8. In-vivo Study

8.1 Introduction

The effectiveness and efficacy of novel developed formulations are predicted using *in vivo* studies that are planned and carried out to determine the efficacy, permeability and other factors. Understanding the fate or behavior of the generated innovative formulation in *in vivo* conditions is made easier with the use of a suitable animal study. As stated in earlier chapters, new formulations of both medications were successfully developed and examined for characteristics best suited for topical distribution both *in vitro* and ex vivo. The focus of this chapter is on evaluating the pharmacokinetic (PK) and pharmacodynamic (PD) effects of the newly developed formulations of Luliconazole and Tavaborole. The *in vivo* tests were conducted to get a better understanding of how these newly created formulations could be able to address issues with topical distribution.

8.2. Materials and Methods

8.2.1. Materials

LUZU cream (1%w/w) was purchased from local pharmacy. EDTA disodium salt was purchased from Himedia private limited, India. Tavaborole topical 5% solution was provided as gift sample by Encube ethicals private limited, India. Various formulations used for study were developed as described in previous chapter 5 and 6.

8.2.2 Pharmacokinetic study

8.2.2.1. Animal study protocol approval

Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India guidelines were used for performing the in-vivo studies(1). Approval was taken from Institutional Animal Ethics Committee of Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara, India for the protocols (Protocol No.- MSU/IAEC/2021-22/2108) of pharmacokinetic study of developed formulations**.**

8.2.2.3 Method

Wistar albino rats were used for performing pharmacokinetic studies (2, 3). (200-270g) were housed in cages placed in an animal room with a constant temperature of 22±3 °C and a fixed 12- hour light-dark cycle. All animals were handled and housed according to the CPCSEA guidelines, Department of Animal Welfare, Government of India. The rats were given standard

chow diet and water ad libitum. 48 rats were allocated in 8 groups randomly. Group animals were fasted 12 hours before starting the experiment. 500 mg (equivalent to 5 mg Luliconazole and 25 mg Tavaborole) formulation was applied on the animals. Group 1 animals were treated with the marketed topical formulation of Luliconazole. Group 2 animals were treated with plain Luliconazole gel. Group 3 animals were treated with nanoemulgel of Luliconazole and group 4 was treated with NLC based gel of Luliconazole. Group 5 animals were treated with marketed topical formulation of Tavaborole and group 6 was treated with Plain Tavaborole Gel. Group 7 animals were treated with nanoemulgel of Tavaborole and group 8 was treated with NLC based gel of Tavaborole. Rats were anesthetized using isoflurane. Blood samples (0.5 ml) were collected from the retro orbital plexus in heparinized microcentrifuge tubes containing heparin at 1, 3, 5, 8, 24 hour from set-1 and 2, 4, 6, 12 hour from set-2 resulting 9 time points (1, 2, 3, 4, 5, 6, 8, 12, 24 hour). The rats were replenished with saline solution. Blood samples were centrifuged at 3500 rpm for 10 min at 4°C and harvested plasma was frozen at –20°C until analysis using HPLC.

Table 8.1: Animal grouping for pharmacokinetic study

Group	Treatment	No. of Animals	
		Set 1	Set 2
1	Marketed topical formulation of Luliconazole	3	3
2	Plain Luliconazole Gel	3	3
3	Nanoemulgel of Luliconazole	3	3
4	NLC containing gel of Luliconazole	3	3
5	Marketed topical formulation of Tavaborole	3	3
6	Plain Tavaborole Gel	3	3
7	Nanoemulgel of Tavaborole	3	3
8	NLC based gel of Tavaborole	3	3
	Total number of animals	48*	

*Not to be sacrificed, rehabilitation were done

8.3 Pharmacodynamic study

8.3.1 Animal study protocol approval

Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India guidelines were used for performing the in-vivo studies. Approval was taken from Institutional Animal Ethics Committee of Institute of Pharmacy, Nirma University, Ahmedabad, India for the protocols (Protocol No.- IP/ PCOL/FAC/34/2023/016) of pharmacodynamic study of developed formulations.

8.3.2 Method:

Sprague Dawley Rats (200-250 g) were housed in cages placed in an animal room with a constant temperature of 22±3°C and a fixed 12- hour light-dark cycle. All animals were handled and housed according to the CPCSEA guidelines, Department of Animal Welfare, Government of India. The rats had given standard chow diet and water ad libitum. The animals were divided into eight groups. Fungal infection was induced using *Candida albicans*. A working culture of *Candida albicans* was grown for 48 hrs at 30°C on sabouraud dextrose agar. The cells were collected, washed and suspended in sterile saline to a final concentration of 10^7 colony forming units (CFU)/ml. Each rat was prepared for induction of infection by shaving approximately 3 cm^2 of dorsal hair using shaving cream. The rats were subcutaneously administered 30 mg of Prednisolone per kg of body weight on the day before and on the day after the inoculation. In the middle of shaved area, 100µl of 10^7 CFU/mL *Candida albicans* suspensions was intradermally injected and injected area was gently rubbed with the help of a sterile swab until no more visible fluid was observed. Flaring of fungal skin infection was observed 72 hrs after induction. After 72 hrs, sufficient growth was observed and the 500 mg formulations were applied topically with the help of a flat brush once daily for six consecutive days after induction, starting from the day of post infection to the rats of respective groups, excluding the animals of model control group. The rats were continuously observed visually for the change in the texture of the skin of the infected area after initiation of the treatment and animals were sacrificed after 10 days from induction using high dose of thiopental sodium followed by clinical and histopathological examination. Clinical signs of fungal infection were monitored and photographs were captured. At the end of the study, skin samples were collected for histopathological analyses (4,5).

Table 8.2: Animal grouping for pharmacodynamic study

Group	Treatment	No. of Animals
1	Model Control animals (Distilled water)	6
2	Standard control Marketed topical Formulation of Luliconazole (1%w/w)	6
3	Test Control Nanoemulgel (Placebo)	6
4	Test control Nanoemulgel of Luliconazole (1%w//w)	6
5	Test control NLC based gel of Luliconazole (1% w/w)	6
6	Standard Control Marketed topical formulation of Tavaborole (5%w/w)	6
7	Test control Nanoemulgel of Tavaborole (5%w/w)	6
8	NLC based gel of Tavaborole (5%w/w)	6
	Total number of animals	48

8.3.3 Collection of Skin samples:

Affected skin area was surgically removed and preserved in 10% formalin. They were then subjected to paraffinization and block formation. 5µm thin sections were stained with H & E stain. Slides were mounted and observed with the help of Optical microscope (10X magnification).

8.4 Result and Discussion

8.4.1 Pharmacokinetic study

HPLC method described in section 3.3.2 & 3.4 of chapter 3 was used for the determination of concentration of Luliconazole and Tavaborole in blood plasma of rats respectively. The data are presented in table 8.3.

Table 8.3: Plasma drug concentration of Luliconazole after applying various formulation of Luliconazole

Time (hr)	Plasma drug concentration of Luliconazole(ng/ml)			
	Marketed topical formulation of Luliconazole	Plain Luliconazole Gel	Nanoemulgel of Luliconazole	NLC containing gel of Luliconazole
0	0	0	0	0
1	80.76±21.4	72.23±33.2	54.32±7.2	63.73±9.3
2	110.32±12.8	131.46±24.7	81.78±13.9	87.43±18.2
3	202.21±51.4	188.63±48.5	122.23±81.6	134.66±50.4
4	198.87±14.6	173.75±87.3	187.42±33.2	191.43±23.2
5	179.66±34.5	150.87±65.8	166.77±65.1	173.32±31.3
6	148.47±13.8	141.61±22.9	150.48±5.3	166.22±40.2
8	121.04±71.6	132.43±71.4	141.87±4.8	147.71±11.1
12	100.87±5.5	118.88±55.3	127.32±7.9	129.11±20.4
24	89.38±28.7	91.72±33.8	96.88±4.58	98.33±62.1

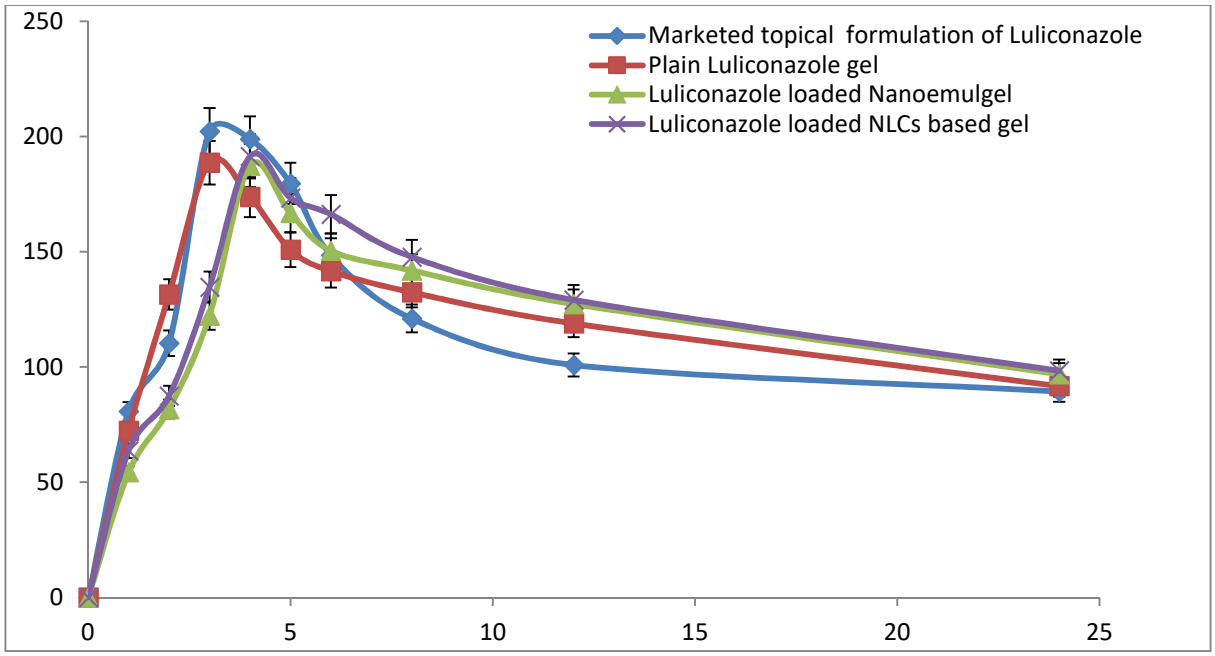


Figure 8.1: Plasma drug concentration of Luliconazole after applying various formulation of Luliconazole

Table 8.4: Plasma drug concentration of Tavaborole after applying various formulation of Tavaborole

Time (hr)	Plasma drug concentration of Tavaborole (ng/ml)			
	Marketed topical formulation of Tavaborole	Plain Tavaborole Gel	Nanoemulgel of Tavaborole	NLC containing gel of Tavaborole
0	0	0	0	0
1	499.21±51.8	431.52±13.6	192.73±14.2	184.07±21.2
2	620.08±11.8	588.29±38.2	289.27±31.5	261.73±35.2
3	751.22±13.5	689.10±19.3	308.45±42.1	294.01±54.5
4	781.87±71.2	652.55±28.7	461.04±11.7	422.87±71.2
5	661.52±31.5	608.93±13.0	524.72±9.95	506.52±31.5
6	592.66±7.6	511.78±22.8	407.83±24.6	432.66±7.6
8	427.59±17.2	418.06±55.1	392.04±22.7	387.59±17.2

12	381.22±23.2	311.25±42.6	328.01±18.4	311.22±23.2
24	250.06±15.8	231.86±21.5	293.21±0.81	297.08±0.11

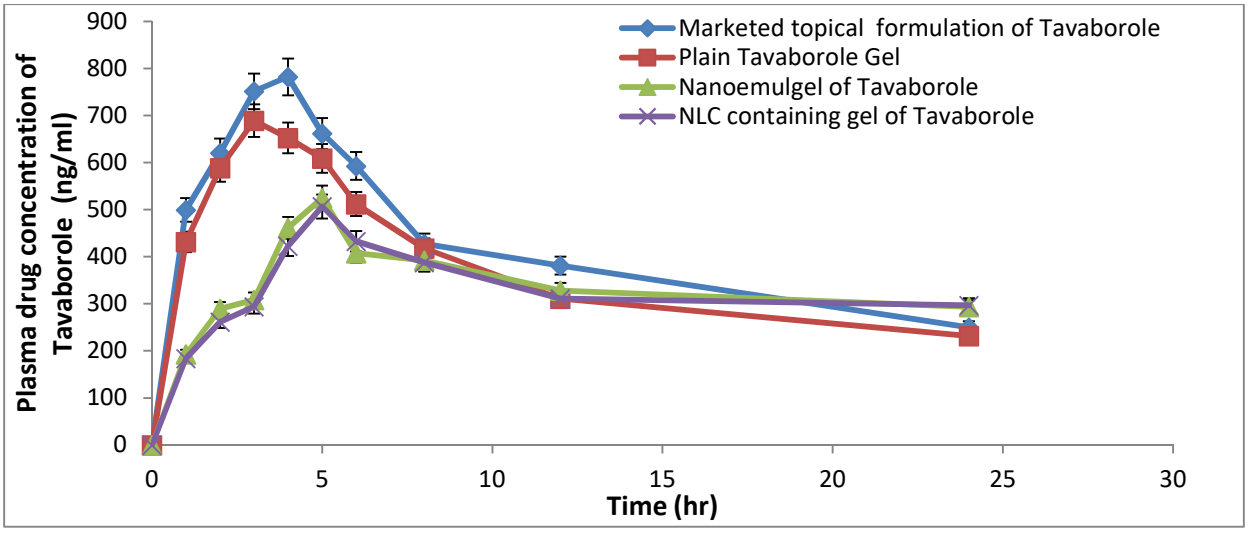
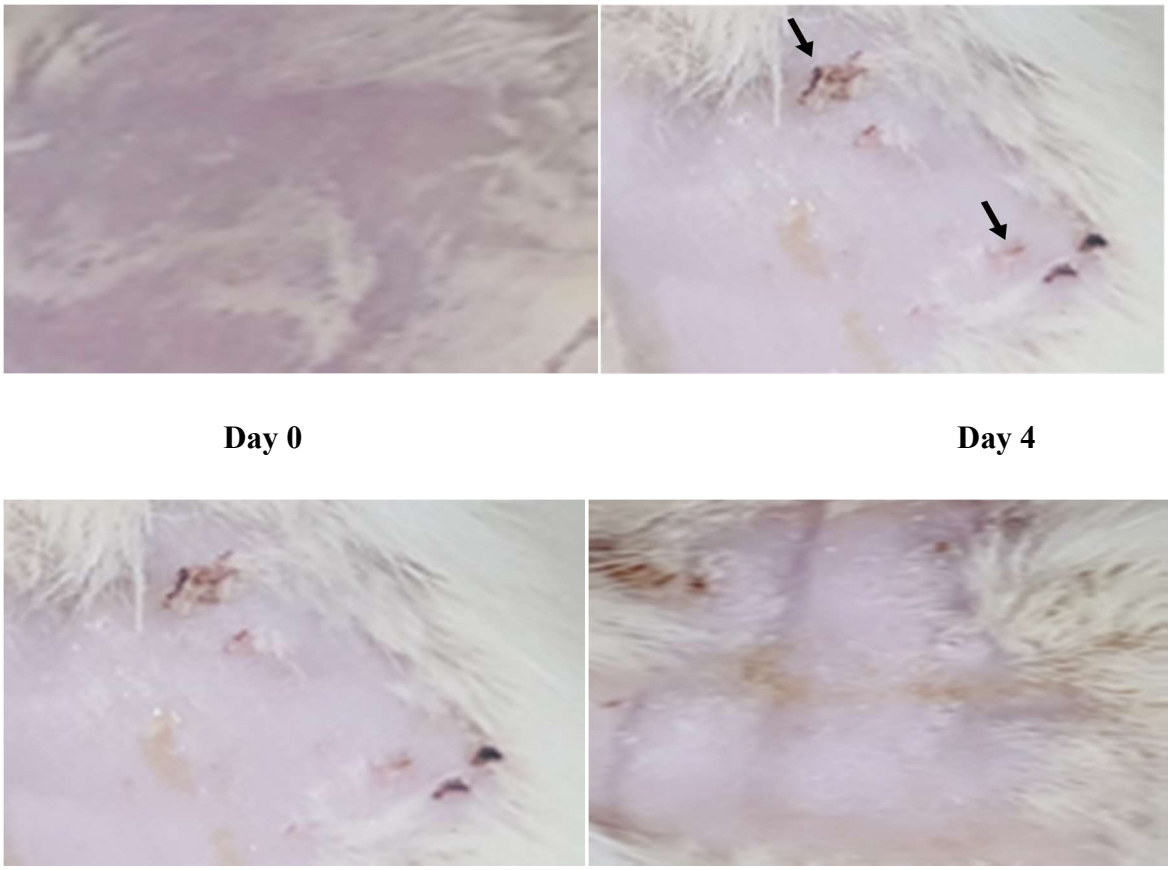


Figure 8.2: Plasma drug concentration of Tavaborole after applying various formulation of Tavaborole

The plasma drug concentrations at different time intervals after application of Luliconazole and Tavaborole formulations are shown in Tables 8.3, 8.4 and figures 8.1 and 8.2 respectively. During the study of 24 hrs maximum 202.21±51.4 ng/ml and 781.87±71.2 ng/ml Luliconazole and Tavaborole were found in the samples, respectively, which confirm that a major portion of the applied drugs is retained in the skin and negligible amounts reaches the systemic circulation. This indicates that the developed formulations are expected to decrease the systemic side effects. It may be due to the lipidic nature of formulations and drugs which enhances the drug deposition in the skin. Enhanced skin retention is expected to present the drug in higher concentrations at the site of infection, i.e skin or nail.

8.4.2 Pharmacodynamic study:

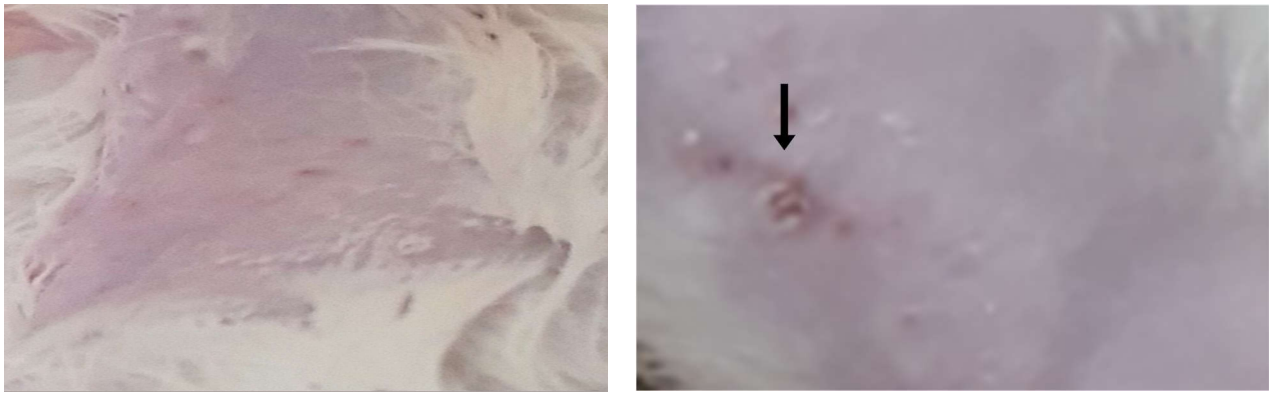
Effect of developed formulations on clinical signs is as following. Initially, before induction no signs of infection were observed. After 72 hr of induction, signs of fungal infection were observed. Eruption, red patches, white patches, small hair like growth of fungus and dark red spots were seen in all groups after induction. After this, treatment was initiated.



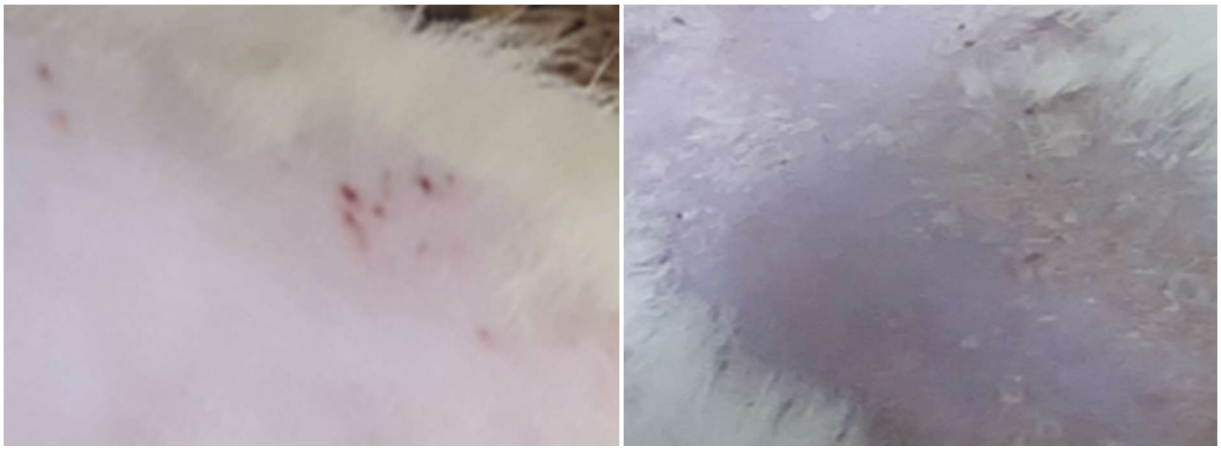
Day 0 **Day 4**

Day 7 **Day 11**

Figure 8.3: Effect of model control on clinical signs of fungal infection

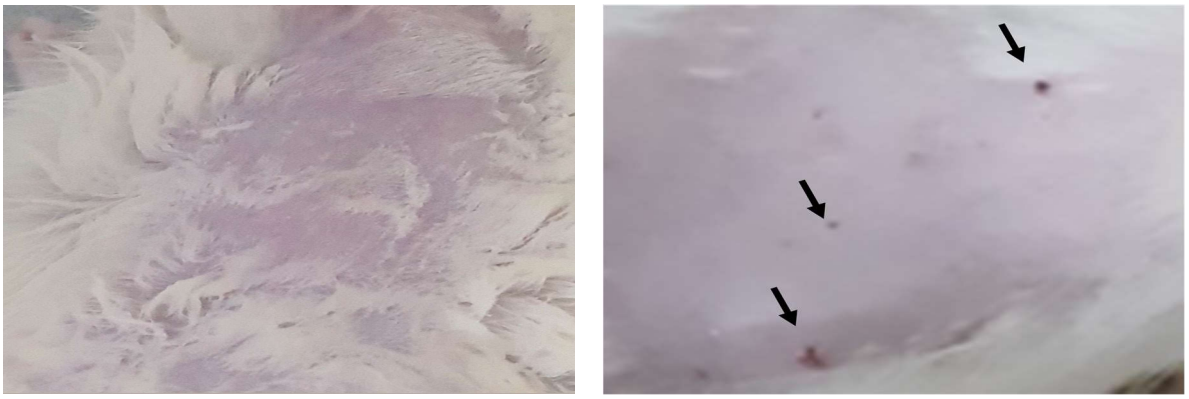


Day 0 **Day 4**

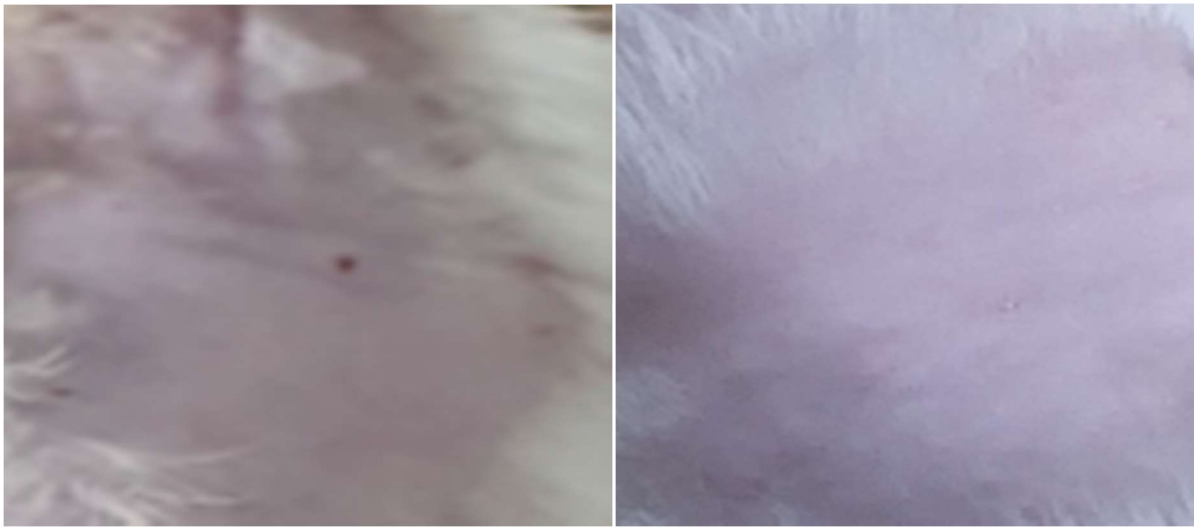


Day 7 **Day 11**

Figure 8.4: Effect of nanoemulgel placebo test control on clinical signs of fungal infection

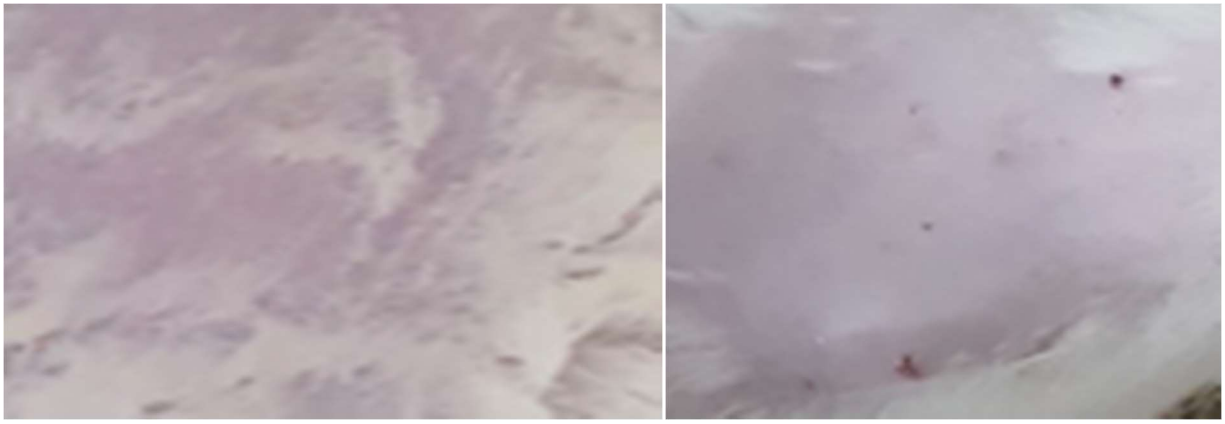


Day 0 **Day 4**



Day 7 **Day 11**

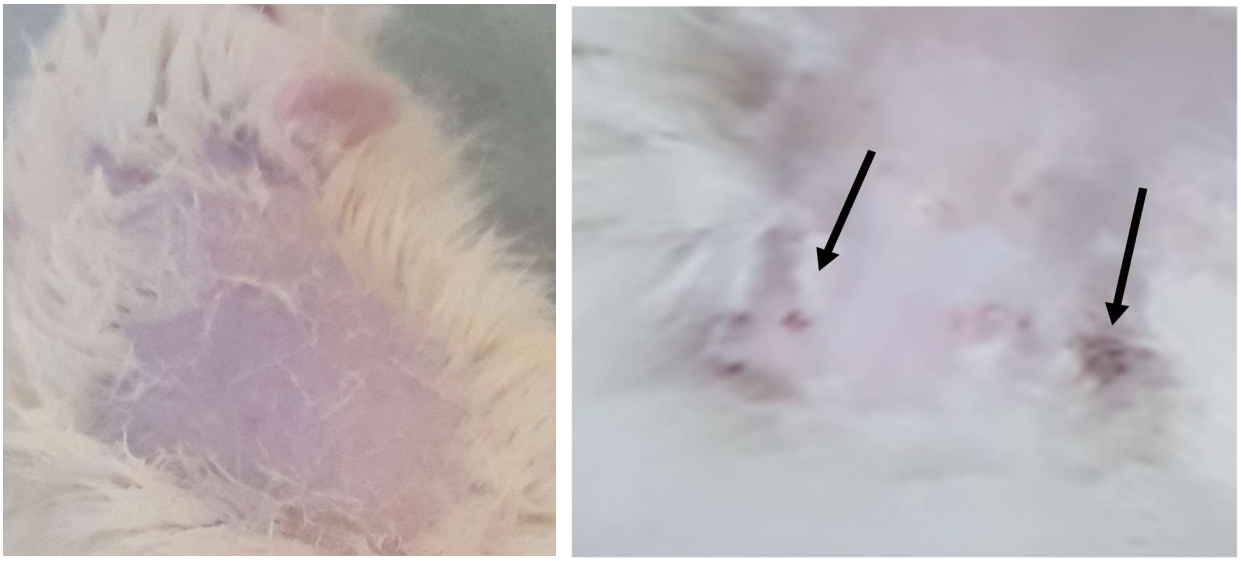
Figure 8.5: Effect of standard control (1% w/w luzu cream) on clinical signs of fungal infection



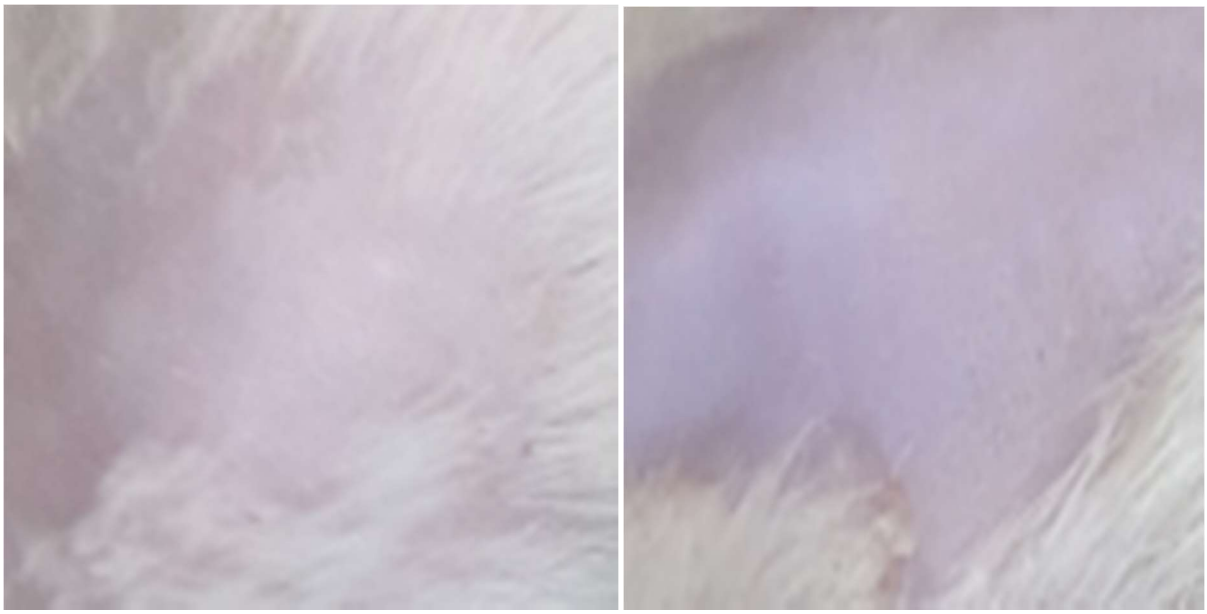
Day 0 **Day 4**

Day 7 **Day 11**

Figure 8.6: Effect of 1% w/w LZ nanoemulgel on clinical signs of fungal infection

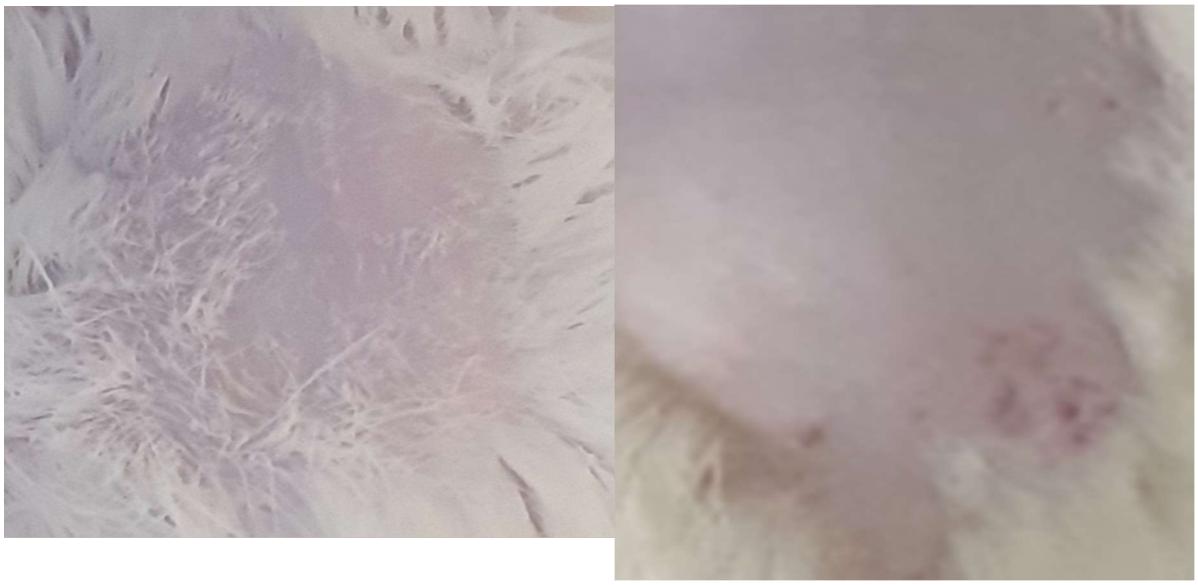


Day 0 **Day 4**

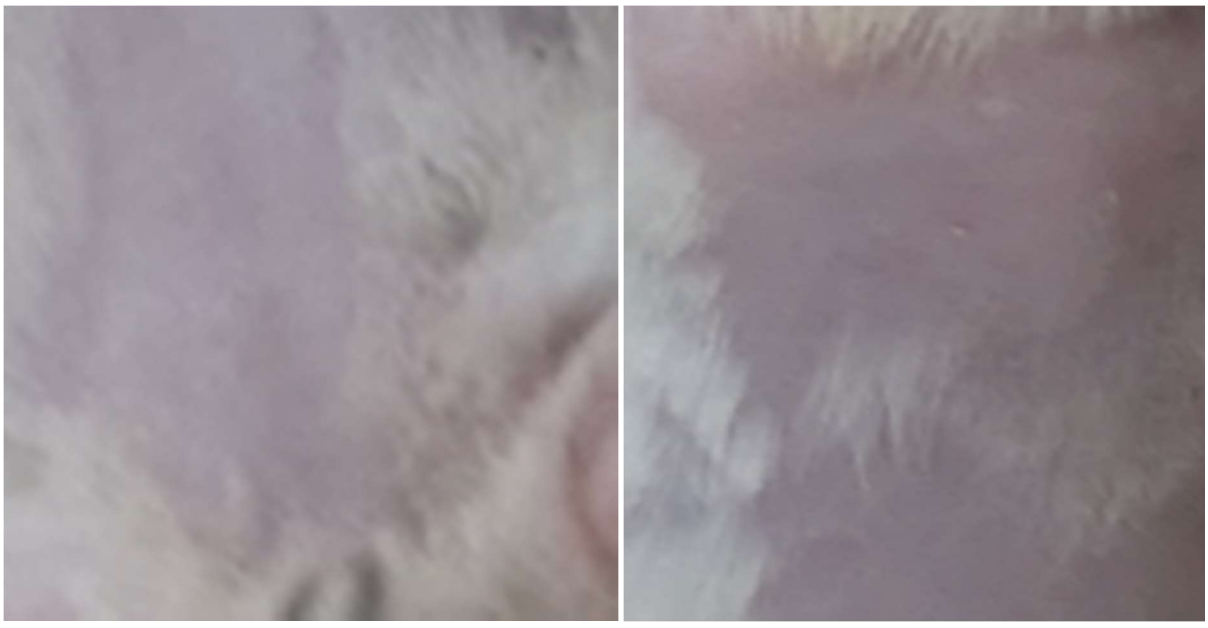


Day 7 **Day 11**

Figure 8.7: Effect of 1% w/w LZ NLCs based gel on clinical signs of fungal infection

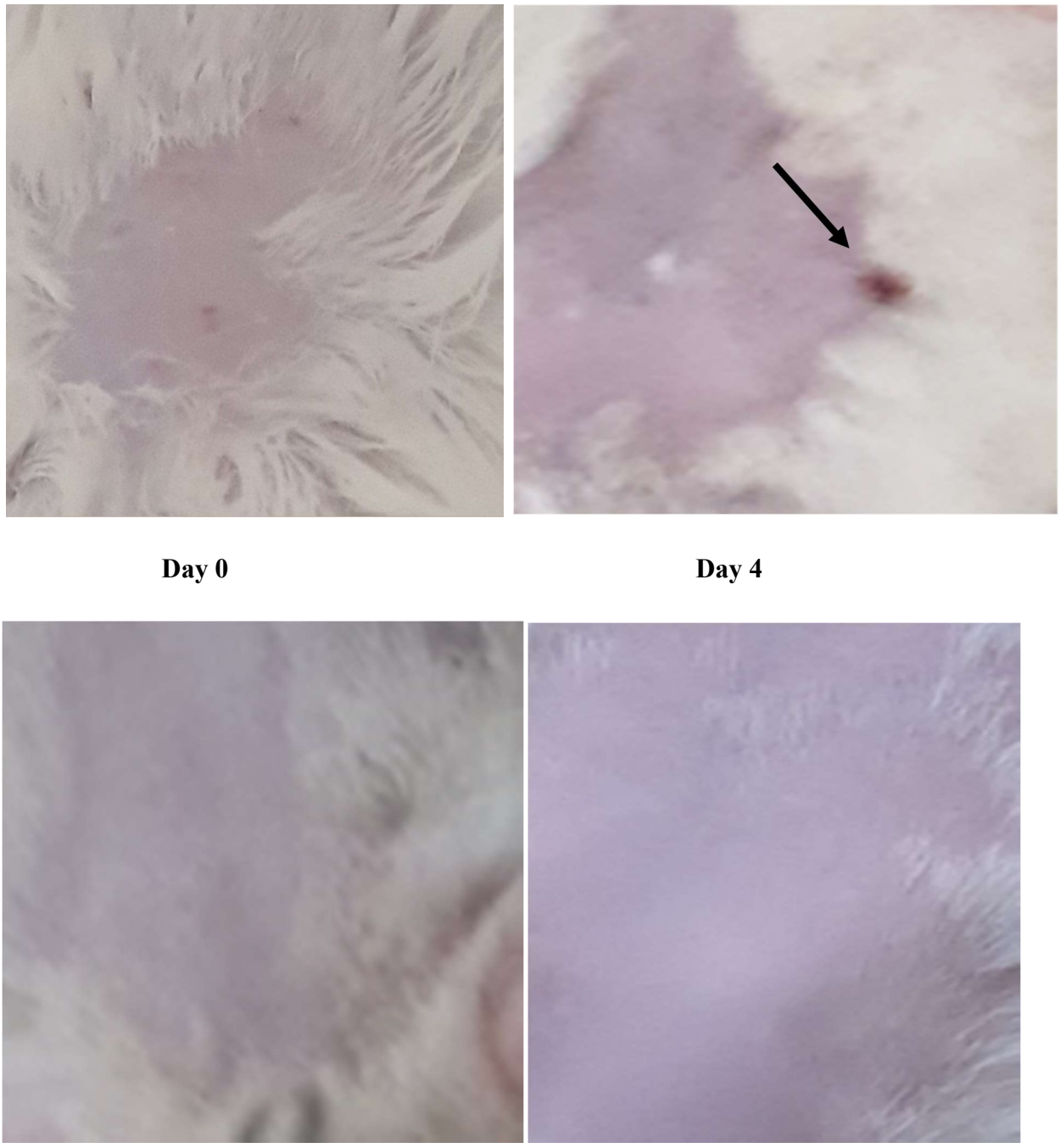


Day 0 **Day 4**



Day 7 **Day 11**

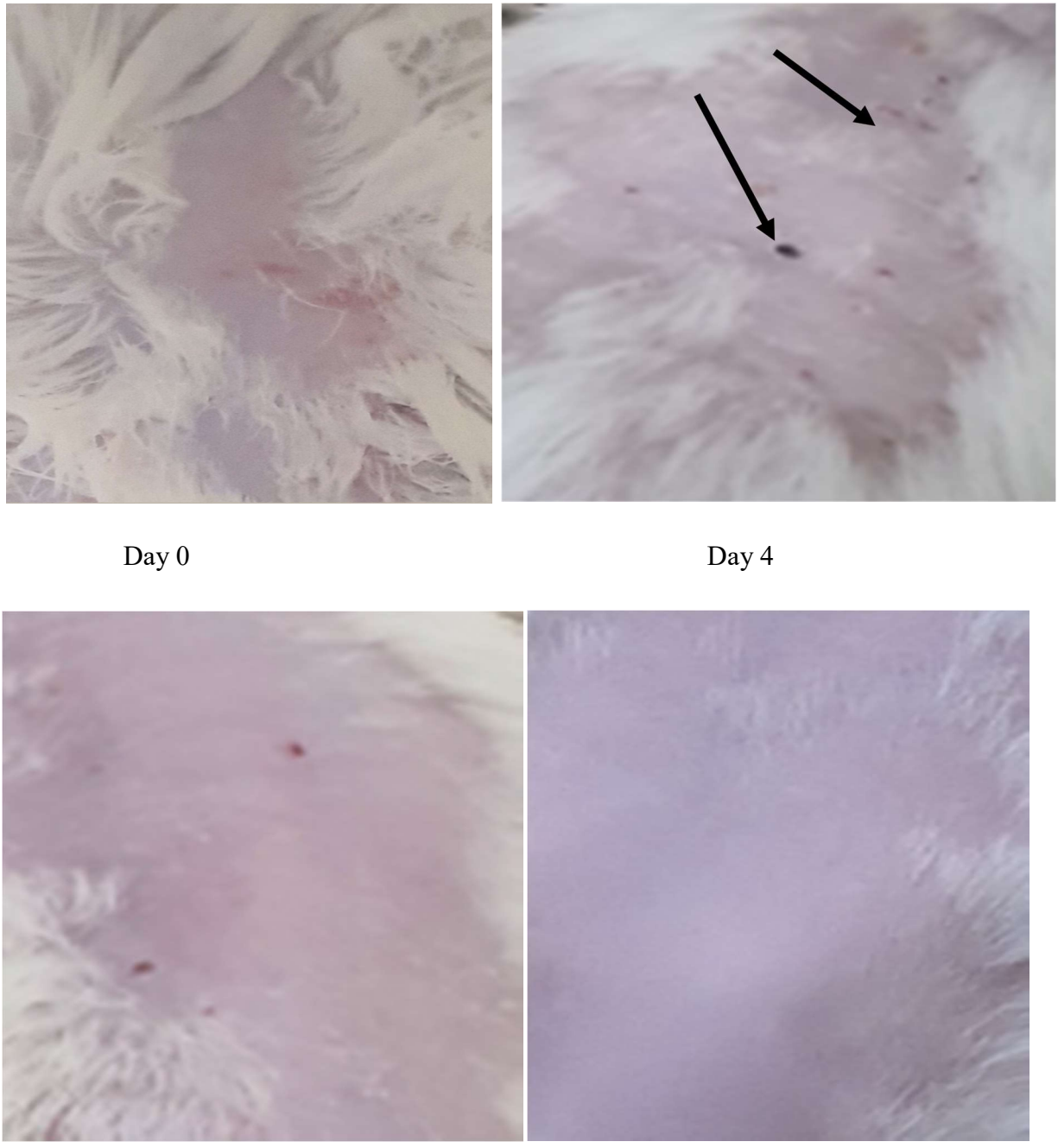
Figure 8.8: Effect of marketed formulation of TB on clinical signs of fungal infection



Day 0 **Day 4**

Day 7 **Day 11**

Figure 8.9: Effect of nanoemulgel of TB on clinical signs of fungal infection



Day 0 Day 4

Day 7 Day 11

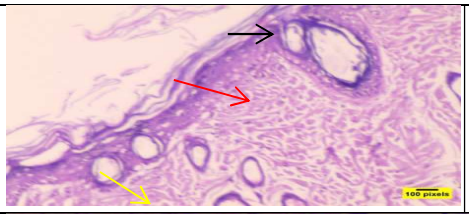
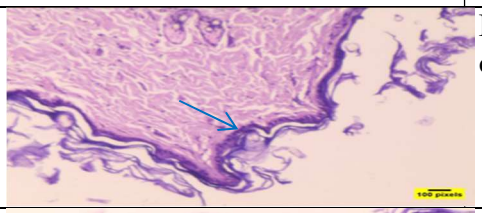
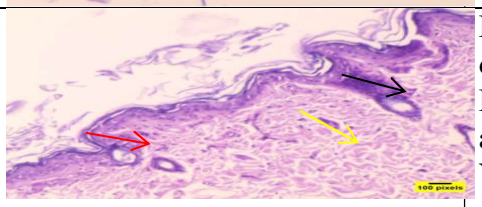
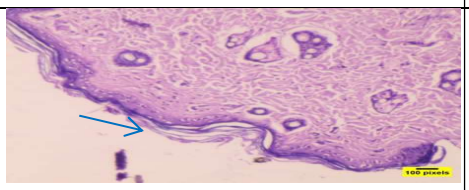
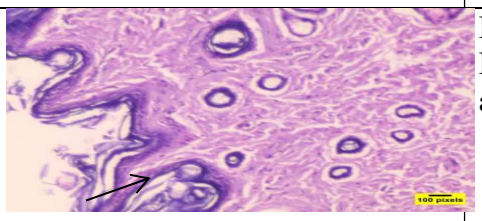
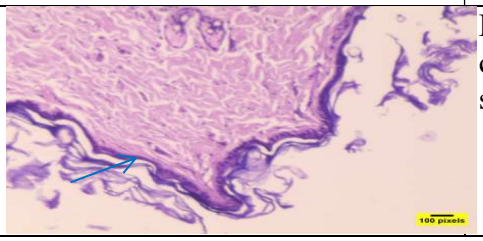
Figure 8.10: Effect of NLCs based gel of TB on clinical signs of fungal infection

Figures 8.3 to 8.10 show the effect of different formulations on clinical signs of fungal infection at different interval like day 0, day 4, day 7 and day 11. In model control group signs of fungal infection were visible till the end of study (figure 8.3). In test control (placebo) group signs of fungal infection were negligible till the end of study (figure 8.4). The effect of standard control marketed formulation of Luliconazole on fungal infection was visible after 7 days of treatment (figure 8.5). The effect of developed nanoemulgel and NLCs based gel of Luliconazole showed 5 days of treatment, and till 7^{th} day all signs of fungal infection were not visible indicating efficient treatment (figure 8.6 & 8.7). For standard control: marketed formulation of Tavaborole, the effect of treatment was visible after 6 days of treatment. After 7th day no clinical signs were visible (figure 8.8). In test control, nanoemulgel of Tavaborole, the effect of treatment was visible after 3 days of treatment and at the end of treatment clinical signs of infection were not visible. (figure 8.9) In test control, NLC based gel of Tavaborole group, the effect of treatment was visible after 2 days of treatment and at the end of treatment clinical signs of infection were not visible (figure 8.10). From the results it can be concluded that developed nanoemulgel of LZ and TB showed faster recovery than developed NLC based gel of LZ and TB. The reason for this outcome is nanoemulgel contains coconut oil which produces synergistic effect (6). To conclude, it can be said that developed formulations are efficient treatment options for the treatment of skin fungal infection.

Histopathological Analysis

From the histopathological analysis, the effect on dermal layer can be observed. In model control and test control placebo groups, black arrow shows signs of inflammation, chronic inflammation in dermal layer, red arrow shows thickening of dermis and focal acanthosis and the yellow arrow shows fungal hyphae in the superficial dermal layer. In standard control, marketed formulation of Luliconazole blue arrow shows thin dermal layer, no signs of chronic inflammation were seen, however acanthosis was seen. In test control, nanoemulgel of Luliconazole, blue arrow depicts that there were no signs of chronic inflammation or acanthosis. In test control, NLC based gel of Luliconazole the black arrow shows that there was slight thickening of dermal layer, mild signs of inflammation and focal acanthosis were seen, but they were less as compared to standard control. In standard control, marketed formulation of tavaborole, the blue arrow shows that dermal layer was thin and there were no signs of inflammation. However, mild focal acanthosis

was seen. In test control nanoemulgel of Tavaborole, the blue arrow shows that dermal layer was thin and there were no signs of inflammation. However, mild focal acanthosis was seen. In test control, NLC based gel of Tavaborole; the blue arrow shows thin dermal layer and no signs of inflammation in superficial dermal layer. To conclude, the fungal infection was visible in model control and placebo. Mild signs of infection were seen in test control NLC based gel of Tavaborole. Nevertheless, all developed formulations demonstrated significant antifungal efficacy indicating efficient therapy for fungal infection (Figure 8.11).

Model Control		Black arrow: Sign of inflammation, chronic inflammation in dermal layer. Red arrow: Thick dermis and focal acanthosis. Yellow arrow: Fungal hyphae in the superficial dermal layer
Marketed formulation of Luliconazole(1%w/w)		Blue arrow: Thin dermal layer, no signs of chronic inflammation, acanthosis is seen.
Nanoemulgel (Placebo, test control)		Black arrow: Sign of lesser inflammation in dermal layer Red arrow: Thick dermis and focal acanthosis Yellow arrow: Fungal hyphae in the superficial dermal layer
Nanoemulgel of Luliconazole 1%w/w (Test control)		Blue arrow: Thin dermal layer, no. signs of chronic inflammation or acanthosis.
NLC based gel of Luliconazole 1%w/w (Test control)		Black arrow: Slightly thick dermal layer. Mild signs of inflammation and focal acanthosis as compared to model control.
Marketed formulation of Tavaborole 5%w/w (Test		Blue arrow: Thin dermal layer, no. signs of chronic inflammation. Focal acanthosis is seen.

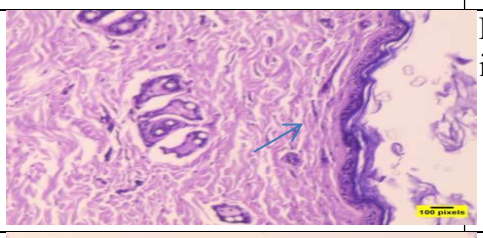
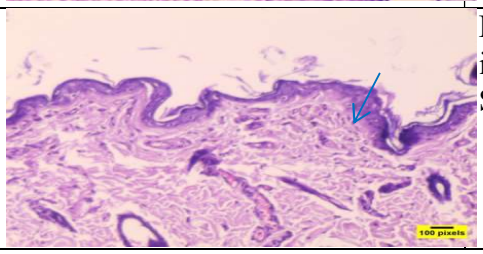
Control)		
Nanoemulgel of Tavaborole 5%w/w (Test control)		Blue arrow: Thin dermal layer, No signs of inflammation. Focal acanthosis is seen.
NLC based gel of Tavaborole 5%w/w (Test control)		Blue arrow: Thin dermal layer, No signs of inflammation. Focal acanthosis is seen. Signs of inflammation are seen.

Figure-8.11: Effect of nano formulation on skin structural alterations induced by fungal infection (H& E stain, all images were captured at 10X magnification and scale bar was 100 pixels.

8.5 References:

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