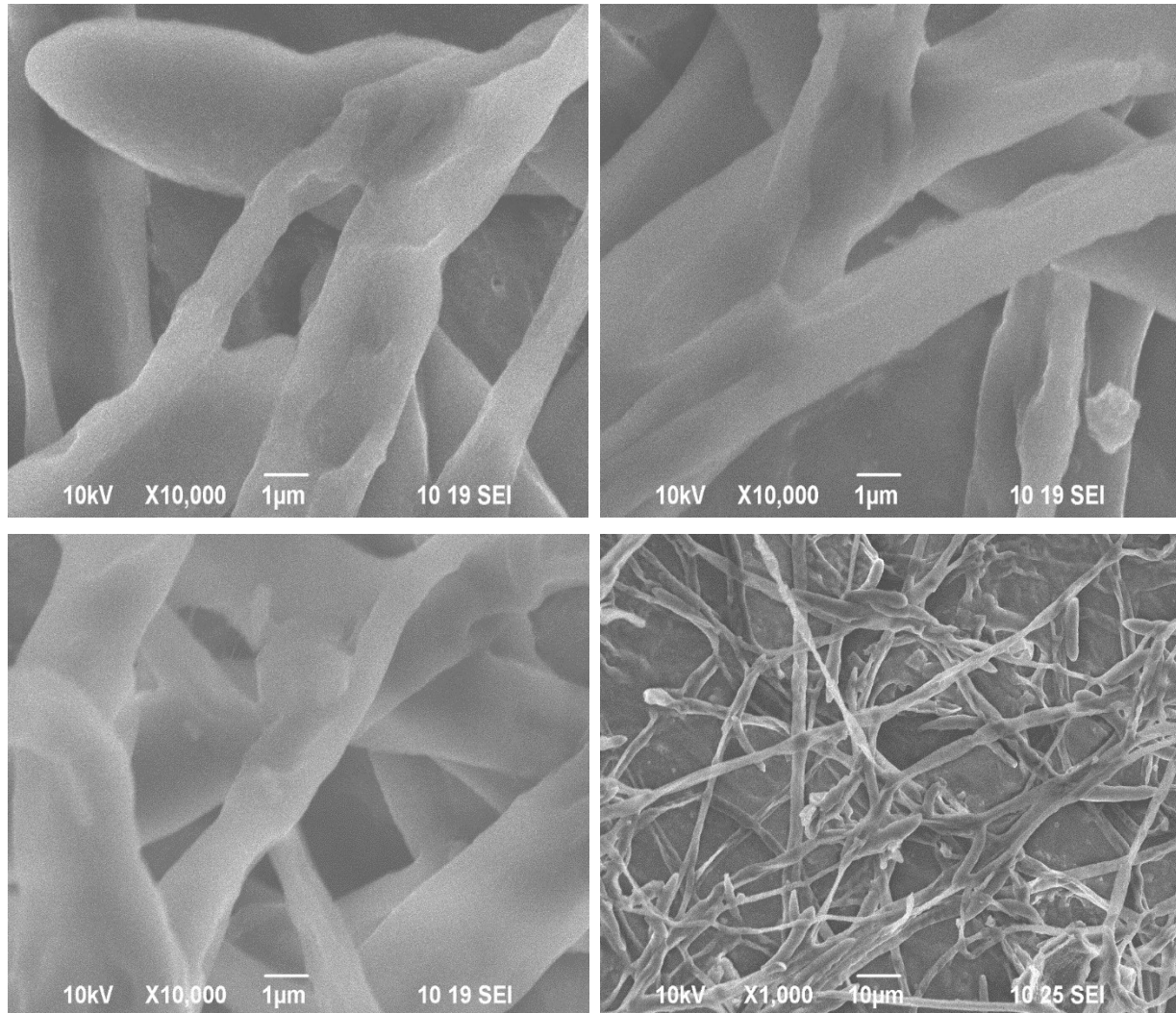
**Figure 7.23** Blank Nail Treated with Sample-5



**Figure 7.24** Blank Nail Treated with Sample-6



**Figure 7.25 Blank Nail**



**Figure 7.26 Nail culture control**

In this experiment, the ventral and dorsal sides of the nails and the surface of the agar plate were observed which assumed the nail bed of patients with onychomycosis. Figure 7.13 to figure 7.18 showed SEM images of culture with treated nails with different samples from S1 to S6. The initial change after treatment with the drug was the formation of fine wrinkles on the surface of the hyphae, eventually, the hyphae were flattened, and after that, no hyphal growth was observed. Figure 7.19 to figure 7.24 showed SEM images of blank nails treated with different samples from S1 to S6. In the control culture grown for 3 days without treatment, normal elongated and branched hyphae with smooth surfaces were observed on the nail. Figure 7.25 showed SEM image of blank nail as negative control and figure 7.26 showed SEM images of nail with culture without treatment as positive control. The initial change observed after

treatment with the formulations was the formation of wrinkles on the surface of hyphae, and eventually, the hyphae were completely crushed, subsequently, the altered hyphae lost their ability to grow. It can be concluded from this study that developed formulations have a strong antifungal activity by inhibiting the ability of fungi to grow and the drug has both excellent nail permeation and retention properties (26, 27).

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