Chapter 8
In-vivo Studies

Dermal Delivery of Protein/Peptide Based Antimicrobial to Treat Secondary Infection in Psoriasis and Eczema

8.1 Animals

BALB/c mice (either sex) weighing 20-25 g were used for the *in-vivo* pharmacodynamic studies. The experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara, with protocol no. MSU/IAEC/2018-19/1832. The animals were housed in a constant temperature (22±2 °C) and a 12-hour fixed light-dark cycle. Animals were fed a standard diet and had free access to water.

8.2 Pharmacodynamic Study

8.2.1 Imiquimod induced psoriatic animal model

Imiquimod-based psoriasis animal model (n=5) was induced on BALB/c mice to evaluate the anti-psoriatic efficacy of the developed Omiganan formulations [1]. Briefly, mice hairs were removed from the dorsal side using Veet® (Reckitt Benckiser Pvt. Ltd., India) before starting the experimental model. Subsequently, the animals were weighed and divided into 7 groups (n=5) (Table 8.1). Psoriatic lesions were developed by applying Imiquad® (62.5 mg; 5% w/w Imiquimod, Glenmark Pharmaceuticals Ltd., India) on each 2 cm^2 dorsal region for six days consecutively except the normal control group. The epidermal thickness was measured at the dorsal site using a vernier caliper. Subsequently, the treatment was started with the formulations from the 3rd day (after 6 h application of Imiquad®) while keeping aside normal and model control groups (received only Imiquad®). In this study, the marketed formulation of Betamethasone Dipropionate gel (Betagel) was used as the standard control [2, 3]. On the 7th day, mice were sacrificed humanely, and blood, skin, and spleen were collected from each mouse. Blood samples were allowed to clot and centrifuged to separate the serum and were stored at – 40 °C till further analysis. Skin and spleen samples were also kept at – 40 °C till further analysis.

Table 8.1 Treatment groups for anti-psoriatic efficacy in Imiquimod induced psoriatic animal model in BALB/C mice

Groups		Administered samples	No. of animals
1.	Normal control	Distilled water	5
2.	Model control	Imiquimod (62.5 mg/day, Topically)	5
3.	Standard control	Betamethasone Dipropionate gel (Betagel) (100 mg/day) + Imiquimod (62.5 mg/day, Topically)	5
4.	Test control -1	Free Omiganan gel (1 mg in 100 mg/day) + Imiquimod (62.5 mg/day, Topically)	5
5.	Test control- 2	Omiganan lotion (1 mg in 100 mg/day) + Imiquimod (62.5 mg/day, Topically)	5
6.	Test control- 3	Omiganan NLC gel (1 mg in 100 mg/day) + Imiquimod (62.5 mg/day, Topically)	5
7.	Test control- 4	Omiganan liposomal gel (1 mg in 100 mg/day) + Imiquimod (62.5 mg/day, Topically)	5

8.2.1.1 Psoriatic scoring

Psoriatic-like skin lesions were evaluated by assigning the PASI (Psoriasis Area Severity Index) score to each animals; a score of 0–4 (0, none; 1, mild; 2, moderate; 3, marked; 4, severe) for erythema, scaling and skin thickening [1].

8.2.1.2 Spleen and body weight

The spleen and body weight were recorded for comparison with the model control group. Notably, the increase in the spleen and normal body weight were assumed as the measure of the release of inflammatory cytokines in psoriatic skin lesions [1].

8.2.1.3 Histopathology

For histopathology investigation, the skin section with appropriate thickness was prepared and stained with hematoxylin and eosin (H & E). Subsequently, the samples

were fixed on the glass slide and observed under the light microscope (Nikon, India), and were compared for the ceding of lesions.

8.2.1.4 Cytokines levels in serum

The serum collected from each animal was further subjected to ELISA for measurement of TNF-α and IL-6 levels [1]. According to the protocol provided by manufacturer, the measurement was performed (Krishgen Biosystems, India). The concentration of TNF-α and IL-6 in the serum was calculated and compared with different groups of animals.

8.2.2 Ovalbumin induced animal model for eczema/atopic dermatitis (AD)

Ovalbumin (OVA) induced animal model (n=5) for eczema/atopic dermatitis was developed on BALB/c mice to examine the anti-eczematic activity of the optimized formulations of Omiganan and DPK 060 [4-6]. Epicutaneous sensitization with Ovalbumin in mice was carried out as per previously reported protocol [4-6]. Briefly, mice hairs were removed from the dorsal side using Veet® (Reckitt Benckiser Pvt. Ltd., India) before starting the experimental model. The animals were weighed and divided into 10 groups (Table 8.2). Subsequently, all animals except the normal control (group 1) were sensitized with Ovalbumin (100µg in 100µL) placed on a 1 x 1 cm^2 patch of sterile gauze, which was secured with a transparent bio-occlusive dressing. Each mouse has a total of three 1-week exposures to the patch. The treatment was initiated with the formulations after the 3^{rd} week onward (for 7 consecutive days) while keeping aside normal control and model control groups (received only Ovalbumin). In this study, the marketed formulation of Betamethasone Dipropionate gel (Betagel) was used as the standard control. On the completion of the study, mice were sacrificed humanely, and blood and skin samples were collected from each mouse. Blood samples were allowed to clot and centrifuged to separate the serum and were stored at – 40 °C till further analysis. Skin samples (1 x 1 cm^2 area) were also kept at – 40 °C till further analysis.

Table 8.2 Different treatment groups for anti-eczematic efficacy in Ovalbumin induced animal model in BALB/C mice

Groups		Administered samples	No. of animals
1.	Normal control	Distilled water	5
2.	Model control	Ovalbumin (100µg in 100µL, Topically)	5
3.	Standard control	Betamethasone Dipropionate gel (Betagel) (100 mg/day) + Ovalbumin (100µg in 100µL, Topically)	5
4.	Test control - 1	Free Omiganan gel (1 mg in 100 mg/day) + Ovalbumin (100µg in 100µL, Topically)	5
5.	Test control - 2	Omiganan lotion (1 mg in 100 mg/day) + Ovalbumin (100µg in 100µL, Topically)	5
6.	Test control - 3	Omiganan NLC gel (1 mg in 100 mg/day) + Ovalbumin (100µg in 100µL, Topically)	5
7.	Test control - 4	Omiganan liposomal gel (1 mg in 100 mg/day) + Ovalbumin (100µg in 100µL, Topically)	5
8.	Test control - 5	Free DPK 060 gel (1 mg in 100 mg/day) + Ovalbumin (100µg in 100µL, Topically)	5
9.	Test control - 6	DPK 060 lotion (1 mg in 100 mg/day) + Ovalbumin (100µg in 100µL, Topically)	5
10.	Test control - 7	DPK 060 NLC gel (1 mg in 100 mg/day) + Ovalbumin (100µg in 100µL, Topically)	5

8.2.2.1 Histopathology

For histopathology investigation, the skin section with appropriate thickness was prepared and stained with hematoxylin and eosin (H & E). Subsequently, the samples were fixed on the glass slide and observed under the light microscope (Nikon, India), and were compared for the ceding of lesions.

8.2.2.2 Cytokines levels in serum

The serum collected from each animal was further subjected to ELISA for measurement of IL-4, IL-6, and TNF-α levels [1]. According to the protocol provided by manufacturer, the measurement was performed (Krishgen Biosystems, India). The concentration of IL-4, IL-6, and TNF-α in the serum was calculated and compared with different groups of animals.

8.2.3 Statistical analysis

The statistical analysis was performed using GraphPad Prism 8.0 (version 8.4.2, GraphPad Software, Inc., USA). The data obtained were analyzed using one-way ANOVA and two-way ANOVA analysis.

8.3 Results and Discussion

8.3.1 Imiquimod (IMQ) induced psoriatic animal model

The efficacy of the Omiganan loaded formulations was assessed in IMQ induced psoriatic animal model in BALB/c mice. Model development progress by IMQ is demonstrated in Fig. 8.1. The scaling and redness to appeared from the 4th day and are enhanced progressively till the 7th day. The IMQ application on the dorsal region leads to epidermal thickening due to hyperplasia of keratinocytes and inflammation (Fig. 8.1) [7, 8]. After the treatment, the skin was examined visually, and there was a decrease in the redness and inflammation in the treated animal groups (Fig. 8.2). Interestingly, the animals treated with Omiganan liposomal and NLC gel demonstrated decreased skin inflammation, and epidermal thickening with no scaly lesions in comparison with free Omiganan gel and lotion treated animals.

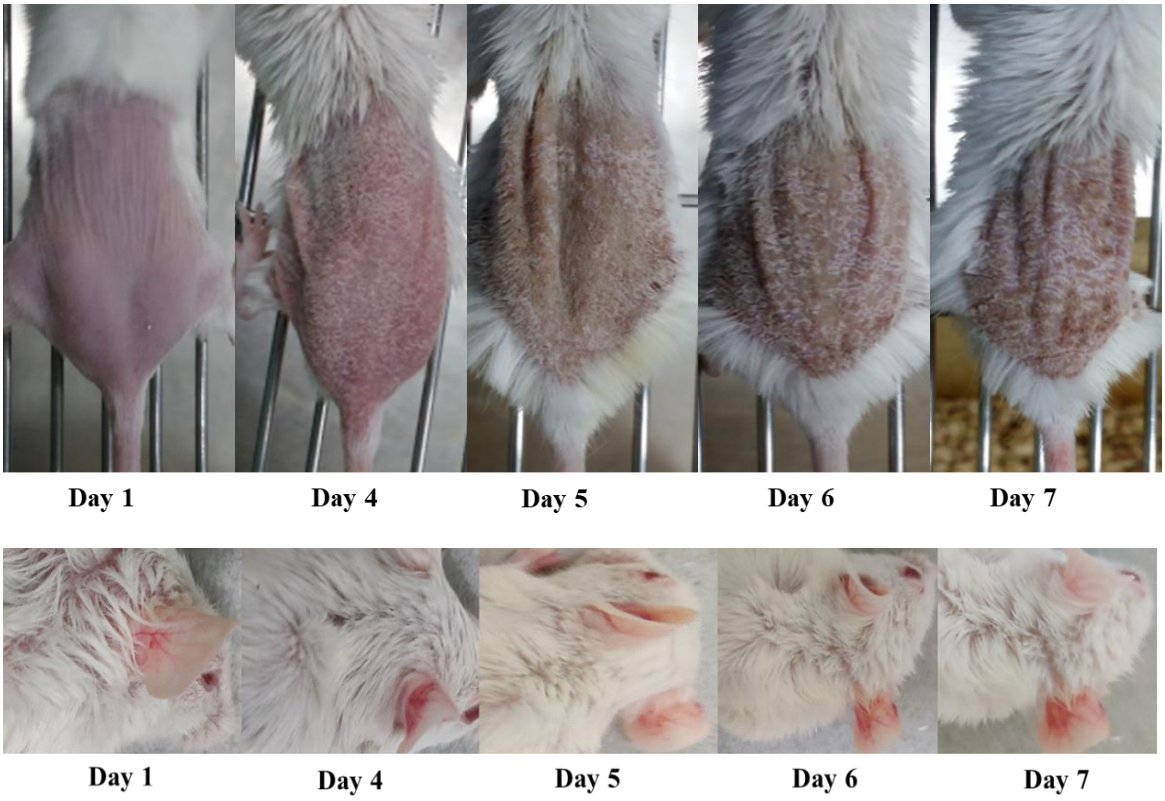

Figure 8.1 Imiquimod induced psoriatic animal model development on the back skin and right ear

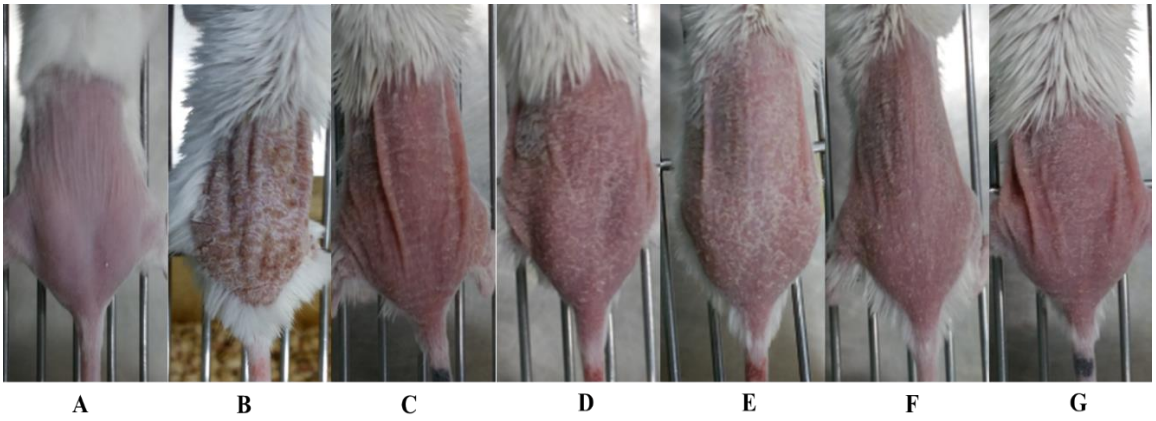

Figure 8.2 Visual analysis of improvement in psoriatic lesions after treatment; A) Normal control, B) Model control (Only Imiquimod), C) Standard control (Betamethasone Dipropionate gel, Betagel), D) Free Omiganan gel, E) Omiganan lotion, F) Omiganan liposomal gel, G) Omiganan NLC gel

8.3.1.1 Psoriatic scoring

PASI was used to examine the effect of the Omiganan treatment. Fig. 8.3 A-D depicts PASI scores for scaling, erythema, and thickening for different animal groups. It was observed that IMQ treated group (model control) demonstrates an increase in the scaling, erythema, and thickening from day 2 and got gradually increased to a cumulative PASI score of 9.6 on day 6. Moreover, treatment groups were able to inhibit the disease progression with a cumulative PASI score of 0, 0.7, 3.1, 2.8, 0.9, and 0.8 for normal control, Betagel (standard control), free Omiganan gel, Omiganan lotion, Omiganan liposomal, NLC gel treated groups, respectively. Omiganan liposomal and NLC based gel formulations found to be more effective in comparison to free Omiganan gel and lotion-based formulations.

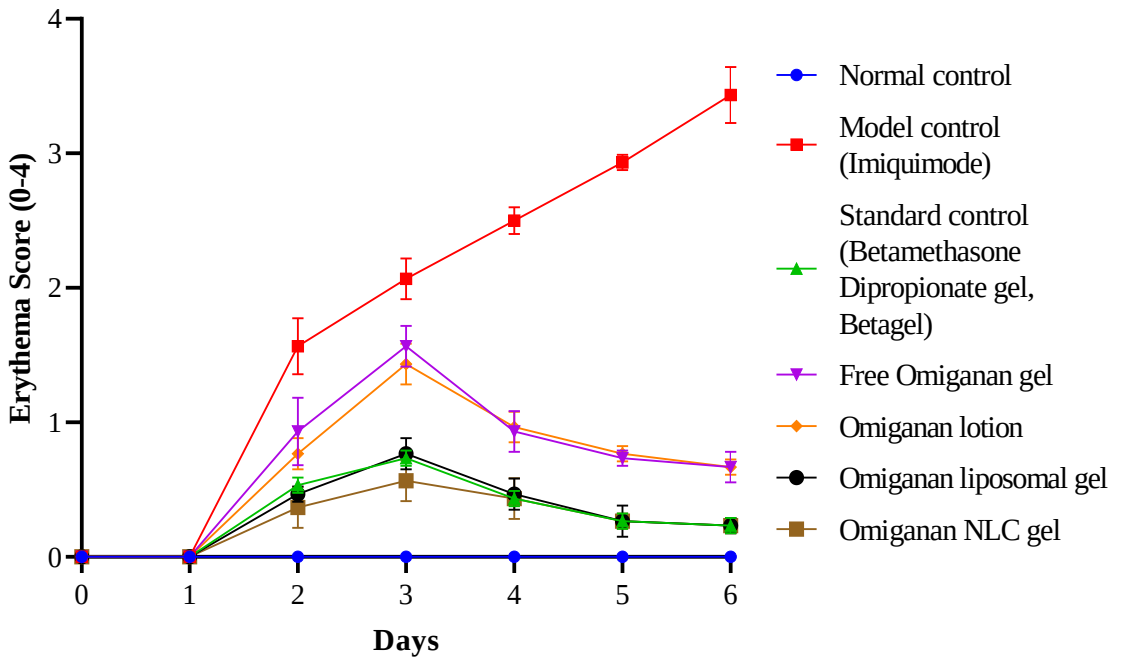

Figure 8.3 A) Erythema score

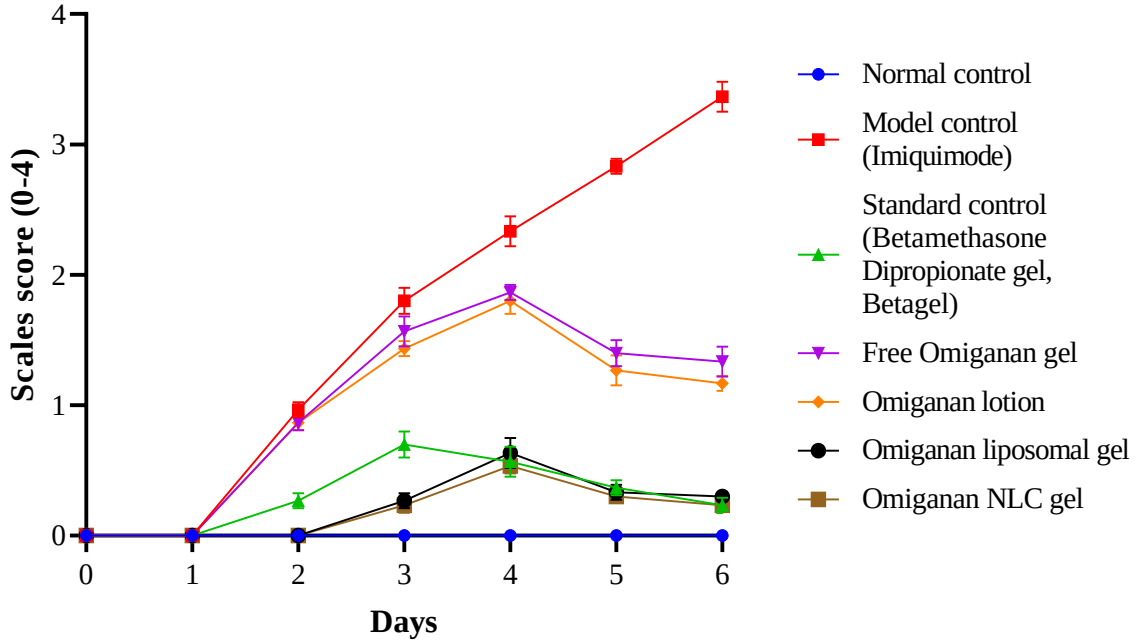

Figure 8.3 B) Scaling score

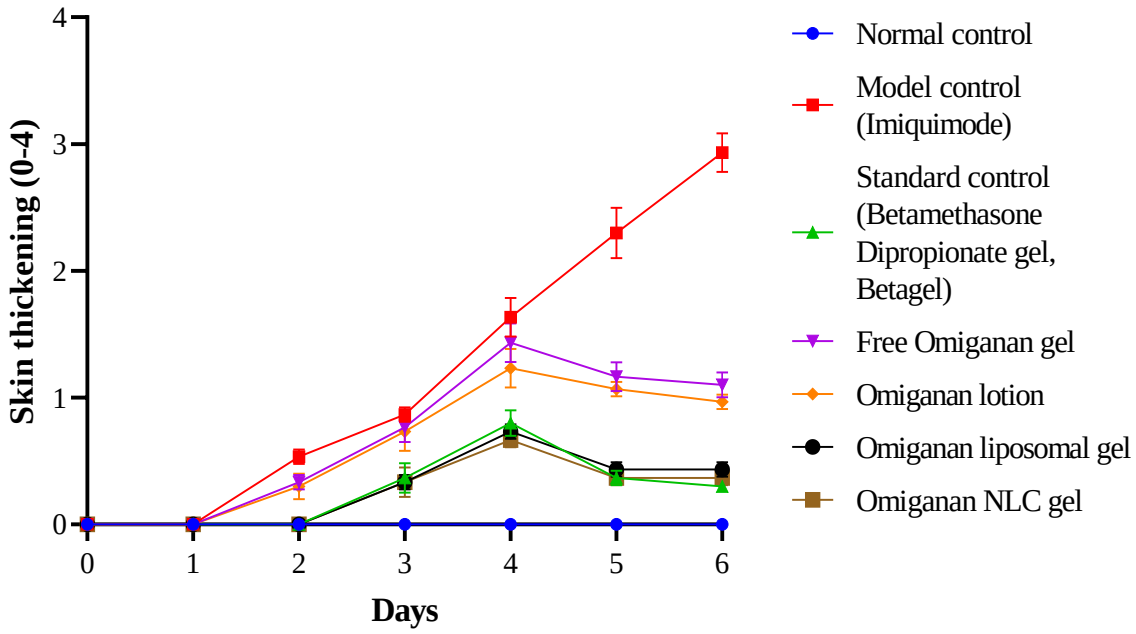

Figure 8.3 C) Skin thickening score

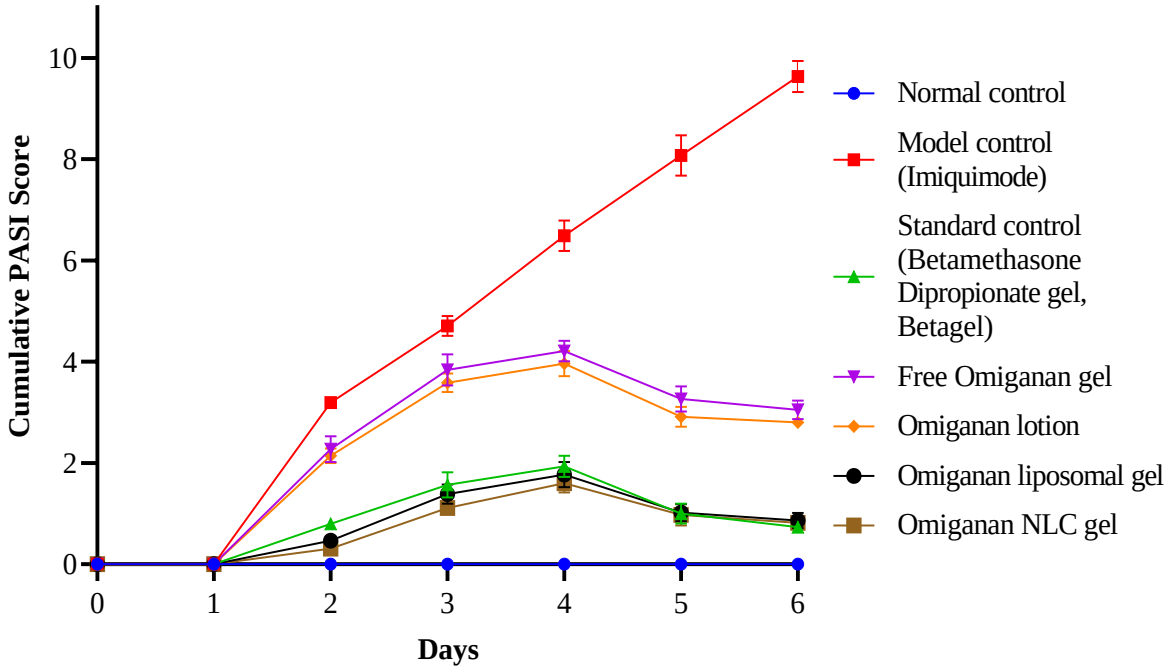

Figure 8.3 D) Total PASI score

8.3.1.2 Spleen weight and body weight

The application of IMQ resulted in a substantial increase in the size and the average spleen weight (Fig. 8.4 and 8.5) of model control animals (320 mg) in comparison to the normal control animals (92 mg). The spleen weights were markedly decreased in Omiganan liposomal (122 mg) and NLC gel (115 mg) treated groups compared to free Omiganan gel (240 mg) and Omiganan lotion (205 mg), thereby showing higher effectiveness of Omiganan liposomal and NLC gel (Fig. 8.5). Further, the normal control group did not significantly change body weight during the study protocol. The average body weight of animals in the model control group (IMQ treated) reduced by 18%. Whereas treatment groups (Omiganan loaded liposomal and NLC gel) did not show any noteworthy change in body weight compared to a standard control group (Fig. 8.6).

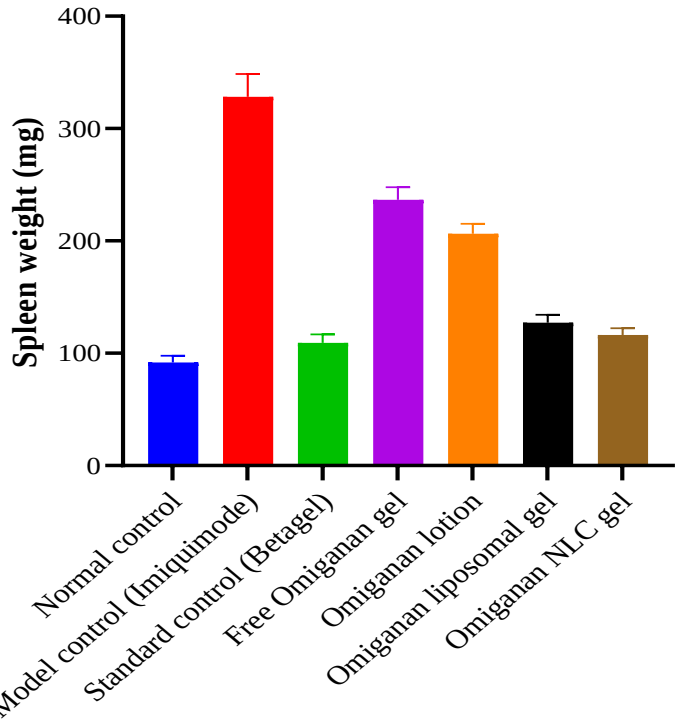

Figure 8.4 % Change in spleen weight

Figure 8.5 Visual analysis and comparison of spleen size after treatment; A) Normal control, B-C) Standard control (Betamethasone Dipropionate gel, Betagel), D) Free Omiganan gel, E) Omiganan lotion, F) Omiganan liposomal gel, G) Omiganan NLC gel, H-I) Model control (Only Imiquimod)

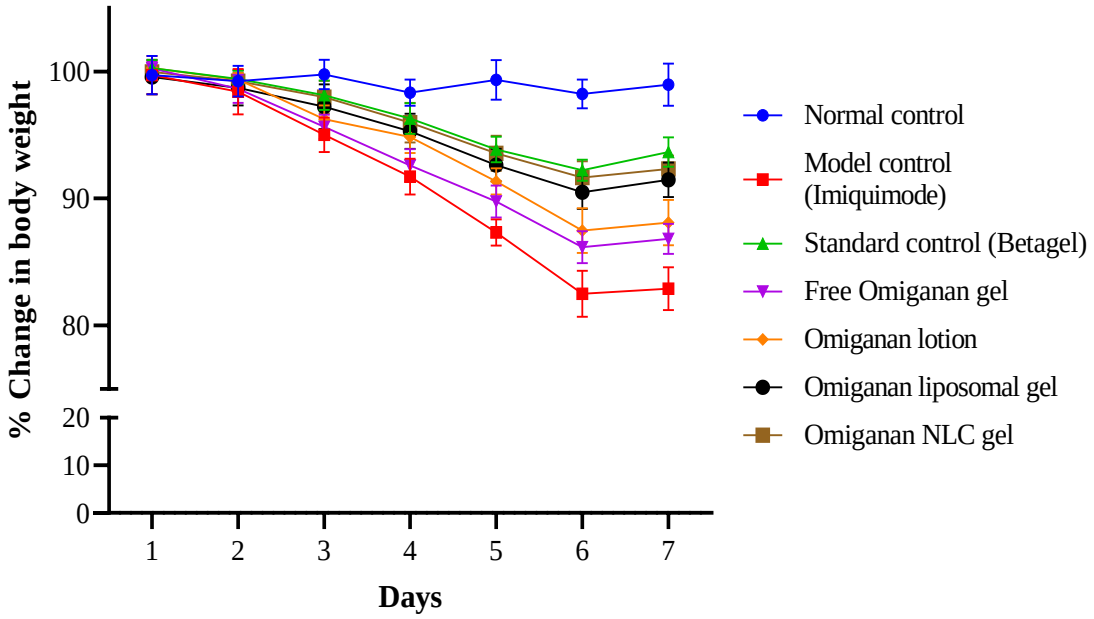

Figure 8.6 % Change in body weight

8.3.1.3 Histopathology

Histopathology of the skin is shown in Fig. 8.7. Acanthosis and hyperkeratosis (epidermal thickening) were observed in the model control group indicated the development of a psoriatic lesions [3, 7]. While the mice treated with Omiganan liposomal/NLC gel showed a marked reduction in epidermal thickening compared to model control and free Omiganan gel and lotion.

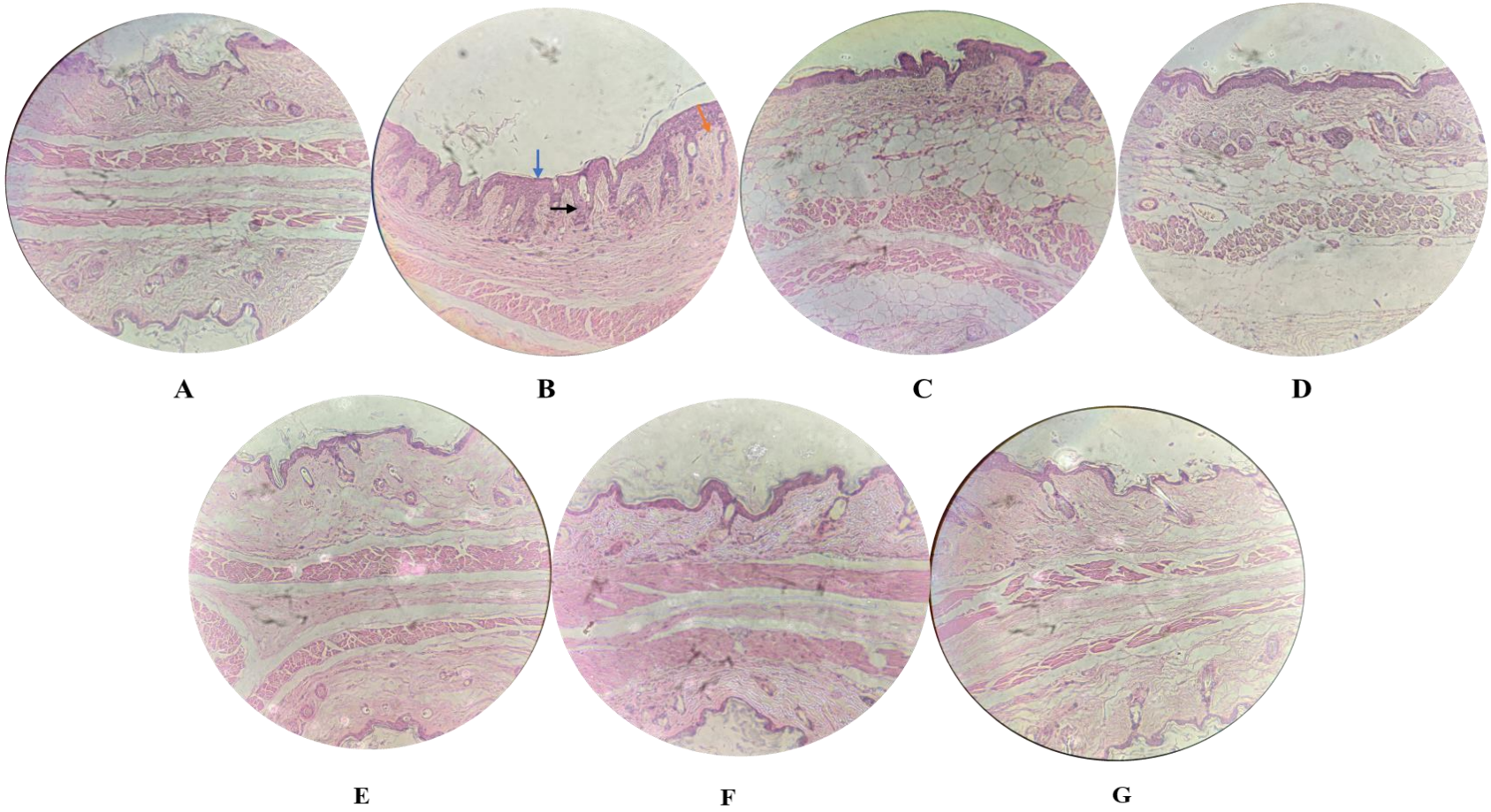

Figure 8.7 Histopathological images of mice skin for different animal groups; A) Normal control, B) Model control (Only Imiquimod); Blue arrow indicates hyperkeratosis, black arrow indicates acanthosis, Orange arrow indicates the presence of inflammatory cells, C) Free Omiganan gel, D) Omiganan lotion, E) Standard control (Betamethasone Dipropionate gel, Betagel), F) Omiganan liposomal gel, G) Omiganan NLC gel

8.3.1.4 Cytokines levels in serum

TNF-α and IL-6 act as key psoriasis mediators amongst the several immune cytokines as they mark the psoriasis beginning when induced by IMQ [7, 8]. Results (Fig. 8.8) demonstrated significantly higher levels of TNF-α and IL-6 in case of model control (TNF-α – 173±16 pg/ml and IL-6 – 877±43 pg/ml) compared to normal control animals (TNF-α – 9±2. pg/ml and IL-6 – 22±4 pg/ml). Later, the treatment with Omiganan liposomal and NLC gel, a substantial reduction in TNF-α levels (~81%) was observed in comparison to the free Omiganan gel (~22%) and lotion (~29%). Similarly, IL-6 levels were also reduced markedly in animals treated with Omiganan liposomal and NLC gel (~90%) compared to free Omiganan gel (~24%) and lotion (~31%). Omiganan liposomal and NLC based gel formulations found to be more effective in comparison to free Omiganan gel and lotion-based formulations.

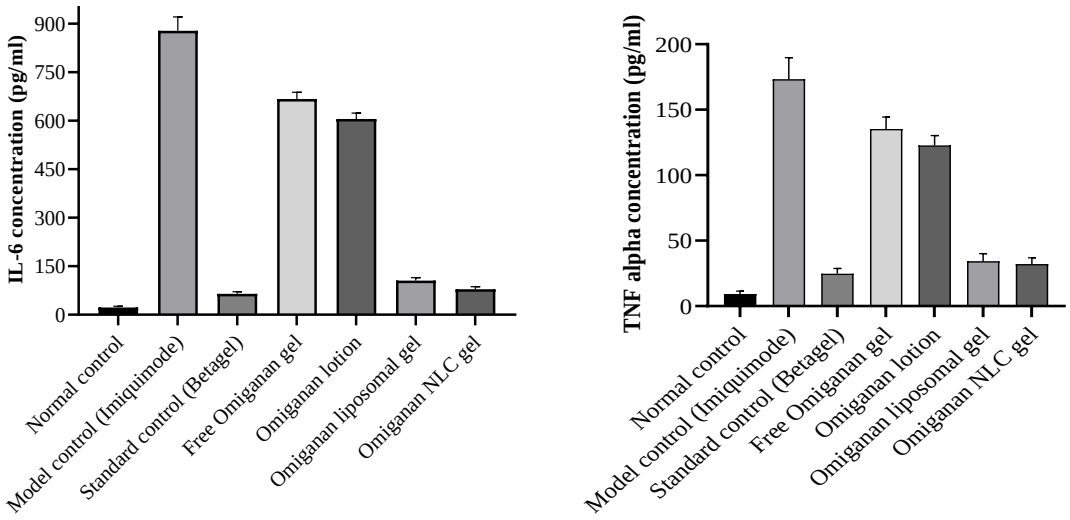

Figure 8.8 IL-6 and TNF- α levels for different animal groups after treatment

8.3.2 Ovalbumin induced animal model for eczema/atopic dermatitis

The efficacy of the Omiganan and DPK 060 loaded formulations was evaluated in OVA-induced eczema/atopic dermatitis animal model in BALB/c mice. AD like-skin lesions were characterized by increased epidermal thickening and dermal infiltration of inflammatory cells (Fig. 8.9) [9, 10]. After the treatment, the skin was evaluated visually, and there was a decrease in inflammation and epidermal thickening of the treated animal

groups (Fig. 8.10). Omiganan liposomal and NLC gel treated animal groups demonstrated a marked decrease in skin inflammation and epidermal thickening in comparison to model control and free Omiganan gel and lotion. Similarly, DPK-060 loaded NLC gel also reduced skin inflammation and epidermal thickening compared to model control and free DPK-060 gel and lotion (Fig. 8.10 & 8.12).

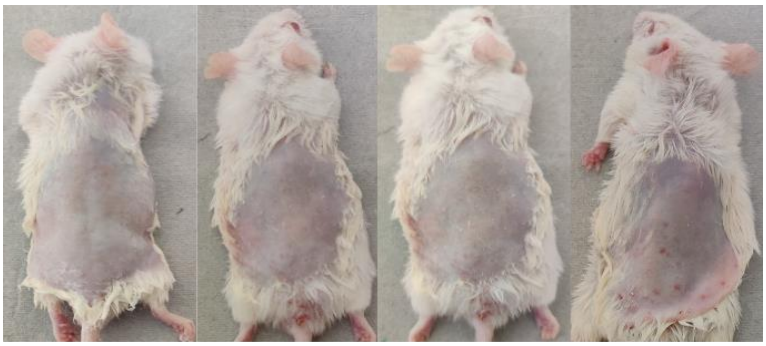

Figure 8.9 Ovalbumin induced eczema animal model development

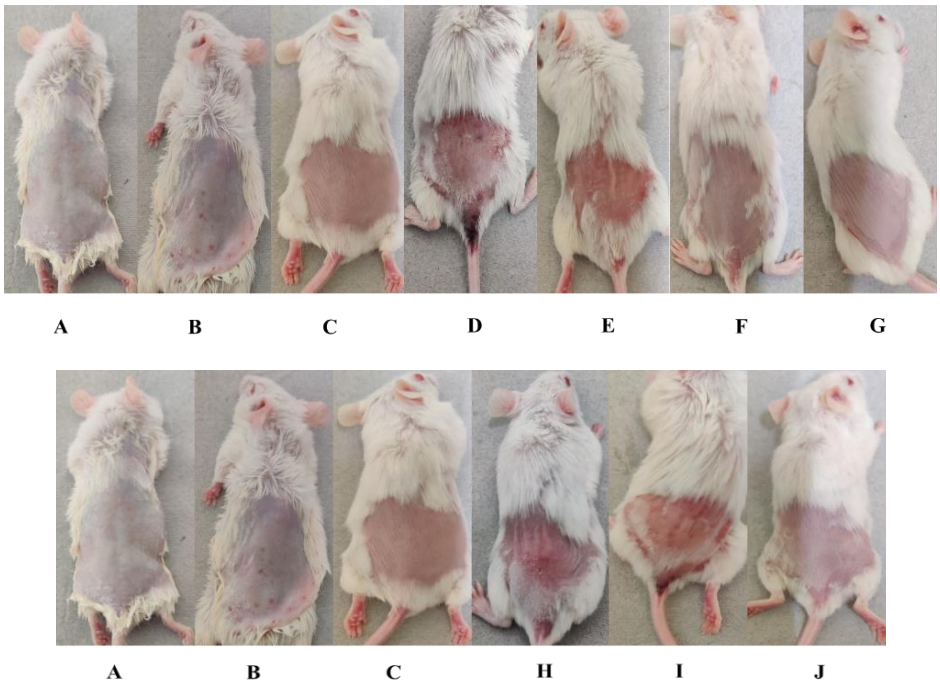

Figure 8.10 Visual analysis of improvement in eczematic lesions after treatment; A) Normal control, B) Model control (Only Ovalbumin), C) Standard control (Betamethasone Dipropionate gel, Betagel), D) Free Omiganan gel, E) Omiganan lotion, F) Omiganan liposomal gel, G) Omiganan NLC gel, H) Free DPK-060 gel, I) DPK-060 lotion, J) DPK-060 NLC gel

8.3.2.1 Histopathology

Histopathology of the AD-like mice skin is shown in Fig. 8.11. Hyperkeratosis (epidermal thickening) was observed in the model control group showed the progress of an AD-like condition [4, 5]. While the mice treated with Omiganan liposomal/NLC and DPK-060 NLC gel demonstrated the restoration of histo-architecture of skin, i.e., normalization of epidermal thickness compared to model control and free Omiganan/DPK-060 gel and lotion (Fig. 8.11 and 8.12).

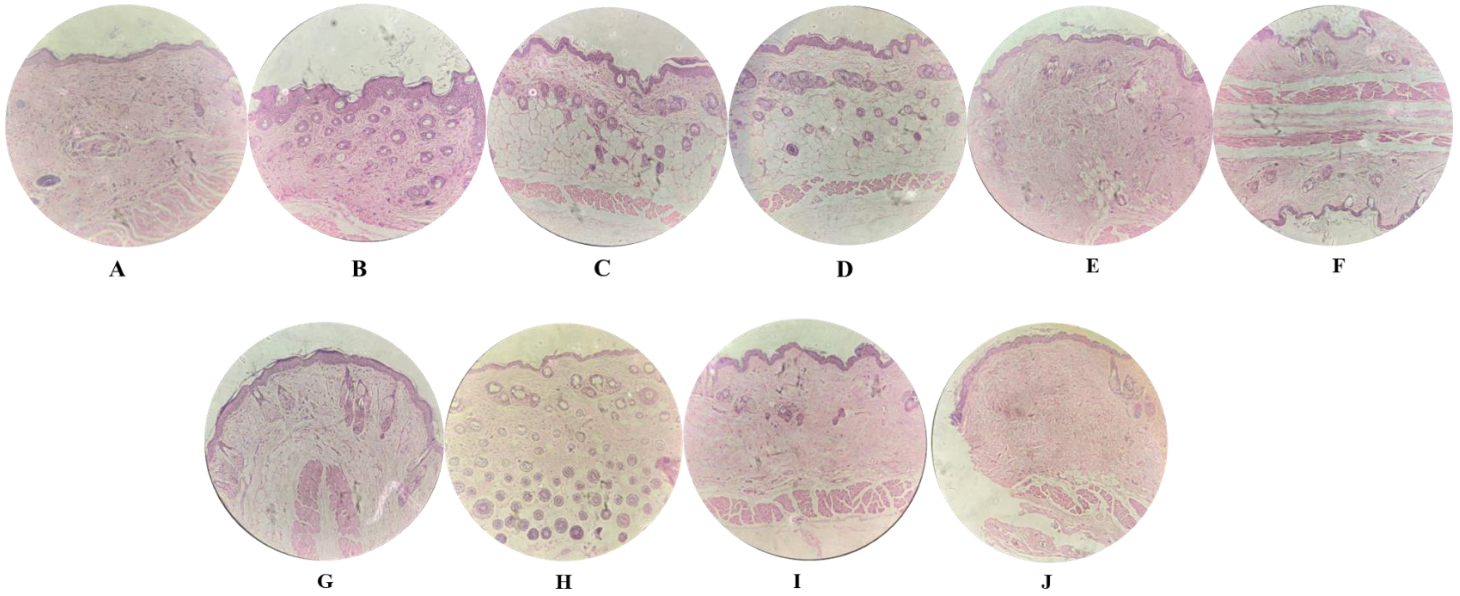

Figure 8.11 Histopathological images of mice skin for different animal groups; A) Normal control, B) Model control (Only Ovalbumin, C) Free Omiganan gel, D) Omiganan lotion, E) Omiganan Liposomal gel, F) Omiganan NLC gel, G) Standard control (Betamethasone Dipropionate gel, Betagel), H) Free DPK-060 gel, I) DPK-060 lotion, J) DPK-060 NLC gel

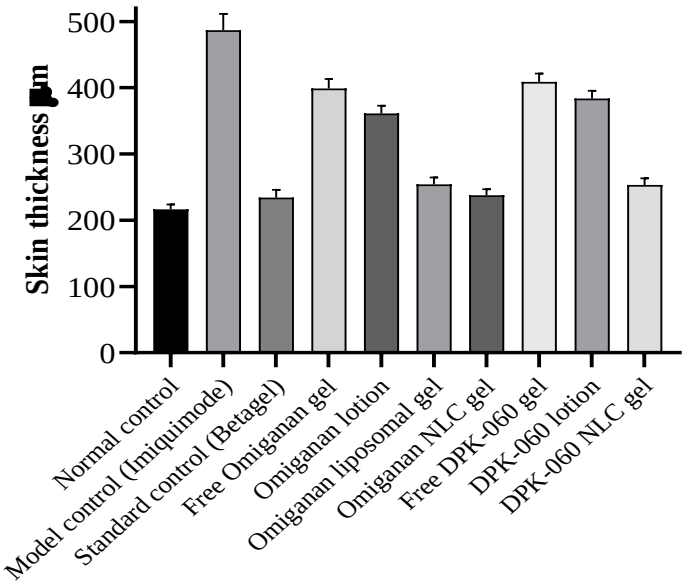

Figure 8.12 Skin thickness for different animal groups after treatment

8.3.2.2 Cytokines levels in serum

IL-4 mainly acts as key mediators of AD amongst the several immune cytokines as they mark the AD initiation when induced by OVA [4, 5]. Additionally, TNF-α and IL-6 levels in the serum were also measured as the markers of inflammation. Results (Fig. 8.13 A-C) demonstrated significantly higher levels of IL-4, TNF-α, and IL-6 in case of model control (IL-4 – 146±11 pg/ml, TNF-α – 149±11 pg/ml and IL-6 – 206±12 pg/ml) compared to normal control animals (IL-4 – 14±3 pg/ml, TNF-α – 16±3. pg/ml and IL-6 – 20±3 pg/ml). Later, the treatment with nano carrier-based gel of Omiganan and DPK-060 (liposomal/NLC gel), a substantial reduction in IL-4 levels (~85%) was observed as compared to the free Omiganan/DPK-060 gel (~20%) and lotion (~26%). Similarly, TNF-α levels (~81%) were also reduced after the treatment with nano carrier-based gel of Omiganan and DPK-060 (liposomal/NLC gel) compared to free Omiganan/DPK-060 gel (~21%) and lotion (~28%). Additionally, IL-6 levels (~86%) were also decrease markedly in animals treated with nano carrier-based gel of Omiganan and DPK-060 (liposomal/NLC gel) in comparison to the free Omiganan/DPK-060 gel (~21%) and lotion (~26%), demonstrating the higher effectiveness of liposomal and NLC based gel formulations in comparison to free Omiganan/DPK-060 gel and lotion-based formulations.

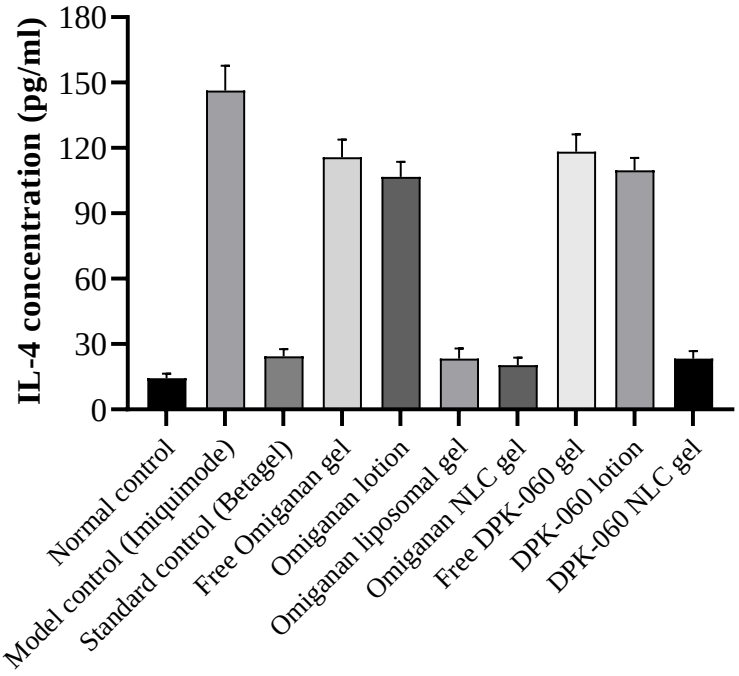

Figure 8.13 A) IL-4 levels for different animal groups after treatment

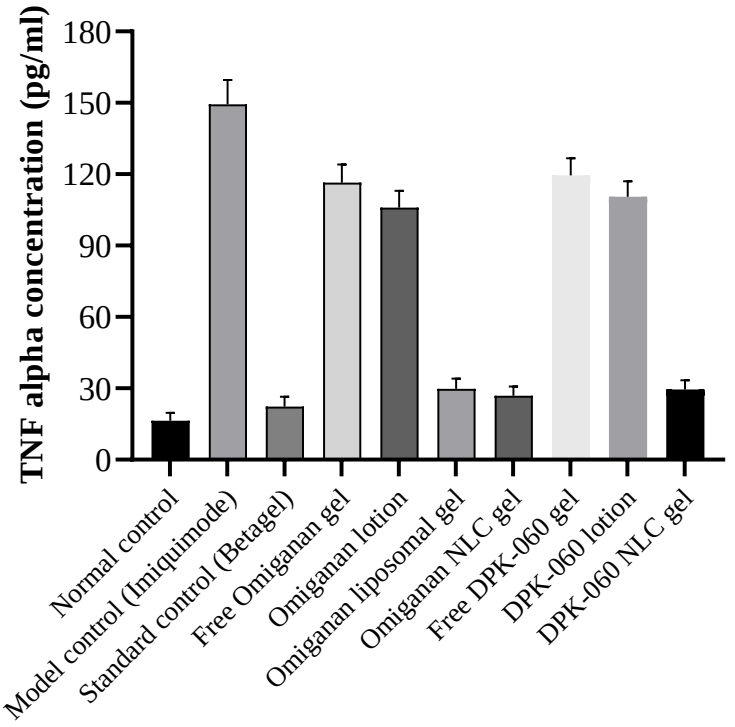

Figure 8.13 B) TNF-α levels for different animal groups after treatment

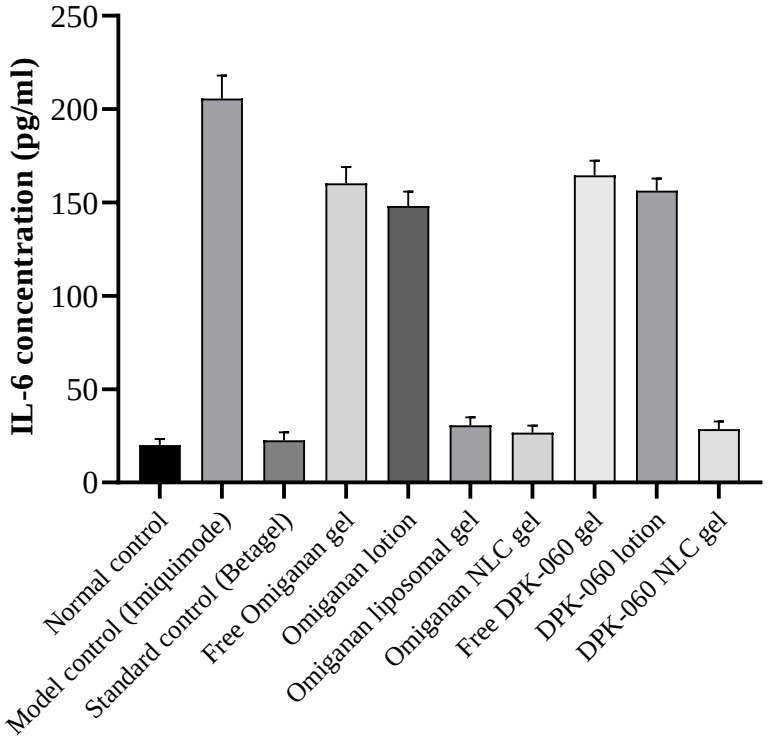

Figure .13 C) IL-6 levels for different animal groups after treatment

8.4 References

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