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Evaluation of in-Vivo Antipsoriatic activity of Tacrolimus and Thymoquinone Co-Loaded Nanostructured Lipid carriers containing Gel

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ABSTRACT

Psoriasis is a chronic inflammatory skin condition requiring effective and targeted treatments. This study evaluates the in-vivo antipsoriatic activity of a gel formulation containing tacrolimus and thymoquinone co-loaded nanostructured lipid carriers (NLCs). The NLC system enhances drug stability, permeation, and skin retention, addressing limitations of conventional therapies. The gel was tested using an imiquimod-induced psoriasis model in mice, demonstrating significant reduction in erythema, scaling, and epidermal thickness compared to control treatments. These findings highlight the therapeutic potential of this innovative NLC-based gel for psoriasis management.

Keywords: Psoriasis, Tacrolimus, Thymoquinone, Nanostructured lipid carriers, Topical gel, In-vivo study

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INTRODUCTION

Psoriasis is a chronic, immune-mediated skin disorder characterized by hyperproliferation of keratinocytes, inflammation, and abnormal differentiation of epidermal cells. Affecting approximately 2–3% of the global population, it significantly impacts patients' quality of life [1, 2]. Topical therapies, including corticosteroids and calcineurin inhibitors like tacrolimus, are often first-line treatments due to their targeted action and reduced systemic side effects [3, 4].

Tacrolimus, a macrolide immunosuppressant, effectively suppresses T-cell activation and cytokine release but is limited by poor skin penetration and potential photodegradation [5]. Thymoquinone (TQ), derived from Nigella sativa, possesses potent anti-inflammatory and antioxidant properties, complementing tacrolimus in managing psoriasis [6–8]. However, its therapeutic efficacy is hindered by low bioavailability and instability [9].

Nanostructured lipid carriers (NLCs) have emerged as a promising drug delivery platform to enhance the stability, permeability, and therapeutic efficacy of bioactive agents. By encapsulating lipophilic and hydrophilic compounds, NLCs overcome solubility challenges, prolong drug release, and improve skin retention [10–12]. Incorporating tacrolimus and thymoquinone into NLC-based gels offers synergistic benefits, optimizing their therapeutic impact while minimizing systemic absorption and side effects [13-15].

This study evaluates the in-vivo antipsoriatic activity of a tacrolimus and thymoquinone co-loaded NLC gel, providing a novel therapeutic approach for effective psoriasis management.

MATERIALS AND METHODS

Preparation of tacrolimus and thymoquinone co-loaded nanostructured lipid carriers-based gel (THQ-NG)

The application of Tacrolimus and thymoquinone co-loaded nanostructured lipid carriers (THQ- NLCs) on the skin is difficult due to their insufficient viscosity. Therefore, the optimized THQ-NLCs were converted into gel with the addition of a gelling agent (Carbopol Ultrez 10; 1% w/w) under constant stirring by placing on a magnetic stirrer maintained at 1000 rpm until the gelling agent fully dispersed. Thereafter, the resulting mixture was left overnight for swelling, followed by

neutralization with triethanolamine addition (0.05% w/w) added dropwise to convert it into a THQ-NLC-gel (THQ-NG). A similar procedure was followed to prepare the THQ-suspension-gel (THQ-SG) (Iqubal et al., 2021).

In-Vivo Antipsoriatic Activity

This study was conducted on imiquimod (IMQ) induced psoriatic *Balb/c* mice to assess the efficacy of THQ-NG as per the reported method (Ghate et al., 2019, Na Takuathung et al., 2018). Initially, the hair from the dorsal skin of animals was re- moved using depilatory cream and examined for any signs of inflammation. Psoriasis- like symptoms were induced in mice by applying 5% IMQ cream at a daily dose of 62.5 mg per mouse for a continuous period of seven days. Mice were monitored daily to visualize the development of psoriatic lesion. Animals were randomly divided into 6 groups, each group containing 4 mice: Where, Group 1, serving as the nor- mal control; Group 2 was subjected to psoriasis induction but left untreated (Toxic); Group 3 was treated with THQ-SG; Group 4 was treated with Group 5 was treated with THQ-NG; and Group 6 was treated with THQ-NG. Then, different formulations at a dosage of 40 mg/cm² (containing 0.1% TAC and 0.3% THQ) were applied to the psoriatic skin using a spatula, and this treatment continued for next seven days. After the seventh day of treatment, animals of different groups were assessed visually for signs of erythema, scaling and thickness and then sacrificed. Thereafter skin, spleen, and liver were removed and preserved in formalin (10% v/v) for histological examinations.

Psoriasis Area and Severity Index (PASI) Scoring

The psoriasis-inflammation evaluation criteria were assessed according to the PASI scoring. Evaluations was conducted to assess and assign scores on a scale from 0 to 4 for erythema, scaling, and thickening. This scale ranges from absence (0) to various levels of severity (1: slight, 2: moderate, 3: marked, 4: severe) for each of these skin characteristics. Then the cumulative PASI score was calculated, which combines the individual scores for erythema, scaling, and thickening. It serves as the definitive indicator of the severity of inflammation, providing a measure of the extent of psoriasis (Pivetta et al., 2018).

Body Weight, Spleen Dimension & weight

Body weight of all the groups were measured initially, after induction of disease and after the end of treatment period. In inflammatory diseases such as psoriasis, the spleen often undergoes an enlargement in size, a response associated with high cytokine production. Hence, the dimension and weight of the spleen were measured (Sahu et al., 2018).

Enzyme-Linked Immunosorbent Assay for TNF-α & IL-6

ELISA experiments were done on skin samples obtained from various treatment groups to quantitatively assess the levels of TNF-α and IL-6 as inflammatory cytokine markers. The skin tissues were chopped and homogenized in phosphate saline buffer solution (pH 7.4) at 3000 rpm using a tissue homogenizer (Remi Elektrotechnik Ltd., Mumbai, India), followed by centrifugation at 3000 rpm for a period of 15 min (Iqbal et al., 2019). The resulting supernatants were separated and stored at a temperature of –80°C until analysis. Finally, the concentrations of TNF-α and IL-6 was measured using kits from Mouse ELISA (Krishgen biosystems, CA, USA) as per the protocol provided by manufacturer.

Histological Assessment of Skin, Spleen, and Liver

In the end, mice were euthanized by CO2 inhalation and the skin excised from their dorsal area, as well as spleen and liver were collected from all experimental groups. These samples were subsequently preserved in formalin (10% v/v), embedded in paraffin, and subjected to Hematoxylin and Eosin staining (H&E) (Khan et al., 2023). Sections measuring 5 µm in thickness were prepared and used for histological analysis. The histological examination of the skin, spleen, and liver sections was conducted under a light microscope

***In-Vivo* Skin Compliance Study**

Firstly, the Balb/c mice were divided into three groups (n = 4). Group 1 served as the control, where no formulation was applied. Group 2 received the application of THQ- NG, while Group 3 was subjected to the application of THQ-SG formulations over a span

of 7 days. The animals underwent daily visual inspections to check for the presence of erythema or edema 24 h after the application of a treatment. Once the treatment period was completed, their observations were recorded and evaluated using a scoring system that ranged from 0 to 4. This scale encompasses categories for evaluating erythema, where 0 signifies the absence of erythema, whereas 1, 2, 3, 4 denoted slight, moderate, marked and severe erythema respectively. Additionally, the scale also included categories for edema, with 0 representing the absence of edema, whereas 1, 2, 3, 4 signifies slight, moderate, marked and severe edema respectively (Mittal et al., 2021).

RESULTS AND DISCUSSION

In-Vivo Antipsoriatic Activity

Psoriasis, an inflammatory skin condition, typically presents as plaques characterized by prominent erythema, scaling, and thickening of skin. An ideal animal model for evaluating anti-psoriatic treatments should replicate both the phenotypic features of the disease and its biochemical context. In this particular investigation, psoriasis was induced by applying IMQ topically for a continuous period of seven days. IMQ, a ligand for Toll-Like receptors (TLR), can induce psoriasis-like lesions on the skin of mice byaffecting the IL-23/IL-17 axis (Van Der Fits et al., 2009). The daily application of IMQon the dorsal area of mice resulted in the emergence of skin abnormalities in the form of inflamed, scaly skin lesion characterized by increased epidermal growth, aberrant cell maturation, the accumulation of neutrophils, the formation of new blood vessels, and the infiltration of cells that mediate immune responses. These manifestations closely mirrored the characteristics of plaque-type psoriasis (Jabeen et al., 2020). The therapeutic effectiveness of THQ-NG was assessed in a research model that induced psoriatic plaques through IMQ application and subsequently the treatment was compared to other treatment groups employing different drugs treatments. The visual representation of animals in various experimental groups after the end of the treatment period is illustrated in **Figure 1.**

Figure 1: The visual representation of animals in various experimental groupsafter the end of the treatment period.

Psoriasis area and severity index (PASI) Scoring

The IMQ-induced psoriasis-like inflammation was evaluated using the PASI scoring, an extensively utilized clinical tool to gauge the severity of psoriasis and evaluate the effectiveness of anti-psoriatic therapies. In our *in-vivo* experiments, we scored the severity of the lesion by visually evaluating the redness, thickness, and scaling of the skin inflammation. Following the initiation of IMQ treatment, mice exhibited initial signs of mild thickening, erythema, and scaling on their dorsal skin, which became apparent on days 2–3. The PASI score reached its peak on day 7. The cumulative PASIscore was determined by summing the scores for erythema, thickness, and scaling of inflammation, each rated on a scale from 0 to 4. The PASI score for erythema, thickness and scaling of inflammation as wells as cumulative score on a scale from (0-4), for all treated groups are shown in **Figure 2.** On the 7^{th} day of IMQ application, all treatment groups displayed scaling, thickness, and erythema with a PASI score falling in the range of 3–4, indicating the development of severe inflammation. In contrast to the toxic control group, all drug treatment groups showed significant reduction in PASIscore of scaling, thickness, and erythema but maximum reduction was observed with THQ-NG ($p<0.0001$) Also as compared to THQ-SG, THQ-NG showed 2.0-, 4.5- and 1.67-fold higher reduction in PASI score of thickness, erythema, and scaling respectively.

Furthermore, **Table 1** presents the comparison between the percentage reduction in the cumulative PASI score after the completion of drug treatment and the cumulative PASI score observed following the onset of the disease. Reduction in the cumulative PASI score represented the healing potential of different formulations. In IMQ treated group, slight increase (2.34%) in cumulative PASI score was observed after induction of disease. In contrast to IMQ treated group,

drug treated groups (THQ-SG, THQ-NG,) showed significant reduction in cumulative PASI score (expressed in %) with maximum reduction with THQ-NG. After the end of treatment, THQ-SG (57.14 %), THQ-NG (39.18 %) showed reduction in cumulative PASI score. The higher % cumulative PASI score reduction of THQ-NG as compared to single drug nanogel formulation (THQ-NG), confirms the enhanced efficacy of THQ, when used in combination. The enhanced efficacy of THQ-NG is attributed to the higher skin penetration and drug retention due to the small size and occlusive characteristics of the formulation. Based on our analysis of PASI scores across all groups, we can infer that the developed formulation, THQ-NG, shows greater promise in terms of both efficacyand safety when compared to THQ-SG.

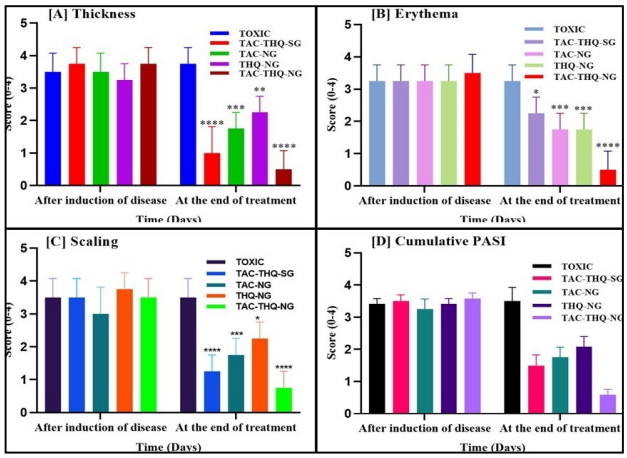

Figure 2: *Images showing effectiveness of different treatment groups on IMQ induced psoriasis based on thickness, erythema, and scaling of skin recorded after induction of disease and end of treatment, on a PASI scale of 0 to 4. [A] Thickness; [B] Erythema; [C] Scaling and [D] Cumulative PASI score.*

Table 1: % Reduction in cumulative PASI of different groups at the end oftreatments.

Treatments	Cumulative PASI score		% Reduction in PASI score
	After disease induction	After end of treatment	
TOXIC	3.42 ± 0.17	3.50 ± 0.43	-2.34
THQ-SG	3.50 ± 0.19	1.50 ± 0.33	57.14
THQ-NG	3.42 ± 0.16	2.08 ± 0.32	39.18

Body weight, Spleen Dimension & Weight

Body weight measurements were recorded for all groups during the treatment period, and changes in body weight relative to the initial body weight were plotted over time, as illustrated in **Figure 3A.** The control group exhibited no significant alterations in body weight throughout the study. While the toxic group displayed a noticeable change in the average body weight of the animals. During the study, there was a decrease in body weight among the drug treatment groups, which included THQ-SG, THQ-NG THQ-NG. However, this decrease in body weight was not statistically significant. Notably, the group labelled THQ-NG showed the least amount of body weight reduction. Spleen enlargement serves as a significant indicator of immunologicaldisorders. The application of IMQ led to the development of an inflammatory condition, resulting in a substantial increase in spleen size and weight due to the release of inflammatory cytokines (Shinno- Hashimoto et al., 2022). The results concerning spleen dimensions and weight are presented in **Figure 3B**, while the specific spleen weights are displayed in **Figure 3C.** In the toxic group, an increase in spleen weight was observed, indicative of the induction of psoriasis following IMQ application. The toxic group exhibited an average spleen weight of 380 ± 17.05 mg. In the drug-treated groups (THQ-SG,THQ-NG), the average spleen weights were measured at 225.5 ± 11.47, 260 ± 12.36, respectively. Notably, the spleen weight of the THQ-NG group closely resembled that of the normal control ($p > 0.05$), with an average weight of 166.75 ± 9.71 mg. Moreover, the induction of psoriasis following IMQ application was confirmed by evaluating the spleen to body weight ratio (SBWR) in mice as reported by Sathe et al., 2019. The SBWR values for various treatment groups were assessed at the conclusion of the experiment, and depicted in **Figure 3D.** In the toxic group, a substantial increase in SBWR was observed compared to the normal group, signifying the successful development of disease. Conversely, the different drug treatment groups yielded a reduction in SBWR, with maximum reduction observed for THQ-NG.

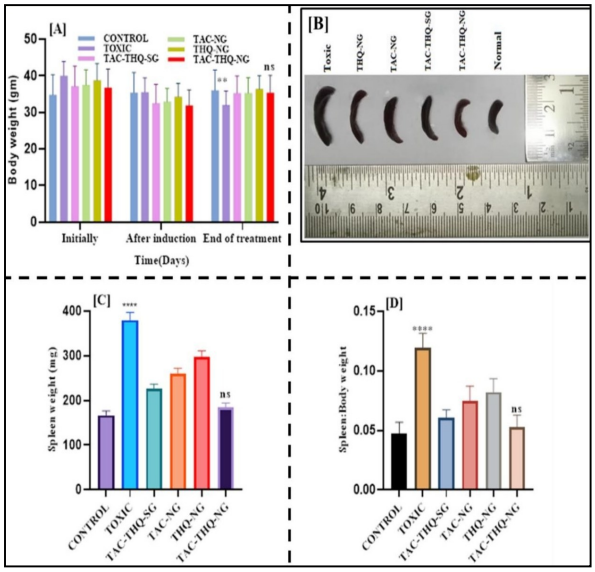

Figure 3: Image showing effect of different formulations on [A] Body weight [B];spleen dimensions [C]; Spleen weight and [D] Spleen: body weight ration of different treatment groups.

Enzyme-Linked Immunosorbent Assay for TNF-α & IL-6

Among the array of immune cytokines, TNF-α and IL-6 are pivotal pro-inflammatory cytokines known for their significant roles in triggering the onset of psoriasis when stimulated by the application of IMQ (Arora et al., 2017). Therefore, a comparative anti-inflammatory efficacy of different drug treated groups was determined. The changes in levels of TNF-α and IL-6 observed in skin homogenate of treated animals belonging to different groups are illustrated in **Figure 4 and Table 2.** As compared to normal group, 3.25 and 3.74-folds increase in levels of TNF-α and IL-6 respectively was observed in case of IMQ treated group (toxic) indicating induction of psoriasis. Following drug treatment, all drug treatment groups revealed a marked decrease in levels of TNF- α and IL-6 levels in contrast with the IMQ-treated group. Further, the level of TNF-α and IL-6 in the THQ-NG treated group was non- significant, when compared to the normal group (without any treatment). There was 2.76 and 2.99 times decrease in TNF-α and IL-6 levels for the THQ-NG treated animalsin comparison to the IMQ-treated group. Furthermore, the THQ-SG group showed 1.84 and 1.97 times decrease in TNF-α and IL-6 levels in comparison to the IMQ-treated group. The higher reduction in cytokines levels on topical treatment with THQ-NG was attributed to higher skin penetration and drug retention. The higher reduction potentialof THQ-NG as compared to single drug NLC-gel for TNF-α and IL-6 level, confirmed the enhanced efficacy of THQ when used in combination. Thus, the study substantiated the enhanced efficacy of nanogel combination (THQ-NG) when compared to their conventional gel (THQ-SG) and single drug-containing nanogel (THQ-NG).

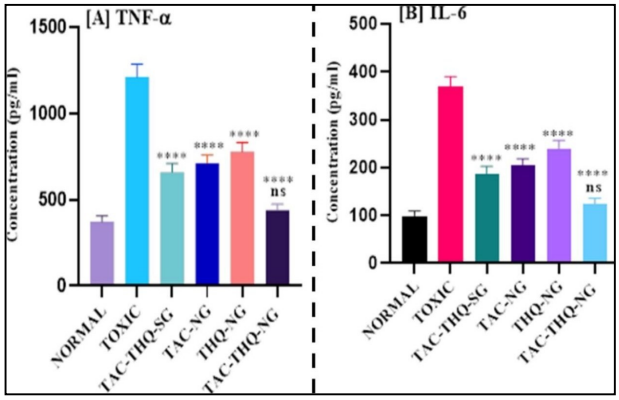

Figure 4: Image showing effect of different treatment groups on inflammatorycytokines levels [A] TNF-α and [B] IL-6 on IMQ induced psoriasis on mice skin.

Table 2: Inflammatory cytokines levels in different animal groups after end oftreatment

Treatment groups	TNF-α (pg/mL ± SD)	IL-6 (pg/mL ± SD)
Normal	373.21 ± 32.79	99 ± 10.41
Toxic	1211.67 ± 75.26	370.25 ± 20.15
THQ-SG	659.74 ± 50.42	187.75 ± 14.93
THQ-NG	777.69 ± 54.03	240.25 ± 17.02

Histological Assessment of Skin, Spleen, and Liver

The H&E-stained histological micrographs of the skin, spleen, and the liver of different treatment groups of Balb/c mice are represented in **Figure 3.** Histologically, normal mouse skin typically displays a well-structured epidermis and dermis, featuring thin layers of the epidermis and normal SC. While, IMQ treated (toxic group) skin displays noticeable epidermal thickening, elongation of epidermal rete ridges, disrupted epidermal differentiation, leukocyte infiltration, and a lack of a granular layer when compared to normal skin (Desai et al., 2013). All the drug treatment groups exhibited a reduction in epidermal thickness and normalized keratinocyte differentiation, with an intact granular layer. However, the group treated with THQ-NG demonstrated the highest efficacy in combating the inflammation induced

by IMQ, when compared to the other groups receiving different treatments. In the histological examination of the spleen, it was evident that the IMQ treatment group represents the rupture of spleen cells due to spleen enlargement, leading to an unclear distinction between the red pulp(appearing pink) and white pulp (appearing blue) regions in the spleen. Conversely, in the normal group (without IMQ treatment), both red pulp and white pulp were clearly distinguishable. Notably, treatment with THQ-NG exhibited the most significant improvement in the restoration of both red pulp and white pulp areas of the spleen in contrast to other drug treatment groups (Kaur et al., 2017). The histological examination of the liver revealed that the group treated with IMQ experienced damage to liver cells (hepatocyte degeneration), fibrotic changes, pyknosis and cellular disintegration. In contrast, the normal group (without IMQ treatment) exhibited no alterations in hepatocyte density. All the groups treated with drugs demonstrated a reduction in hepatocyte degeneration. Notably, animals treated with THQ-NG experienced the most significant improvement in hepatocyte regeneration compared to other drug treatment groups (Pukale et al., 2020).

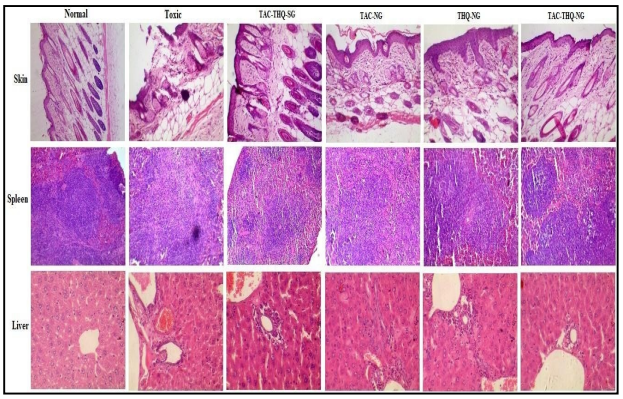

Figure 3: The histological visual representation of skin, spleen and liver ofdifferent treatment groups of Balb/c mice after end of treatment period.

In-vivo Skin Compliance Study

Topical therapy associated with adverse effect like skin irritation, specifically erythema, considerably restricts its clinical application. This challenge is exacerbated by the fact that many conventional dosage forms, including creams, lotions, and gels, often fail to mitigate the irritation caused by topical application. A hypothesis was formulated that the encapsulation of drugs within NLCs could avoid drug contact withthe SC, potentially leading to a reduction in erythema episodes (Tripathi et al., 2018). Also, marketed formulation is associated with skin irritation (skin burning, itching) when used topically (Jain et al., 2019). Therefore, the skin compliance studies of THQ- NG and THQ-SG on healthy mice skin was done to analyze the compatibility of the developed formulation with skin. The irritation potential was assessed on the basis of scores attained for erythema and edema for both formulations as represented in **Table 3.** The application of THQ-NG did not show any sign of erythema and edema while in the case of THQ-SG, slight erythema was observed which was due to TAC in its free form. This suggests that the excipients utilized in the NLCs-gel, as well as the encapsulation of drugs within the NLCs, do not pose an irritation risk. The individual primary irritancy index (PII) for THQ-NG and THQ-SG was 0.0 and 1.25 ± 0.50 respectively. The skin compliance study indicated that TAC-THQ-NG exhibited no signs of erythema and edema, even after repeated application for 7 days as compared to THQ-SG which showed a sign of slight erythema. Therefore, it can be inferred that THQ-NG is safe for topical application.

Table 3: Skin compliance score of mice of different group treated with THQ-NG and THQ-SG.

Animals (Balb/c)	Control		THQ-SG		THQ-NG	
	Erythema	Edema	Erythema	Edema	Erythema	Edema
1	0	0	1	0	0	0
2	0	0	2	0	0	0
3	0	0	1	0	0	0
4	0	0	1	0	0	0
Score ± SD	0 ± 0.0	0 ± 0.0	1.25 ± 0.50	0 ± 0.0	0 ± 0.0	0 ± 0.0
PII	0		1.25		0	

Erythema or edema scale: 0, No erythema/edema; 1, slight; 2, moderate; 3, marked; 4,severe

CONCLUSION

The *in-vivo* anti-psoriatic efficacy was assessed in a research model that induced psoriasis in Balb/c mice through IMQ application. The IMQ-induced psoriasis-like inflammation was evaluated using the PASI scoring an extensively utilized clinical tool to evaluate the effectiveness of anti-psoriatic therapies. In contrast to the toxic group, all drug treatment groups showed significant reduction in PASI score of scaling, thickness,

and erythema but maxi- mum reduction was observed with THQ-NG. THQ-NG showed 2.0, 4.5-fold higher reductions in PASI score of thickness, erythema, and scaling respectively as compared to THQ-SG. Further, THQ- NG showed significant reduction (83.80%) in % cumulative PASI score. Therefore, based on PASI scoring it was concluded that THQ-NG showed improved efficacy as compared to THQ-SG and other treatment groups. The study showed decrease in body weight, which was not statistically significant, with least decrease in BW observed with THQ-NG. The spleen size and weight of THQ-NG treated group closely resembled to the normal control group. Further, THQ-NG treated groups yielded a maxi- mum reduction in SBWR and cytokines levels (TNF-αand IL-6). Based on above findings, we can infer that the THQ-NG showed greater promise in terms of both efficacy and safety as compared to TAC-THQ-SG and other drug treatment groups. Based on the histological examination of different organs (skin, spleen and liver), it can be concluded that THQ-NG is safe and effective for topical application. The *in-vivo* skin compliance study indicated that THQ-NG is safe for topical application as compared to THQ-SG which showed a sign of slight erythema.

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