Summary

Omiganan is a novel cationic peptide (12 amino acid) and an analog of indolicidin. Omiganan possesses antimicrobial activity against various gram-positive and gram-negative micro-organism including fungi. Apart from its antimicrobial effect, Omiganan also has anti-inflammatory activity. These antimicrobial and anti-inflammatory properties make Omiganan a promising agent for treating eczema/atopic dermatitis and psoriasis. Furthermore, the positive phase II clinical results were obtained in patients with AD (mild to moderate) with Omiganan 1% gel. However, there is no significant decrease in the *S. aureus* payload in patients treated with Omiganan 0.5 % and 1% gel may be due to the proteolytic degradation of Omiganan and poor permeation profile of free Omiganan gel.

DPK-060 is a synthetic 17 amino acid peptide, structural derivative from the human protein kininogen. DPK-060 mainly acts by membrane disruption mechanism along with immunomodulation, thus demonstrating strong broad-spectrum antimicrobial activity against both gram +ve and -ve bacteria, including *methicillin-resistant S. aureus (MRSA) in-vitro* and *in-vivo*. Moreover, the safety and effectiveness of 1% DPK-060 in a PEG based ointment has been evaluated in phase II clinical trial (NCT01522391) to treat AD patients. Additionally, the positive results were obtained in these clinical trials but found not statistically conclusive due to the instability of DPK-060 as a drug substance in the formulation. Further, nanotechnology-based formulations of DPK-060 have been developed to improve the functionality, stability, and release profile of DPK-060.

To overcome the aforesaid concerns associated with dermal administration of peptides, lipid-based nano-carriers such as liposomes and NLCs have been formulated and characterized. Liposomes and NLCs possess a natural affinity for skin lipids. Moreover, they offer enhanced permeation of drug(s), may be attributed to improved interaction with the skin, an occlusion, and sustained release characteristics. Also, in the case of hydrophilic molecules, the high lipidic content can lead to an occlusion and film development on skin, make possible improved skin hydration owing to decreased trans-epidermal water loss, helping the diffusion of active ingredients into the SC. This would lead to overcome the challenges of conformational instability, low permeability across stratum corneum, partitioning in a different stage of the subdermal region and proteolytic degradation of

peptides. Moreover, these nanocarriers also provides the controlled release for prolong time period and decreasing the frequency of administration, leading to improved patient compliance.

Analytical method development

Gradient HPLC methods were validated for quantifiable estimation of Omiganan and DPK-060 in various *in-vitro* and *ex-vivo* experimental analysis. The calibration plots of Omiganan and DPK 060 in a mixture of acetonitrile and tri-fluro acetic acid (0.1%) at 220 nm λmax were generated using gradient HPLC method and used to estimate the % drug entrapment in developed nanocarriers (liposomes and NLCs). The LOD and LOQ values were found to be 0.062 µg/ml and 0.208 µg/ml for Omiganan and 0.033 µg/ml and 0.111 µg/ml for DPK-060 by developed gradient HPLC method, indicates the accuracy and sensitivity of the methods.

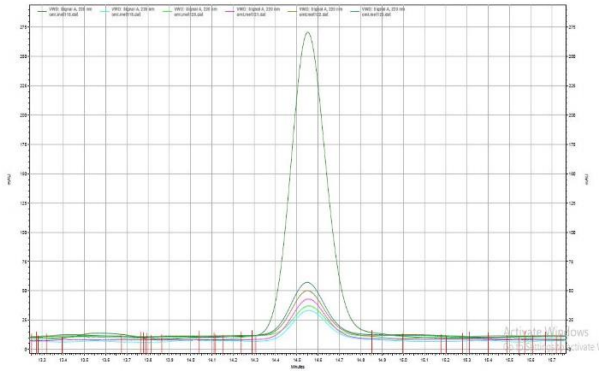


Figure 1 Overlay plot of Omiganan by HPLC method

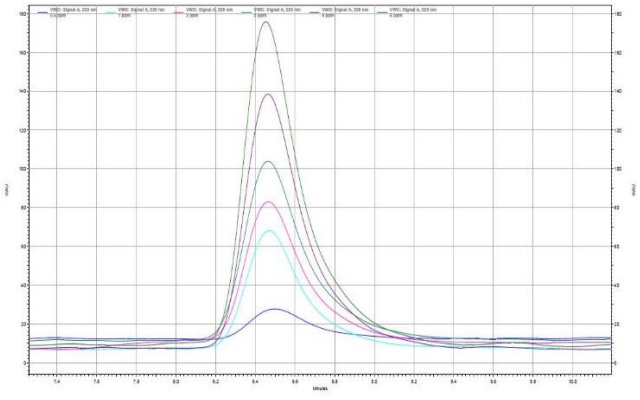


Figure 2 Overlay plot of DPK-060 by HPLC method

Preformulation studies

Authentication of Omiganan and DPK-060 and their compatibility with excipients were evaluated in pre-formulation studies. Omiganan and DPK-060 were authenticated based on the molecular weight obtained from LC-MS spectra with that available in literatures. The compatibility among peptides and excipients were analyzed by FT-IR and the results indicated the compatibility between excipients and peptides. Additionally, the solubility of Omiganan and DPK-060 were evaluated for selection of lipids/oils and surfactants to prepare NLC based formulations.

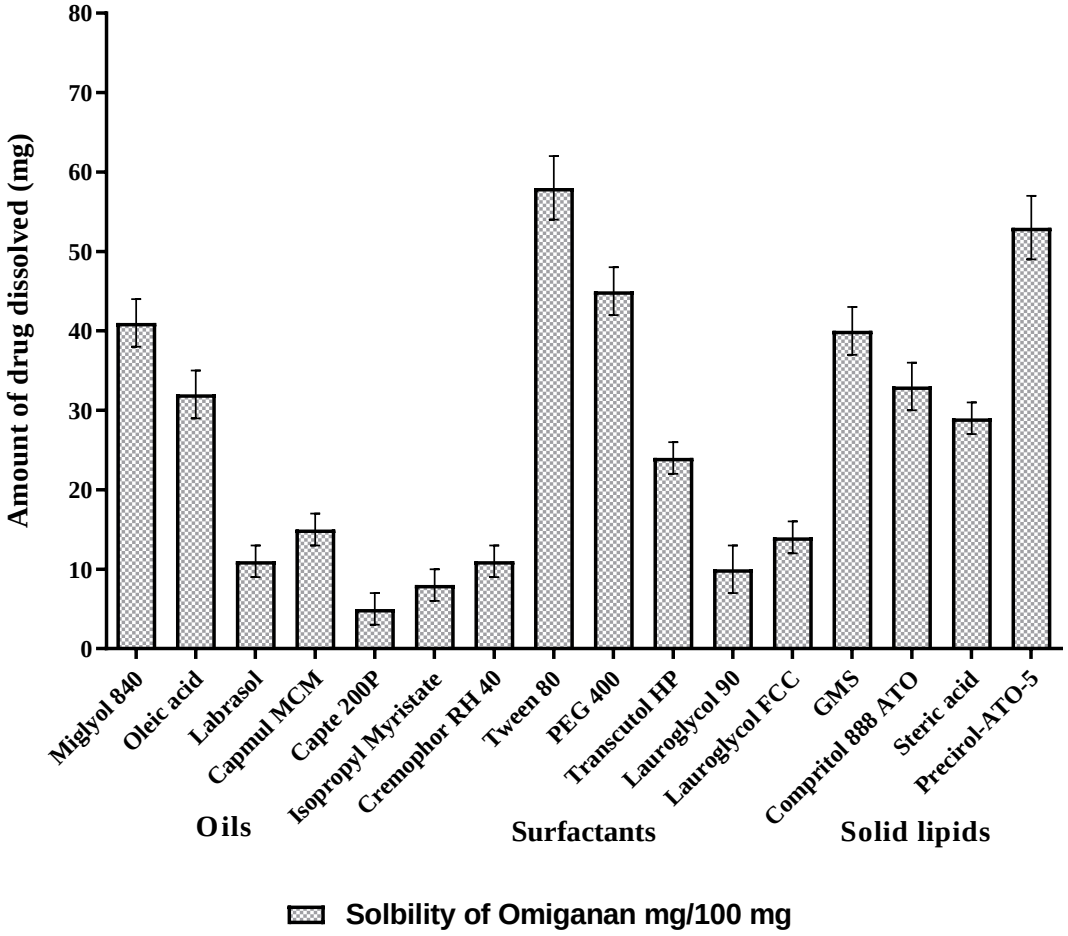


Figure 3 Solubility of Omiganan in various excipients

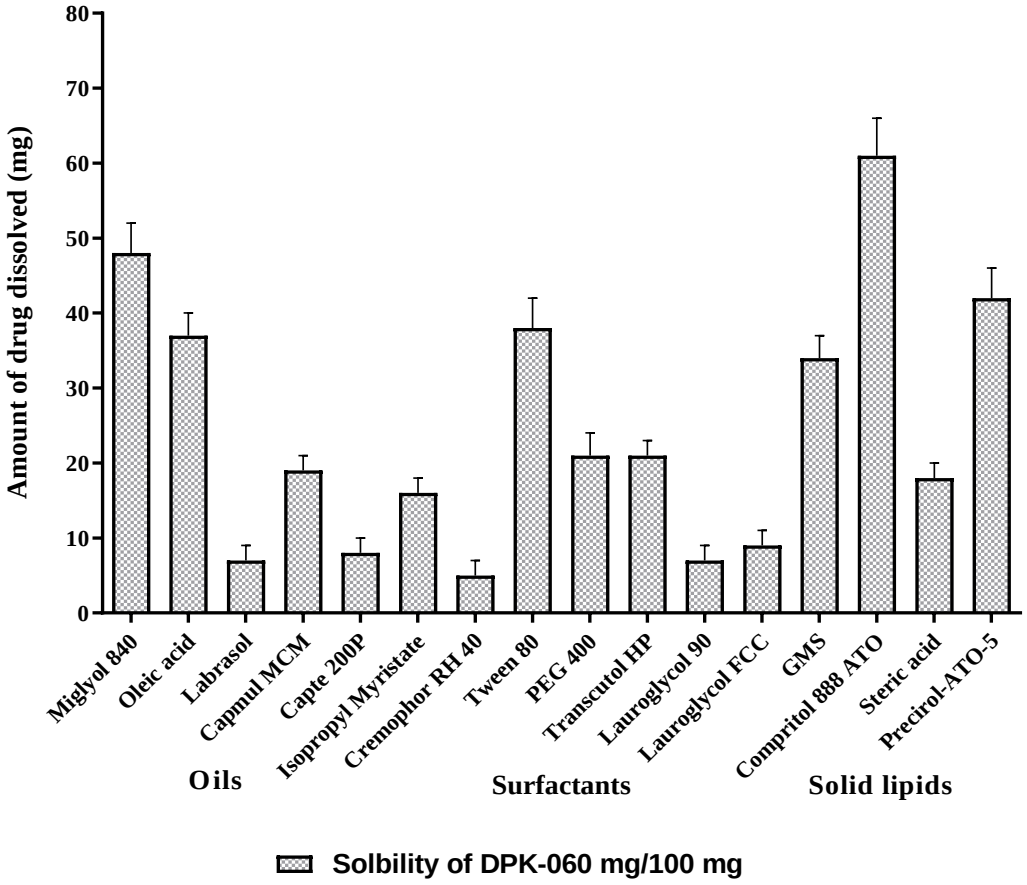


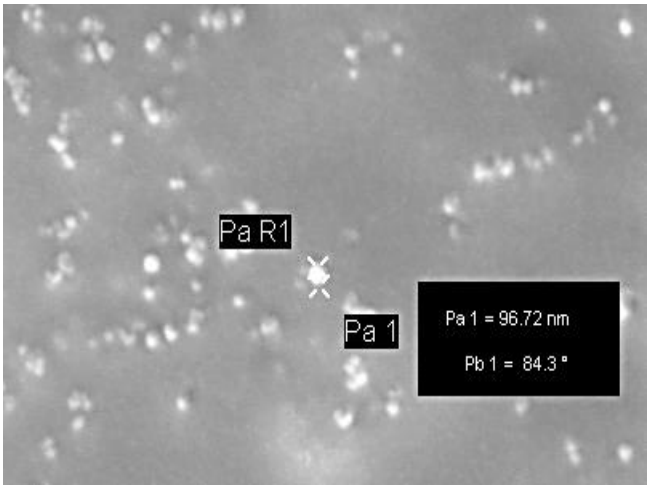
Figure 4 Solubility of DPK-060 in various excipients

Omiganan and DPK-060 loaded NLCs and NLC gel

Out of the several available methods for preparation, melt-emulsification method was chosen for NLC preparation of both the peptides. Additionally, high pressure homogenization technique was used for size reduction of the NLCs. The QbD approach was adapted in the formulation development of NLCs. Based on the scientific, industrial and regulatory aspects, QTPP elements and their targets were established and demonstrated in Ishikawa diagrams. Based on the preliminary investigation, critical material attributes (CMA) such as lipid concentration, surfactant/cosurfactant concentration, homogenization cycles were identified and their relationship with critical quality attributes (CQA) i.e., % drug entrapment and particle size were exhaustively investigated using Box-Behnken Design. Stat-Ease Design Expert Software 13.0. was used to generate BBD design matrix with 17 experimental. The response surface plots (2D and 3D) were generated for both

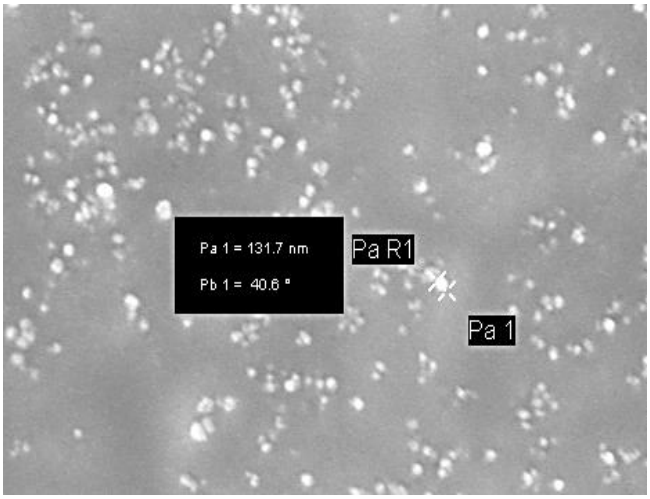
Omiganan and DPK-060 NLCs showed that amount of lipids and homogenization cycles have the maximum effect on the % drug entrapment and particle size considering $P < 0.05$ as a level of significance. Numerical optimization for achieving maximum % drug entrapment and minimum particle size resulted optimization solutions with desirabilities of 0.911 and 0.945 for Omiganan and DPK-060 NLCs, respectively.

The optimized composition of Omiganan NLC was predicted to have Omiganan 1%w/w, lipid concentration of 3.13%w/w (Precirol-ATO-5 and Miglyol-840), Smix concentration of 10%w/w (Tween 80 and PEG 400) and homogenization at 10,000 psi for 10 cycles with % drug entrapment of 79.49 % and mean particle size of 115.7 nm. Similarly, the optimized composition of DPK-060 NLC was predicted to have DPK-060 1%w/w, lipid concentration of 4.0%w/w (Compritol 888 ATO and Miglyol-840), surfactant concentration of 13.08%w/w (Tween 80 and egg lecithin) and homogenization at 10,000 psi for 10 cycles with % drug entrapment of 85.20 % and mean particle size of 128.6 nm. Additionally, the gel-based formulation was developed and loaded with Omiganan and DPK-060 (1%w/v) and optimized Omiganan/DPK-060 NLCs (1%w/v) to improve the viscosity of the formulation so as to enhance the skin retention. Carbopol 934P, a polyacrylic acid based gelling agent was used to prepare gel base. The optimized composition of NLC based gel was Omiganan/DPK-060 free drug/NLCs (1%w/v), Carbopol 934P (1.2%w/v), propylene glycol (4%w/v) and methyl paraben (0.2%w/v) and propyl paraben (0.02%w/v). the pH was adjusted to ~6.5 by drop wise addition of 10% v/v Sodium hydroxide solution.



Scale of 2 µm

Figure 5 SEM image of the developed Omiganan loaded nano-lipid constructs



Scale of 2 µm

Figure 6 SEM image of the developed DPK-060 loaded nano-lipid constructs

Optimized Omiganan and DPK-060 NLCs/NLC based gels were than characterized for shape and surface morphology, zeta potential, viscosity, spreadability and pH. The zeta-potential of Omiganan and DPK-060 loaded NLCs was found to be -16.1 mV and -22.5 mV, respectively. The charge was found sufficient enough to keep the particles dispersed via repulsive forces. Additionally, results of SEM analysis showed the spherical shape of NLCs. The viscosity of free Omiganan gel and Omiganan NLC gel was found to be 12.49 ± 0.367 Pa.S and 15.42 ± 0.37 Pa.S, respectively. Similarly, he viscosity of free DPK-060 gel and DPK-060 NLC gel was found to be 13.10 ± 0.292 Pa.S and 16.22 ± 0.451 Pa.S,

respectively. Optimized NLC gel of Omiganan and DPK-060 showed good spreadability and with pH of 6.5, suitable for dermal administration.

Omiganan loaded Liposomes

Omiganan loaded liposomes were formulated by reverse-phase evaporation technique. Additionally, probe sonication was used in the size reduction of the liposomes. The QbD approach was adapted in the formulation development of liposomes. Based on the scientific, industrial and regulatory aspects, QTPP elements and their targets were established and demonstrated in Ishikawa diagrams. Based on the preliminary investigation, critical material attributes (CMA) such as lipid concentration, sonication time, and sonication amplitude were identified and their relationship with critical quality attributes (CQA) i.e., % drug entrapment and vesicle size were exhaustively investigated using Box-Behnken Design. Stat-Ease Design Expert Software 13.0. was used to generate BBD design matrix with 17 experimental. The response surface plots (2D and 3D) were generated for Omiganan liposomes showed that amount of lipids, sonication time and have substantial effect on the % drug entrapment and vesicle size considering $P < 0.05$ as a level of significance. Numerical optimization for achieving maximum % drug entrapment and minimum vesicle size resulted optimization solution with desirability of 0.876. The optimized composition of Omiganan liposomes was predicted to have Omiganan 1%w/w, lipid concentration of 98.4 mg/ml (HSPC, DPPC, soya-lecithin, and cholesterol – ratio: 5.5:3:1:0.5), and sonication time and amplitude of 60 sec and 70%, respectively. % Drug entrapment and vesicle size of optimized Omiganan liposomes were found to be 71.77 % and 120.5 nm, respectively. Additionally, the gel-based formulation was developed and loaded with optimized Omiganan liposomes (1%w/v) to improve the viscosity of the formulation so as to enhance the skin retention. Carbopol 934P, a polyacrylic acid based gelling agent was used to prepare gel base. The optimized composition of Omiganan liposomal gel was Omiganan liposomes (1%w/v), Carbopol 934P (1.2%w/v), propylene glycol (4%w/v) and methyl paraben (0.2%w/v) and propyl paraben (0.02%w/v). the pH was adjusted to ~6.5 by drop wise addition of 10% v/v Sodium hydroxide solution.

Optimized Omiganan liposomes and liposomal gel were than characterized for shape and surface morphology, zeta potential, viscosity, spreadability and pH. The zeta-

potential of Omiganan loaded liposomes was found to be -17.2 mV, sufficient enough to keep the particles dispersed via repulsive forces. Additionally, results of cryo-TEM analysis showed the spherical shape and smooth surface of liposomes. The viscosity of Omiganan liposomal gel was found to be 14.05 ± 0.420 Pa.S. Optimized liposomal gel of Omiganan showed good spreadability and with pH of 6.5, suitable for dermal administration.

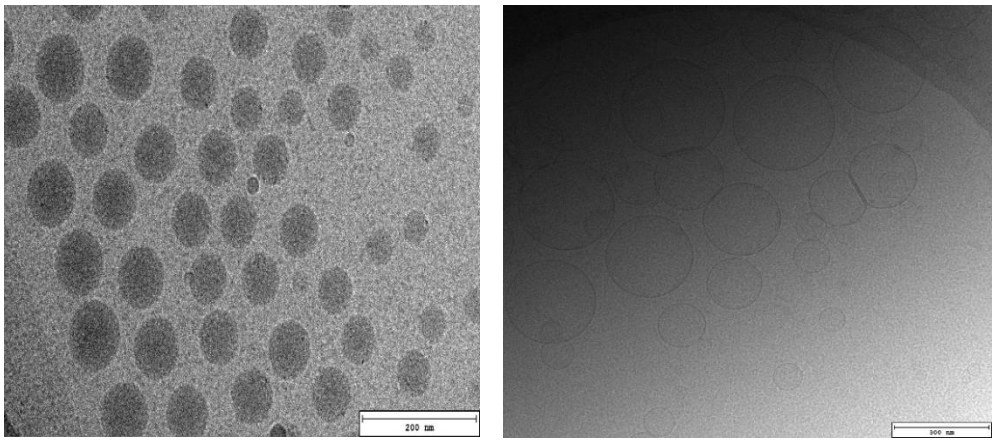


Figure 7 Cryo-TEM images of the developed Omiganan loaded liposomes

Omiganan and DPK-060 lotion

Lotion based formulations of Omiganan and DPK-060 were developed and characterized for viscosity, spreadability, pH and thermodynamic stability. The viscosity of Omiganan and DPK-060 lotion was found to be 5.79 ± 0.158 Pa.S and 6.20 ± 0.192 Pa.S respectively, lower in comparison with the developed nanocarrier gel based formulations of Omiganan and DPK-060. Optimized Omiganan and DPK 060 lotion was stable under the given stress conditions. Additionally, no coalescence and cracking were observed throughout the thermodynamic stability assessments.

In-vitro and *ex-vivo* studies

The optimized formulations of Omiganan and DPK 060 were subjected to various *in-vitro* and *ex-vivo* studies i.e., *in-vitro* drug release, *in-vitro* cytotoxicity and cellular uptake, *in-vitro* antimicrobial efficacy, *ex-vivo* drug permeation and skin retention studies.

Results of i*n vitro* drug release studies of Omiganan loaded formulations demonstrated that around 90% Omiganan was released from the lotion (89.73 ± 2.82) and

free Omiganan gel (91.71 ± 3.01) within the 1h whereas around 77% of Omiganan was released from the liposomal gel (78.04 ± 2.26) and NLC gel (76.66 ± 2.49) after 6 h, offers the sustained release of Omiganan. The quick equilibrium was observed from lotion and free Omiganan gel due to the hydrophilic nature of Omiganan. The regression coefficient of korsmeyer- Peppas model for Omiganan liposomal gel and NLC gel was found to be 0.991 with value of n (release exponent) 0.59 and 0.67 for Omiganan liposomal and NLC gel respectively, i.e., $0.45<n<0.89$ indicates anomalous diffusion/non-fickian diffusion i.e., combination of both diffusion and erosion-controlled rate release. While, the results of in-vitro release studies of DPK 060 loaded formulations demonstrated around 84% of DPK 060 was released from the lotion (87.64 ± 2.61) and free DPK 060 gel (90.49 ± 3.20) within 1h whereas around 72% of DPK 060 was released from NLCs after 6 hr, offers the sustained release of DPK 060. The quick equilibrium was observed from lotion and free DPK 060 gel due to the amphiphilic nature of DPK 060. The regression coefficient of korsmeyer- Peppas model for DPK 060 NLC gel was found to be 0.993 with value of n (release exponent) 0.48, i.e., $0.45<n<0.89$ indicates anomalous diffusion/non-fickian diffusion.

The viability of cells treated with free Omiganan solution, Omiganan lotion, and Omiganan loaded liposomes and NLCs were found in this order; free Omiganan < Omiganan lotion < Omiganan liposomes < Omiganan NLCs, significantly higher than positive control Triton X100 (<25%). While, he viability of cells treated with free DPK 060 solution, DPK 060 lotion, and DPK 060 loaded NLCs were found in this order free DPK 060 < DPK 060 lotion < DPK 060 loaded NLCs, significantly higher than positive control Triton X100 (<25%). The developed formulations of Omiganan and DPK 060 have no potential toxic effects on the 3T3-fibroblast cells and are found to be safe for dermal delivery.

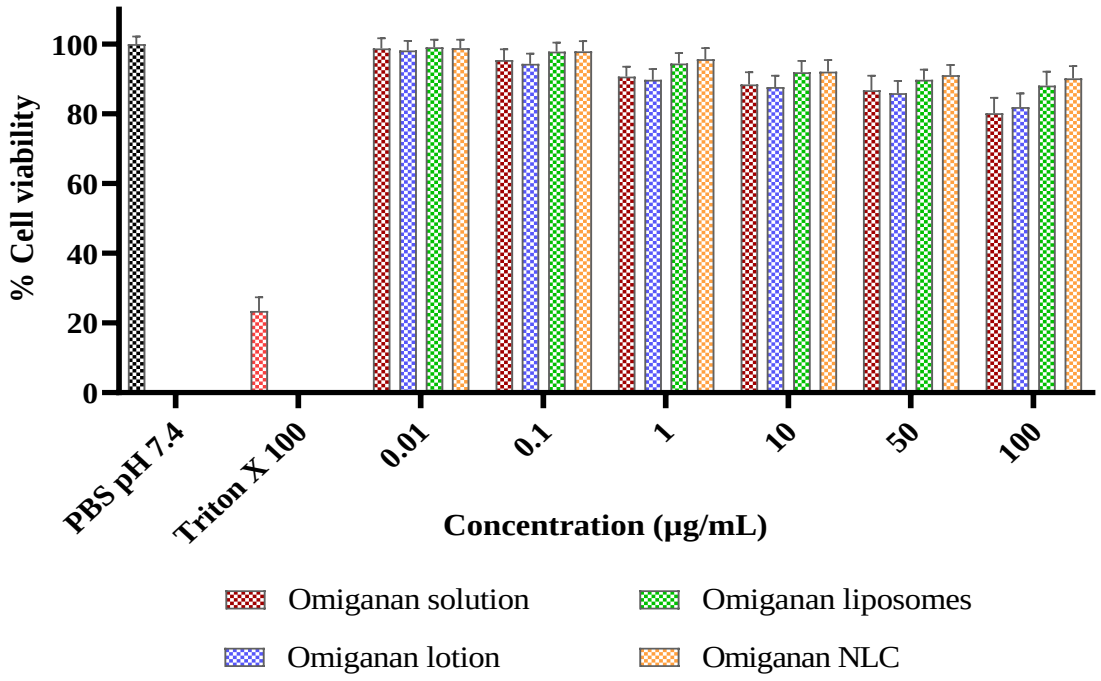


Figure 8 *In-vitro* cell viability data for Omiganan formulations in 3T3-fibroblast cells

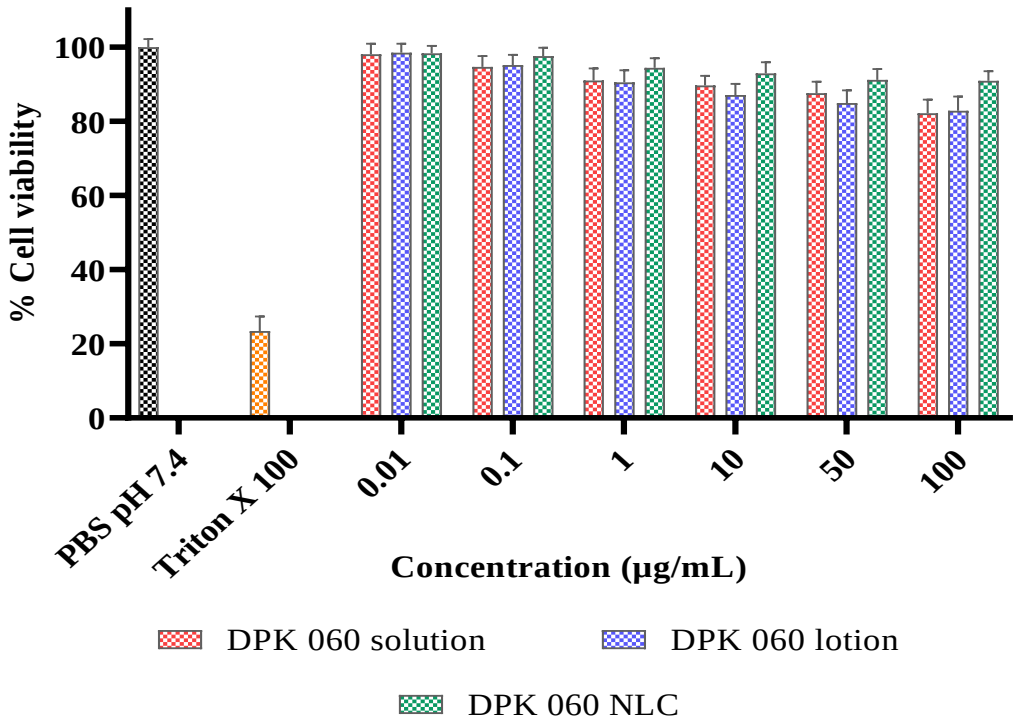


Figure 9 *In vitro* cell viability data for DPK-060 formulations in 3T3-fibroblast

The results of cellular uptake studies of Omiganan and DPK 060 loaded formulations demonstrated higher cellular uptake of the Omiganan loaded liposomal and NLC gel in comparison to free Omiganan gel and Omiganan lotion after 4 h. While the results of cellular uptake studies of the DPK 060 formulations demonstrated higher cellular uptake of DPK 060 NLC gel in comparison to free DPK 060 gel and DPK 060 lotion after 4 h. The increase in the cell uptake indicated the enhanced internalization of the Omiganan and DPK 060. This may be correlated that the enhanced cell uptake increases the therapeutic efficacy of Omiganan and DPK 060.

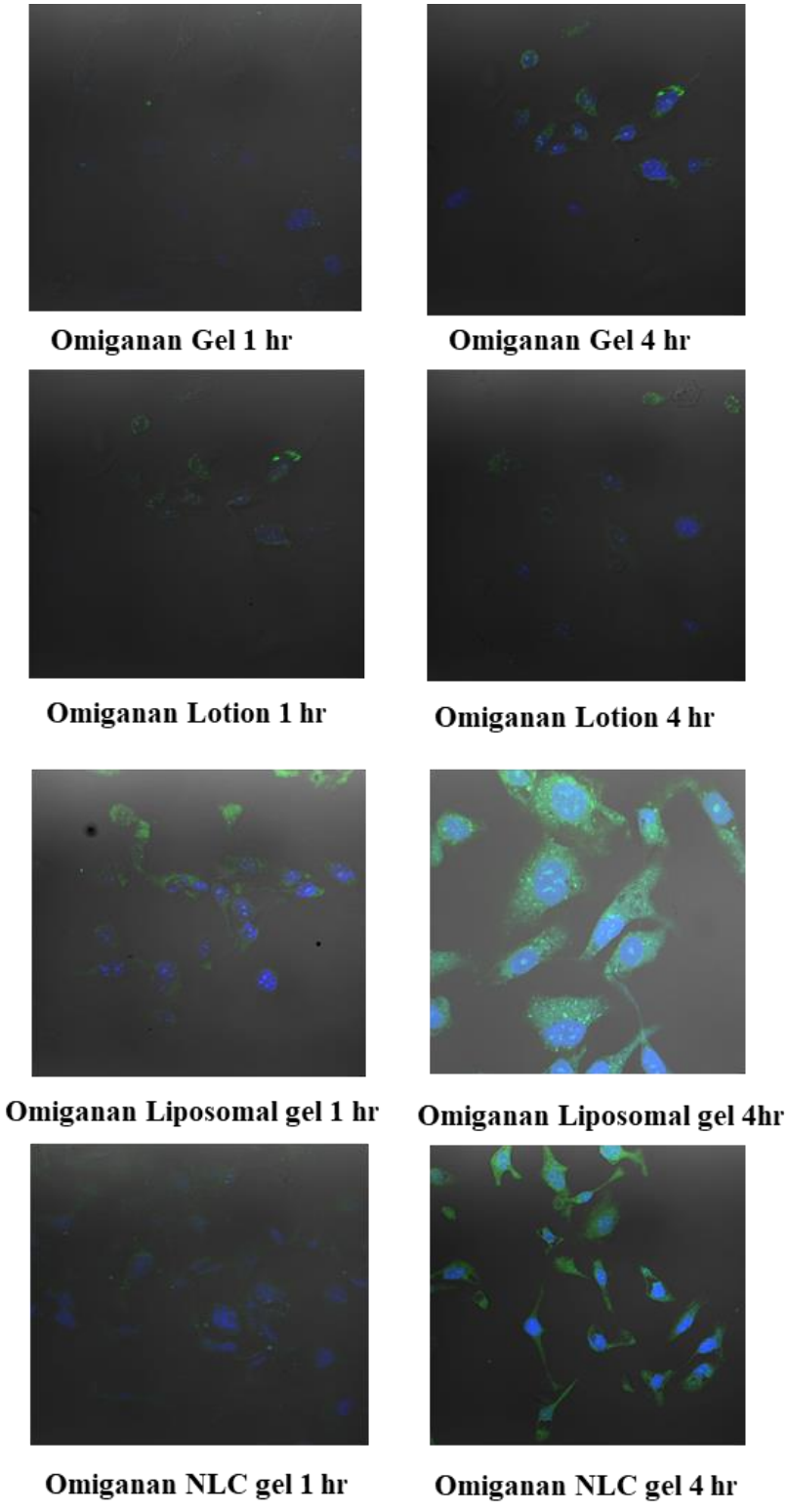


Figure 10 *In vitro* cell uptake of Omiganan formulations in 3T3-fibroblast cells

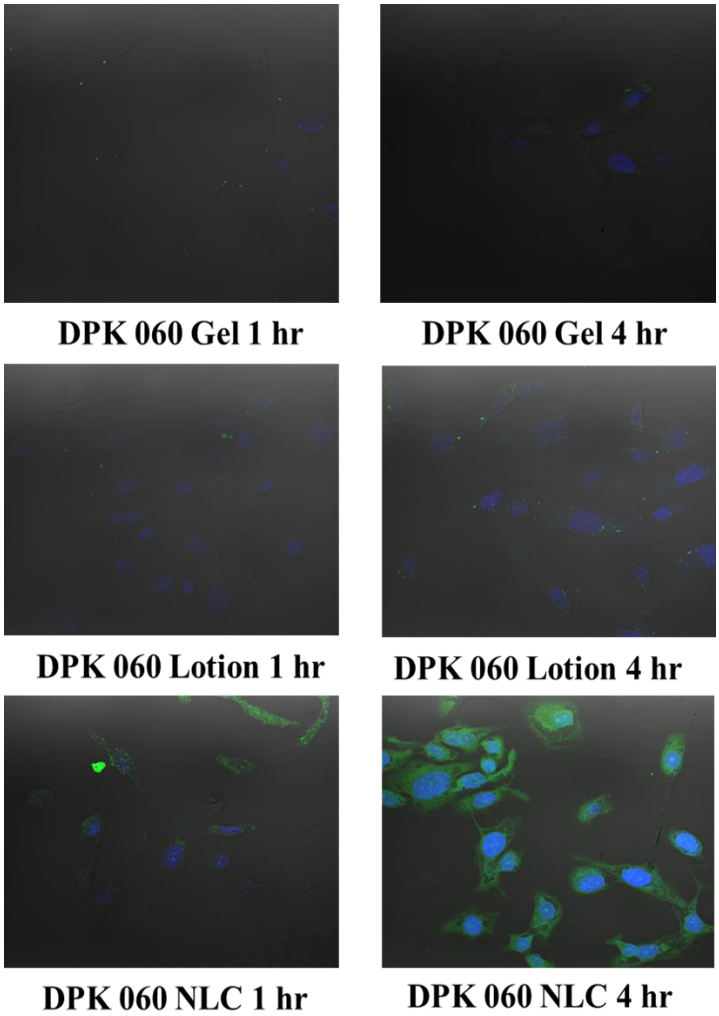


Figure 11 *In vitro* cell uptake of DPK 060 formulations in 3T3-fibroblast cells

Omiganan loaded formulations exhibited a MIC in the range of 12-16 µg/mL and 32-64 µg/mL against *S. aureus* and *C. albicans* respectively, in agreement to previously reported MICs. While DPK 060 loaded formulations exhibited a MIC in the range of 2-4 µg/mL and 128-160 µg/mL against *S. aureus* and *C. albicans* respectively, in agreement to previously reported MICs. The results indicate that both of these peptides have strong antibacterial activity against *S. aureus* while less potent to the strain of *C. albicans*.

The results of hemocompatibility study of Omiganan and DPK 060 loaded formulations demonstrated the compatibility of the Omiganan and DPK 060 loaded formulations with the RBCs as compared to the RBCs treated with Triton x-100 (*hemolytic* agent) showed the rupture/lysis of RBCs.

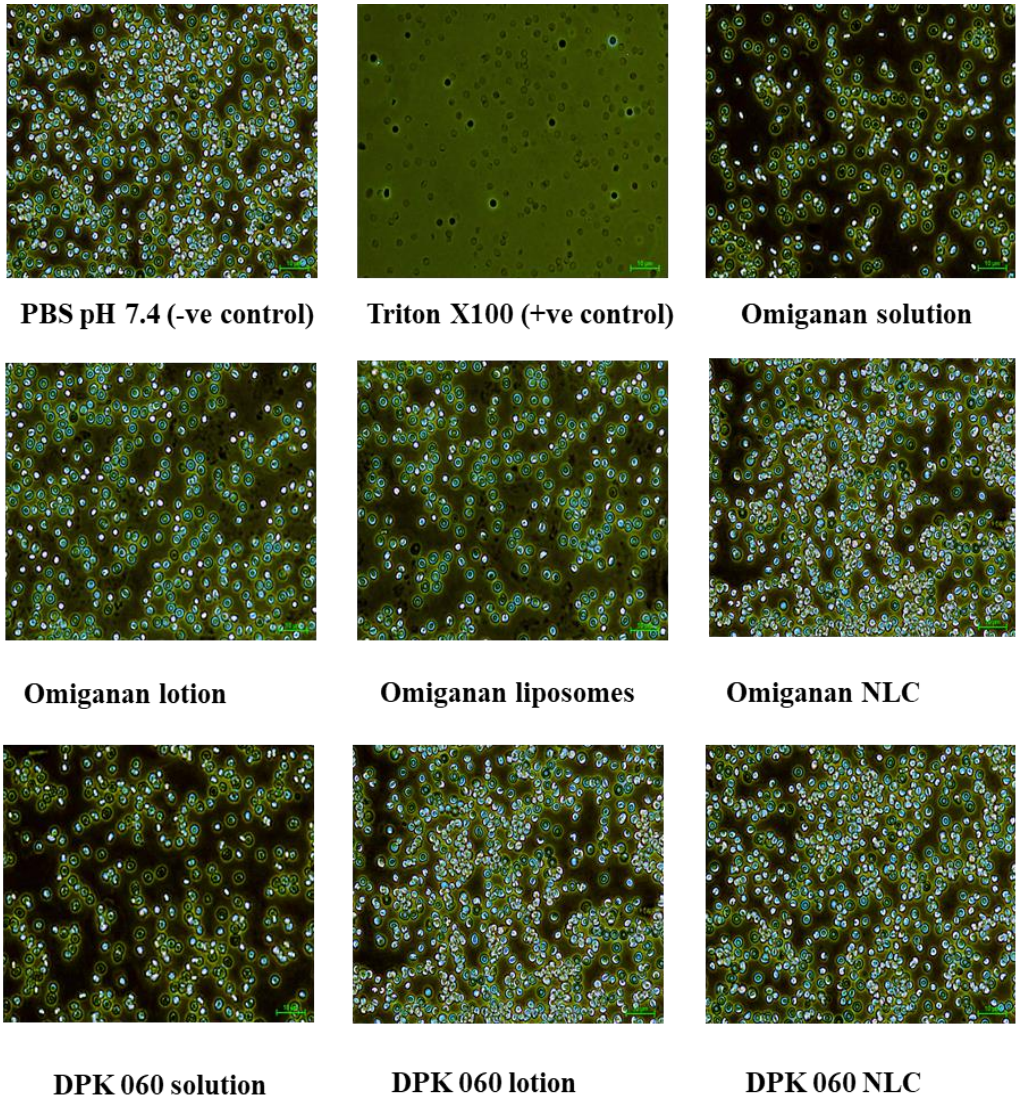


Figure 12 Results of hemocompatibility study of Omiganan & DPK 060 formulations

The kinetics of degradation by trypsin was studied for Omiganan and DPK 060 loaded formulations in order to assess the intrinsic susceptibility of Omiganan/DPK 060 at the predefined ratio. Omiganan (free drug solution and lotion) demonstrated moderate resistance (around 40 % Omiganan was degraded at 20 %w/w trypsin concentration) against the trypsin induced proteolytic degradation. However, the ability of the formulation to shield Omiganan seems to be high when the Omiganan was encapsulated/loaded in to the liposomes or NLCs (around 10 % Omiganan was degraded at 20 %w/w trypsin concentration). Whereas DPK-060 (free drug solution and lotion) showed high

susceptibility to trypsin in comparison with Omiganan. 5% w/w of trypsin was adequate for total degradation of the DPK 060 after 4 h of incubation. Whereas, DPK 060 loaded NLC showed a limited but noteworthy protection against trypsin in comparison with the DPK 060 solution and lotion.

The % cumulative amount of drug permeated from Omiganan and DPK 060 nano-formulations after 12 h were found to be 3 folds, respectively higher for NLC based gel compared to free drug gel and lotion. Additionally, Results demonstrated that around 87% of Omiganan was retained on the skin from the lotion and free Omiganan gel whereas around 60% of Omiganan was retained on the skin from the liposomes and NLCs gel. While for DPK 060, results demonstrated that around 89% of DPK 060 was retained on the skin from the lotion and free DPK 060 gel whereas around 68% of DPK 060 was retained on the skin NLCs gel.

Ex-vivo fluorescence microscopic results demonstrated nsegligible fluorescence in sections of skin treated with free Omiganan and DPK 060 gel and lotion. The formulations can be organized in an order of increasing fluorescence for Omiganan formulations: free Omiganan gel<Omiganan lotion<Omiganan liposomal/NLC gel. While for DPK 060 loaded formulations: free DPK 060 gel<DPK 060 lotion<DPK 060 NLC gel. The fluorescence microscopic data were found in-line with the ex vivo skin permeation and deposition data where maximum fluorescence was observed in section of skin treated with Omiganan and DPK 060 loaded NLC gel.

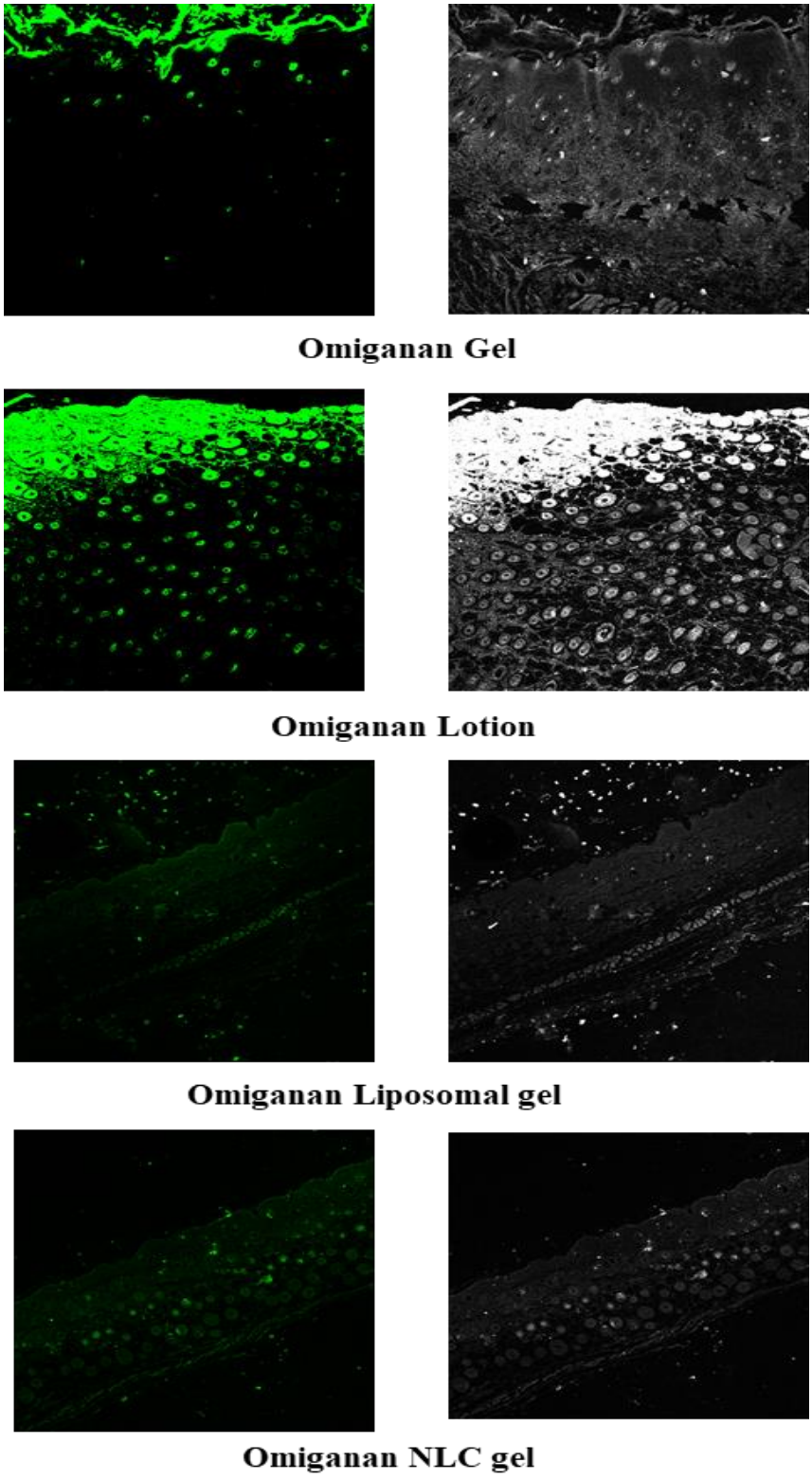


Figure 13 Fluorescence microscopic images of mice skin sections after 6 h of treatment with Omiganan formulations

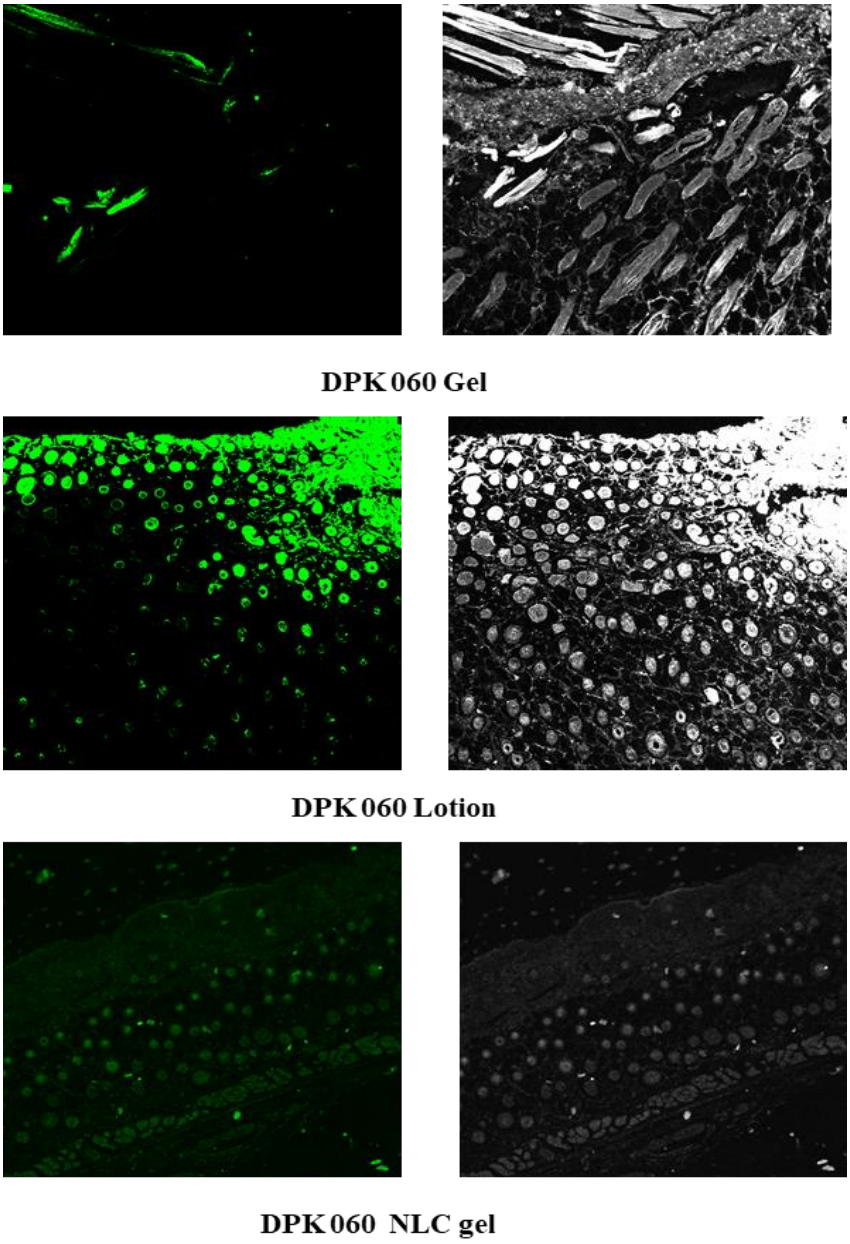


Figure 14 Fluorescence microscopic images of mice skin sections after 6 h of treatment with DPK 060 formulations

In-vivo studies

BALB/c mice (either sex) weighing 20-25 g were used for *in-vivo* pharmacodynamic study.

The effectiveness of the Omiganan loaded formulations was examined in an imiquimod (IMQ) induced psoriatic animal model in BALB/c mice. Omiganan liposomal

and NLC gel treated group demonstrated maximum decrease in epidermal thickening and skin inflammation with no scaly lesions in comparison to free Omiganan gel and lotion. 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, in comparison to a cumulative PASI score of 9.6 of model control group (IMQ only) on day 6. The spleen weights were significantly reduced 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. Moreover, the average body weight of animals in model control group was reduced by 18%. Whereas, treatment groups (Omiganan loaded liposomal and NLC gel), did not display any noteworthy change in body weight in comparison to standard control group. Omiganan liposomal/NLC gel showed marked reduction in epidermal thickening during histological examination with compared to model control and free Omiganan gel and lotion. After the treatment with Omiganan liposomal and NLC gel, substantial reduction in TNF-α levels (~81%) was observed as compared 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 the free Omiganan gel (~24%) and lotion (~31%).

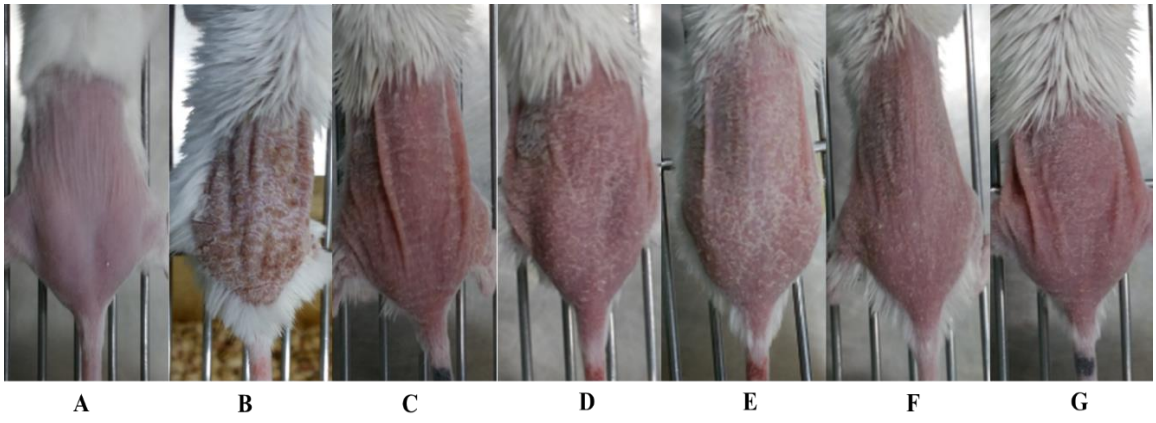


Figure 15 Visual analysis of improvement in psoriatic lesions after treatment; A) Normal control, B) Model control (Only Imiquimod), C) Standard control (Betamethasone

Dipropionate gel, Betagel), D) Free Omiganan gel, E) Omiganan lotion, F) Omiganan liposomal gel, G) Omiganan NLC gel

Figure 16 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)

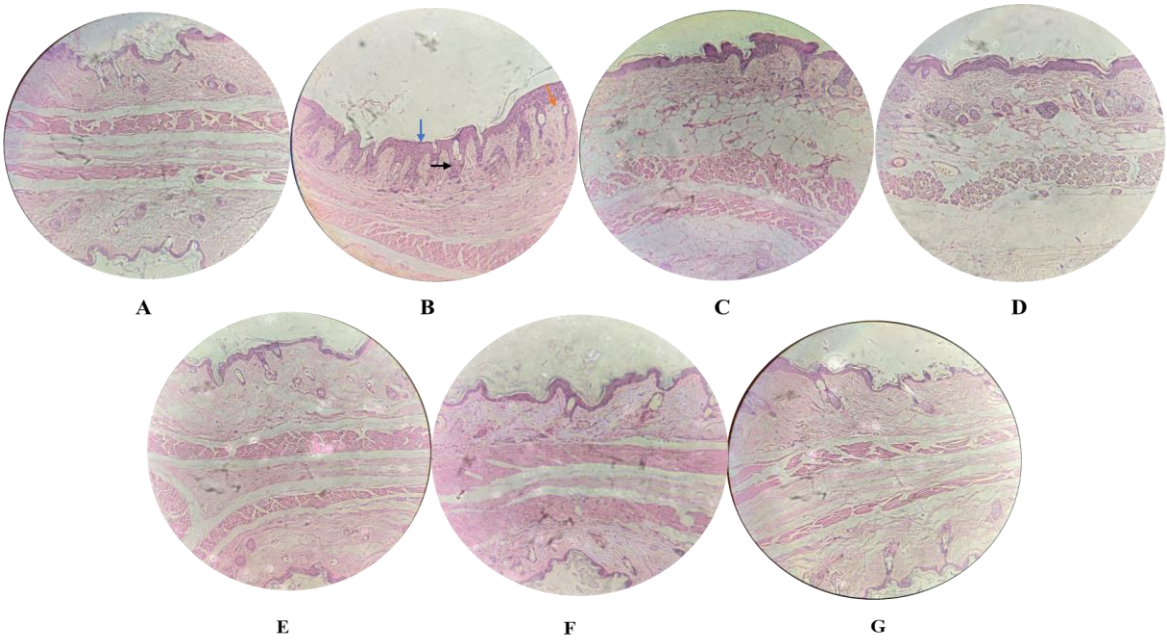
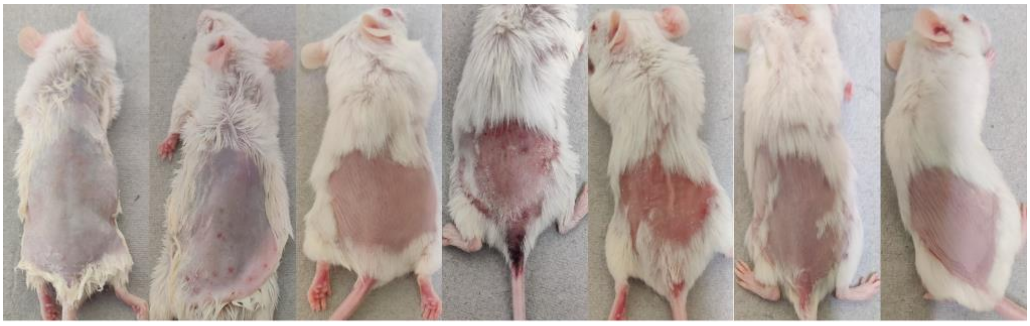


Figure 17 Histopathological images of mice skin for different animal groups; A) Normal control, B) Model control (Only Imiquimod); Blue arrow indicates hyperkeratosis, black arrow indicates acanthosis, Orange arrow indicates the presence of inflammatory cells, C) Free Omiganan gel, D) Omiganan lotion, E)

Standard control (Betamethasone Dipropionate gel, Betagel), F) Omiganan liposomal gel, G) Omiganan NLC gel

The efficacy of the Omiganan and DPK 060 loaded formulations were evaluated in OVA induced eczema/atopic dermatitis animal model in BALB/c mice. Omiganan liposomal and NLC gel treated group demonstrated maximum decrease in epidermal thickening and skin inflammation in comparison to model control and free Omiganan gel and lotion. Similarly, DPK-060 loaded NLC gel also demonstrated reduction in skin inflammation and epidermal thickening compared to model control and free DPK-060 gel and lotion. In histopathological evaluation, the mice treated with Omiganan liposomal/NLC and DPK-060 NLC gel clearly 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. Additionally, after the treatment with nanocarrier based gel of Omiganan and DPK-060 (liposomal/NLC gel), 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 nanocarrier based gel of Omiganan and DPK-060 (liposomal/NLC gel) in comparison with free Omiganan/DPK-060 gel (~21%) and lotion (~28%). Additionally, IL-6 levels (~86%) were also decrease markedly in animals treated with nanocarrier based gel of Omiganan and DPK-060 (liposomal/NLC gel) compared to the free Omiganan/DPK-060 gel (~21%) and lotion (~26%).



A B C D E F G

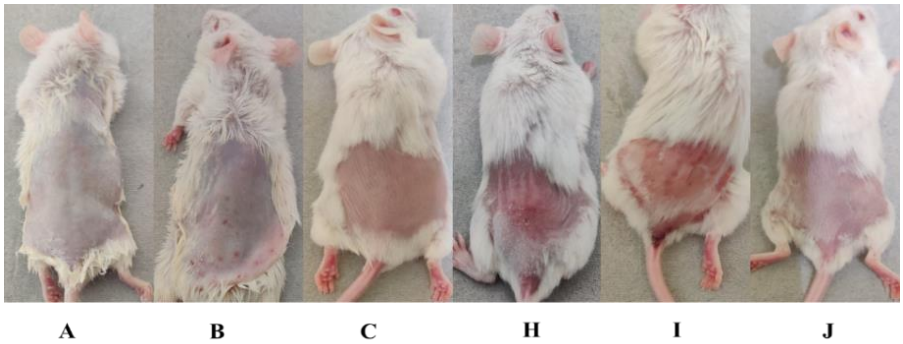


Figure 18 Visual analysis of improvement in eczematic lesions after treatment; A) Normal control, B) Model control (Only Ovalbumin), C) Standard control (Betamethasone Dipropionate gel, Betagel), D) Free Omiganan gel, E) Omiganan lotion, F) Omiganan liposomal gel, G) Omiganan NLC gel, H) Free DPK-060 gel, I) DPK-060 lotion, J) DPK-060 NLC gel

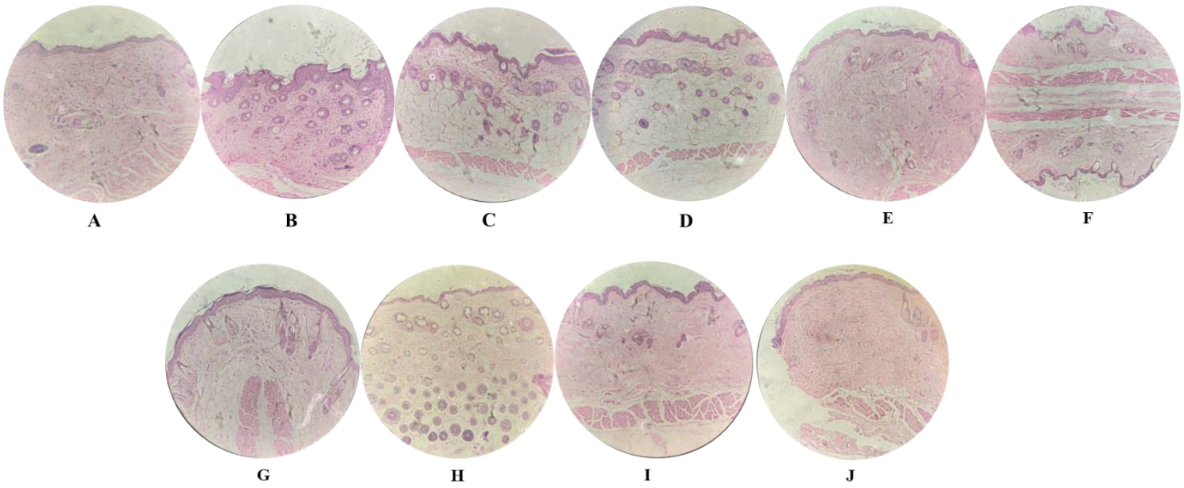


Figure 19 Histopathological images of mice skin for different animal groups; A) Normal control, B) Model control (Only Ovalbumin, C) Free Omiganan gel, D) Omiganan lotion, E) Omiganan Liposomal gel, F) Omiganan NLC gel, G) Standard control (Betamethasone Dipropionate gel, Betagel), H) Free DPK-060 gel, I) DPK-060 lotion, J) DPK-060 NLC gel

Conclusion

The aim of this study was to overcome the challenges associated with dermal delivery of Omiganan and DPK-060 i.e., proteolytic degradation, poor permeation profile, twice-a-day application, etc. via development and characterization of their suitable dermal formulations. The nanocarrier based formulations of Omiganan and DPK-060 were successfully prepared and optimized to have maximum drug entrapment and minimum particle/vesicle size. The *in-vitro* characterization revealed uniform size distribution, spherical shape and favorable zeta potential, pH, viscosity and spreadability along with the prolonged release profile and higher cellular uptake. Additionally, Omiganan and DPK-060 loaded nanocarrier gel-based formulations were demonstrated potent antibacterial activity and resistance to proteolytic degradation. These formulations have no cytotoxic potential and are hemocompatible. *Ex-vivo* study revealed enhanced permeation of Omiganan and DPK-060 loaded nanocarrier gel-based formulations. Moreover, the results of pharmacodynamic studies demonstrated strong anti-psoriatic potential of Omiganan loaded nanocarrier gel-based formulations in Imiquimod induced psoriatic animal model. While, strong efficacy was also observed in Ovalbumin induced atopic dermatitis/eczema animal mode by both Omiganan and DPK-060 nanocarrier gel-based formulations.

In a nutshell, the optimized nanocarrier gel-based formulations of Omiganan and DPK-060 were found to possess potent antibacterial and anti-inflammatory activities along with quality attributes in desired range as defined in QTPP and therefore seems suitable for once daily application in psoriasis or eczema.