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Clobetasol Propionate and Pramoxine Hydrochloride Loaded Nanolipid Carrier Gel for the Treatment of Atopic Dermatitis

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ABSTRACT

Aim: To prepare and evaluate Clobetasol Propionate (CP) and Pramoxine Hydrochloride (PH) loaded nanolipid carrier (NLC) based gel for improved skin permeation for the treatment of Atopic Dermatitis.

Methodology: CP and PH loaded NLCs were prepared by the melt emulsification ultra-sonication technique. *In vitro, ex vivo,* and *in vivo* studies in the atopic dermatitis animal model of formulated drug-loaded NLC (DNLC) gel were evaluated. Dermal pharmacokinetic parameters were evaluated by Phoenix WinNonlin software.

Results and discussions: DNLC gel was prepared successfully. Skin permeation and retentive property of DNLC gel showed that CP from DNLC had a permeability flux of 5.88 µg/cm2/h and an enhancement ratio of 1.92 compared to CP drug solution, while PH from DNLC gel had a permeability flux of 9.52 µg/cm2/h and an enhancement ratio of 1.62 compared to PH drug solution. Dermal pharmacokinetic parameters were determined using WinNonlin software. CP retained at 24h in stratum corneum, epidermis, and dermis was 4.25 ± 0.02µg, 75.77 ± 0.01µg, and 32.04±0.012µg, respectively, while PH retained at 24h was 11.82 ± 0.003µg, 344.0± 0.05µg, and 172.85± 0.040µg, respectively. More CP was retained from the DNLC gel than marketed CP cream. In vivo animal studies confirmed DNLC gel's effectiveness in treating atopic dermatitis compared to commercial cream and individual drug-loaded DNLC gel, decreasing induced disease on par with marketed CP cream. Epidermal thickness, Immunoglobin E (IgE), and Absolute Eosinophil Count (AEC) showed the greatest reduction in the DNLC gel treatment group.

1.0 INTRODUCTION:

Atopic dermatitis (AD) is a chronic relapsing skin disease characterized by severe itching, dry skin, and local skin inflammation affecting 15-20% of children and 1-3% of adults worldwide. The disease has a distinct acute and chronic phase that differs in histological and immunological profile [1]. The prevalence of eczema and other allergic symptoms is increasing globally, especially among children, making AD a significant global concern [2]. The condition often relapses and requires long-term management, significantly impacting the quality of life of individuals [3].

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Notably, AD frequently marks the beginning of the atopic march, causing patients to be more susceptible to developing allergic rhinitis and asthma due to immune sensitization and impaired skin barrier [4]. AD is influenced by genetic, immunologic, and environmental factors, making it a complex pathological condition that requires timely intervention to prevent further progression. Understanding its complex pathophysiology opens new avenues for treatment and management of the condition [5]. Innovative topical treatments, such as silver-nanolipid complexes, have shown promise by combining antimicrobial action with barrier-restoring properties [6]. Microemulsion-based gels and nanoparticles have demonstrated improved delivery and efficacy of corticosteroids like clobetasol propionate, offering potential for enhanced treatment outcomes [7,8,9]. The heterogeneous signs and triggers of AD underscore the need for personalized management strategies and proactive treatment approaches [10,11].

Topical calcineurin inhibitors and corticosteroids are part of current therapeutic options; however, their use has been limited due to various drawbacks, such as burning sensation, redness, and allergic responses. Additionally, the main concern has been systemic absorption, which can lead to breathing difficulties, facial swelling, and a systemic immune-suppressive impact, all of which increase the risk of malignancies [12, 13]. Menthol, pramoxine, and capsaicin are compounds that function as local numbing medications to temporarily block the transmission of itch signals from the skin to the central nervous system [14]. Pramoxine belongs to the class of drugs known as local anesthetics and works by numbing the skin to block pain and itch sensations [15].

The use of permeation enhancers in topical vehicle systems improves medication penetration over the skin; however, the use of such chemical enhancers may be detrimental, as many are irritants [16]. As a result, a topical carrier free of chemical enhancers to aid medication absorption through the skin is preferred. Colloidal medication delivery techniques are projected to outperform traditional topical administration systems for Clobetasol propionate (CP). Solid lipid nanoparticles (SLNs), microspheres, lipid nano carriers, lecithin/chitosan nanoparticles, and microparticulate drug delivery systems have all been designed and tested for the topical administration of CP [16,17,18]. The use of permeation enhancers in topical vehicle systems enhances drug permeation across the skin, but the employment of such chemical enhancers could be harmful, as many of them are irritants [16]. Therefore, a topical vehicle without chemical enhancers to facilitate drug permeation through the skin is desirable. Colloidal drug delivery systems are expected to be a

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better alternative for conventional topical delivery systems of Clobetasol propionate (CP). Delivery systems such as solid lipid nanoparticles (SLNs), microspheres, lipid nano carriers, lecithin/chitosan nanoparticles, and microparticulate drug delivery systems have been developed and evaluated for the topical delivery of CP [16,17,18]. They offer many advantages, such as not requiring organic solvents during preparation, stability against drug hydrolysis, and relative stability during storage. However, due to their rigid structure, erratic gelation propensity, particle development, and unexpected polymeric transitions, the potential of solid-lipid nanocarriers as drug carriers was restricted by drug expulsion during storage. Nanostructured lipid carriers were developed to address the challenges with solid lipid nanoparticles. At normal temperatures, the NLCs remain solid, even when a significant amount of liquid lipids is present. Compared to SLNs, the combination of solid and liquid lipids creates a less structured matrix with greater imperfections that can accommodate more drug molecules and achieve higher entrapment efficiency. Additional advantages over SLNs include reduced toxicity, medication protection, and a higher level of stability during storage. NLCs are less prone to have unexpected gelation due to their reduced water content, which is a key concern with SLNs [19]. Nanolipid carriers' ability to exchange lipids with the outermost layer of the stratum corneum after adhering to the skin makes them an attractive choice for topical drug delivery. NLCs are composed of both solid and liquid lipids, resulting in structures with enhanced drug loading, altered drug release patterns, and improved stability features [20, 21]. NLCs also significantly improve skin hydration and exhibit occlusive properties due to the reduction in transepidermal water loss; this occlusive trait can aid in skin hydration and medication penetration. [22, 23].

CP is a potent corticosteroid that reduces inflammation and redness in inflammatory conditions like eczema and dermatitis. Pramoxine hydrochloride blocks the voltage-gated sodium channel, thereby reversibly numbing the sensation of itch. The combination of both drugs can provide substantial symptomatic relief by addressing both inflammation and pruritus. Currently, no marketed formulations exist for the combined dosage form of CP and PH. This study aimed to prepare a topical NLC-based gel formulation of CP and PH that can enhance skin retention and permeation and to evaluate its potential in managing AD through an in vivo animal study in comparison to a marketed cream of CP. The NLCs developed in our previous work were incorporated into Carbopol 934 gel to achieve increased contact time and a consistency suitable for topical application. The prepared DNLC gel was evaluated for physicochemical properties,

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rheological studies, *in vitro* drug release, *ex vivo* porcine skin permeation, *ex vivo* porcine skin retention, rhodamine-tagged DNLC gel skin permeation studies, and *in vivo* studies on Wistar rats, followed by histopathological investigations and serum IgE and AEC level measurements to assess the disease-mitigating properties of DNLC gel compared to the marketed formulation of CP, CP-loaded NLC gel, PH-loaded NLC gel, and NLC gel without drug.

2.0 MATERIALS AND METHODS:

2.1 Materials

Clobetasol propionate was supplied by Mahima Life Sciences, Haryana, India. Pramoxine hydrochloride, triethanolamine, and 2,4-dinitrochlorobenzene were purchased from Sigma-Aldrich Co. LLC. Stearic acid, Tween 80, propylparaben, and propylene glycol were obtained from Nice Chemicals, Kochi. Oleic acid and Carbopol 934 were procured from Loba Chem. Pvt. Ltd, Mumbai. Poloxamer F68 was shipped from Research-Lab Fine Chem Industries, Mumbai. Soya lecithin was obtained from Hi Media Laboratories Pvt. Ltd., Mumbai. Millipore water was used during this study.

2.2 Animals

Wistar rats weighing 250g–300g were obtained from the central animal house facilities at the Amrita Institute of Medical Sciences and Research Centre (AIMS), Kochi, India. The animals were housed in the central lab animal facility, AIMS, under standard laboratory conditions with a 12-hour light/dark cycle at 25 ± 2°C, provided with standard animal feed and water ad libitum. All experimental procedures were approved by the Institutional Animal Ethical Committee (IAEC), AIMS, with ref. no. IAEC/2017/3/18.

2.3 Preparation of Optimized Drug-Loaded Nanostructured Lipid Carrier (DNLC)

Drug-loaded NLCs were prepared and optimized using the melt emulsification ultrasonication technique based on our previous work (Dixit C Mohan et al, 2018) [24]. In brief, oleic acid was used as the liquid lipid and stearic acid as the solid lipid. The required quantities of medicines, liquid lipids, and solid lipids—constituting the lipid phase—were weighed. The aqueous phase contained surfactants, co-surfactants, stabilizers, and distilled water. After heating both phases in a water bath at 80°C for 20 minutes, the aqueous phase was added dropwise to the lipid phase on

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a magnetic stirrer at 750 rpm while maintaining the temperature at 80°C. The resulting primary emulsion was then subjected to probe sonication at 70% amplitude with 6-2 pulses for 15 minutes, followed by cooling in an ice bath to produce an NLC dispersion.

2.4 Characterization of DNLC

2.4.1 Particle size and zeta potential

The particle size, polydispersity index (PDI), and zeta potential of the nanoparticles were measured by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK). Before measurements, the samples were diluted with Milli-Q water to measure the particle size and with conductivity-adjusted Milli-Q water to measure the zeta potential [24].

2.4.2 Scanning electron microscopy (SEM)

The surface morphology of DNLC was examined by SEM (VEGA3 TESCAN) by placing the sample on metal studs with double-sided conductive tape and observed at an accelerated voltage of 20 kV [23]. The DNLC dispersion was diluted with distilled water, after which a drop of this sample was placed on the slide and allowed to dry.

2.4.3 Transmission electron microscopy (TEM)

To further confirm the morphology of DNLC, TEM studies were carried out by negative staining method using HR-TEM, Tecnai G2F20 (Germany) equipped with a CCD camera (4K × 4K) image resolution operating at an accelerated voltage of 200 kV. 5µl of diluted DNLC was placed on a copper grid (carbon-coated) and allowed to dry for 12h at room temperature [24]. The sample was prepared by diluting DNLC with distilled water and placing a drop of 100 µL on a carbon-coated copper grid and allowing it to dry for 12 hours at room temperature.

2.4.4 Entrapment efficiency (EE)

The entrapment efficiency of DNLC was determined by the ultracentrifugation method. A volume of 5ml of DNLC was centrifuged (Hermle labortechnik GmbH, Germany) at 10000rpm for 30mins. The supernatant and pellet were collected; the pellet was dispersed with methanol [24].

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The amount of drug present in the supernatant was determined by UV spectrophotometry by the following equation:

$$EE\% = \frac{Wi - Wf}{Wi} \times 100$$

Where,

W_i: Amount of drug added initially

W_f: Amount of drug detected in the supernatant/pellet after centrifugation of the formulation

2.5 Preparation of DNLC gel

DNLC gel was prepared by mechanical stirring. Carbopol 934 was used as a gel-forming polymer; the required amount of carbopol was weighed and allowed to soak in a few mL of distilled water for 4 hours, after which it was placed under a mechanical stirrer [25]. Freshly prepared DNLC solution was added drop by drop into the beaker under constant stirring, carefully observing for uniform mixing of the polymer and DNLC dispersion. Propylparaben was added as a preservative, Triethanolamine was added to neutralize carbopol and to achieve topical consistency. Carbopol gels were prepared at two concentrations, 1% w/w and 1.5% w/w.

2.6 Characterization of Gel Formulations

2.6.1 Homogeneity, grittiness, pH determination, and Spreadability

The prepared gels were tested for homogeneity by visual inspection after the gels had been set in the container. They were tested for their appearance and the presence of any aggregates. The prepared gels were evaluated microscopically for the presence of particles under a light microscope. pH was measured using a digital pH meter, which had been calibrated before the experiment. The bulb of the electrode was inserted into the gel, and the reading was noted. The experiment was performed in triplicate. To determine the spreadability, 1 g of the gel was placed on a horizontal glass plate, and a second glass plate was placed on the gel such that the gel was sandwiched between the glass plates. Then, a standard weight of 125 g was placed on top of the upper glass plate for 1 minute. The upper plate was then removed, and the diameter of the gel was measured [26]. The formula then calculated spreadability:

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$$S = d^2 \times \pi \div 4$$

Where S is Spreadability and d is the diameter of the gel in mm^2.

2.6.2 Rheological evaluation

Rheological evaluation was carried out on DNLC gels and NLC gels (NLCs without drug). The rheological properties were analyzed using a Brookfield rheocalc V 32 rheometer (Brookfield USA) using cone and plate geometry. Each sample was equilibrated at 28^0C before every measurement, and data analysis was done with the Brookfield rheocalc 2.010 application software. Viscosity vs. shear rate, as well as the viscoelastic modulus and phase angle vs. frequency, were also investigated; the measurement was triplicated for each system [27].

2.7 Drug content analysis of Drug-Loaded NLC Gels (DNLC Gels)

0.5 g of DNLC gel was accurately weighed and dissolved in 100 mL of pH 5.5 phosphate buffer solution in a volumetric flask. The volumetric flask was kept for 4 hours in an orbital shaker. The solution was then passed through a filter paper and filtered. 1 mL of this solution was then taken into a 10 mL volumetric flask, and the final volume was made with pH 5.5 phosphate buffer solution [28]. The absorbance was measured at 240nm for clobetasol propionate and 224nm for pramoxine hydrochloride. Phosphate buffer solution was used as a blank.

2.8 *In-vitro* drug release from DNLC gel

The release of CP and PH from DNLC gels was studied by the cellophane membrane method (Sigma Aldrich, India) at pH 5.5. Previously activated cellophane membrane was tied to one end of the open-end tube, and 0.5g of DNLC gel was placed through the other end [29]. The open-end tube with DNLC gel was suspended in the receptor compartment containing 30 mL of PBS ethanol mixture (7:3) as release medium. This experimental setup was retained on a magnetic stirrer maintained at 50 rpm and 37^0C. Sink condition was maintained throughout the experiment. At predetermined time intervals, samples were withdrawn from the receptor compartment and were replaced with an equal amount of fresh release medium. The amount of CP and PH in the release medium was determined using a UV-Visible spectrophotometer set at 240nm and 224nm, respectively. The experiments were carried out in triplicate. Kinetic modelling of data was performed.

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2.9 *Ex vivo* porcine skin studies

Ex vivo skin studies were conducted using porcine ear skin, which is considered a suitable model for permeation experiments. Pig ear skin was obtained from the local slaughterhouse and cleaned by washing with water. Hair present on the skin surface was removed using a scalpel blade. The underlying fatty layer was removed, and the skin was washed with normal saline and kept at -20^{0}C for further use [30]. The experiment was conducted on a Franz diffusion cell apparatus; the surface area of skin exposed to formulation was 2.54 cm2, and the receptor chamber was filled with 7 mL of PBS ethanol mixture (7:3). The temperature was maintained at 32^{0}C, and the receptor chamber was stirred at 400 rpm.

2.9.1 Skin permeation studies

Skin samples were properly hydrated by soaking in pH 5.5 PBS for half an hour, then sandwiched between the donor and receptor compartments of the Franz diffusion cell. 0.5 g of DNLC gel was applied on the skin on the donor side. At predetermined time intervals, samples were collected from the receptor compartment and replaced with equal amounts of buffer-ethanol mixture. The amounts of CP and PH permeated were measured using a UV-Visible spectrophotometer set at 240 nm and 224 nm, respectively. The permeation experiment lasted 24 hours. The experiment was conducted in triplicate. The steady-state flux (J) was calculated from the slope of the graph, and the permeability constant (P) was determined using Fick's first law of diffusion. The enhancement ratio (E) was calculated from the steady-state flux. [30, 31].

2.9.2 Drug retention studies

The amount of drug retained in each layer of skin (stratum corneum, epidermis, and dermis) was determined. Skin samples were sandwiched between the donor and receptor compartments of the Franz diffusion cell apparatus. The skin samples were collected at predetermined time intervals and washed with PBS solution to remove the DNLC gel remaining on the skin surface. The dried skin samples were cut into 1 cm^2 pieces. This piece was then fixed using tissue freezing medium and was cryo-sectioned with a microcryotome (Leica CM 1505 S) into stratum corneum (25µm), epidermis (75µm), and dermis (1 mm). CP and PH retained within the skin layers were extracted by incubating the samples overnight in methanol in a shaking incubator at 37^{0}C, after which they were homogenized and centrifuged [30,31]. The amounts of CP and PH retained were determined

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using a UV-Visible spectrophotometer set at 240 nm and 224 nm, respectively, and are expressed as µg/cm² of skin area.

2.9.3 Dermal pharmacokinetic parameters

The data obtained from *ex vivo* skin permeation studies were used to determine dermal pharmacokinetic parameters such as Cmax, Tmax, AUC, AUMC, and MRT. These parameters were determined by Phoenix Winnonlin ® [32]

2.9.4 Rhodamine-tagged DNLC gel skin retention study

To determine the depth of penetration and retention of DNLC in the formulation, a skin permeation experiment was conducted. The rhodamine-tagged DNLC gel was prepared by mixing rhodamine and DNLC gel on a magnetic stirrer overnight. 100µl of rhodamine was mixed with 1 g of DNLC gel. The method used was the same as that of the previously explained permeation experiment, except that everything was performed in the dark to prevent the photolytic deactivation of rhodamine dye. After 24 h, skin samples were removed and cryosectioned. The cryosectioned skin samples were placed on Poly-L-lysine (PLL) coated glass slides, and fluorescence was detected using an Olympus-BX-51 fluorescent microscope [33].

2.10 *In vivo* studies

In-vivo animal studies were performed on male albino Wistar rats (250g – 300g) for 28 days to evaluate the disease-mitigating property DNLC gel formulation.

2.10.1 Induction of atopic dermatitis in an animal model

2. 4-dinitrochlorobenzene (DNCB) was used to cause atopic dermatitis. Animals were anesthetized with isoflurane, their back hair was shaved, and 0.2 mL of DNCB in a 4:1 vehicle of acetone and olive oil was applied topically to a premarked 8 cm2 region. Atopic dermatitis-like lesions formed following 4 administrations of DNCB with a 2-day gap between each treatment, the first two being sensitization (0.2ml, 1% DNCB) and the last two being challenge (0.2ml, 0.2% DNCB). [34,35].

2.10.2 Atopic dermatitis studies

The animals were divided into 7 groups (n=6). Group 1 was an untreated control, group 2: CP and PH loaded NLC, group 3: conventional CP cream (0.05%), group 4: NLC gel treatment, group 5:

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CP NLC gel treatment, group 6: PH NLC gel treatment, and group 7: normal control. Each group, except the normal control, was induced with atopic dermatitis, while group 1 was not subjected to any treatment. Treatment for other groups was started on the 14th day after the disease was induced to a severe condition, and the treatment period was for 14 days. One fingertip dose of the formulation was applied evenly on the area premarked on the 14th, 17th, 21st, and 24th day. To retain the disease during the treatment period, once every 3 days, DNCB was administered. The animals were euthanized on the 28th day; the skin was collected and stored in 10% neutral phosphate-buffered formalin. Investigative global atopic dermatitis (IGAD) scoring was measured for each group every day by 3 individuals, among whom 1 was not made aware of the experiment to obtain an unbiased blind scoring [36]. The area of atopic dermatitis disease induced was measured using ImageJ software.

2.10.3 IgE and absolute eosinophil count (AEC)

Blood from the animals was collected from the retroorbital plexus on day 0, day 14, and day 28 to determine the amount of IgE and AEC in the plasma [37].

2.10.4 Histological analysis

The skin samples collected at the end of euthanasia were treated with isopropanol and xylene and then embedded in paraffin. 5µm-thick sections were cut using a microtome, stained with hematoxylin and eosin, mounted using DPX, and observed under an optical microscope equipped with a computer-controlled camera. The histological changes of all skin samples in comparison with the control animals were evaluated with the help of a veterinary pathologist, and the thickness of the epidermis was measured using the Fiji Image J software.

2.12 Statistical analysis

The data was analyzed using Student's *t-test* calculator, Graph Pad software, and QuickCalcs, and $p<0.05$ was considered to be significant.

3.0 RESULTS AND DISCUSSION

3.1 Characterization of DNLC

3.1.1 Particle size and zeta potential

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Determining particle size is important in nanostructured carriers because the particle size contributes to the interfacial area. Nano carriers having a smaller particle size can easily penetrate into the deeper layers of skin, providing enhanced drug partitioning and adsorption. There is, however, no consensus in the literature on the exact size ranges of nano formulations [38]. The particle size and zeta potential of DNLC were determined by the dynamic light scattering technique using Zetasizer. The average particle size and zeta potential were found to be 66.95 ± 5.27 nm and -42.86 ± 4.66 mV, respectively, which were in accordance with our previous work (Dixit et al). This particle size can be attributed to the technique adopted for the preparation of DNLC, and also increased the concentration of surfactant aids in the reduction of particle size. The stability of NLC dispersion can be represented by zeta potential. It is reported that Nano dispersion with a zeta potential of <-60mV has excellent stability [39]. DNLC exhibits a zeta potential of -42.86 ± 4.66 mV, possibly due to the usage of soya lecithin. The anionic components of lecithin may have contributed to the negative charge [40]. The polydispersity index (PDI) measures the size distribution of nanoparticles in the dispersion. Monodispersed samples with uniform particle size distributions have PDI values ranging from 0.1 to 0.7, whereas PDI values more than 0.7 indicate that the dispersion has a broad size distribution. The prepared DNLC dispersion had a PDI value of 0.25, indicating that it is monodispersed, with uniform particle size. [39].

3.1.2 SEM and TEM

SEM and TEM studies were carried out to further determine the size and morphology of DNLC. The measurement using zetasizer does not measure the actual size but only estimates the size of NLC according to the light scattering pattern and intensity; hence, SEM and TEM analysis are useful [41]. TEM result (Fig. 1A) shows a particle having a size less than 120nm. SEM result (Fig. 1B) shows NLC as aggregates, which could be the reason for the particle size between 100nm to 150nm. The aggregation might have occurred due to the sample preparation for SEM analysis.

3.1.3 Entrapment Efficiency

Entrapment efficiency was calculated to determine the amount of CP and PH entrapped in the NCL. The average EE of CP and PH in NLC was found to be 75.47 ± 3.26% and 87.75 ± 2.14%, respectively. This EE could have been achieved due to the incorporation of drugs into the lipid phase, where the solid lipid was melted in the presence of liquid lipid. This caused disarray in the crystal order, causing imperfect lattice formation, which is a characteristic feature of NLCs that

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can help in the accommodation of drug molecules [42]. EE also depends on the nature of drugs; CP and PH are both lipophilic drugs. The use of soya lecithin in the preparation of DNLC also influences the entrapment of drugs. It is reported that lecithin reduces the possibility of drug loss and provides more space for the drug [43].

3.2 Preparation of DNLC gel

DNLC dispersions lack the consistency required for topical use; thus, DNLC must be incorporated into a carrier to give a suitable form for topical treatment [44]. Carbopol gel acts as a secondary carrier for medicines and a medium for DNLCs. Carbopol gels are suitable for nanoparticle inclusion [45], allowing the NLC to permeate the skin during a longer contact duration. Soaking carbopol makes it smooth and viscous, and mechanical stirring loosens the polymer chains. Triethanolamine neutralizes carbomer, allowing it to thicken to the desired consistency for topical application. Table 1 shows the formula for making the gel.

3.3 Characterization of gel formulations

3.3.1 Homogeneity, grittiness, pH determination, and spreadability.

Physiochemical properties such as homogeneity, grittiness, pH, and spreadability of DNLC gel and plain gel were studied, and the results are presented in Table 2. It was observed that all the gel formulations were homogeneous without the presence of any gritty particles and exhibited a pH range of 5.8 to 6.2, which falls within the physiologically acceptable range for skin application. This pH range helps maintain the natural acidic mantle of the skin and minimizes the risk of irritation.[46]. As the gel strength increases, the spreadability decreases, which was seen in Table 3. The addition of DNLC further increased the spreadability, which is due to the lipid nature of the DNLC dispersion.

3.3.2 Rheological evaluation

Rheological evaluation is essential to determine the flow properties of the system since it is intended for topical application. The plot of elastic modulus (G`), viscous modulus (G``), and phase angle vs. frequency (Figure 2A) has revealed that G` is greater than G`` (G`>G``), which is ideal for a topical gel. The value of phase angle being less than 10^0 is indicative that the formulation has gel-like behaviour and does not flow unless a shear is applied [47,48]. The plot of

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viscosity vs. shear rate (Figure 2 B) revealed that the gel formulations (DNLC gel and plain gel) have shear-thinning behavior, which is that with an increase in the shear rate, the viscosity of the formulation decreases. The shear-thinning behavior is considered essential for topical gels to be extruded easily from the container.

3.4 Drug content analysis of DNLC gels

It is essential to determine the amount of CP and PH present in one fingertip unit of gel (i.e., 0.5g of DNLC gel) because *in vitro* drug release studies, *ex vivo* skin studies, and in vivo studies require the knowledge of drug content in each application. It was found that 0.221 ± 0.012 mg of CP and 4.670 ± 0.093 mg of PH were present in one fingertip unit of gel (i.e., 0.5g).

3.5 *In-vitro* drug release from DNLC gel

In vitro drug release from the DNLC gel was assessed using the dialysis membrane method at 37^0C and 50 rpm. The drug release pattern from the DNLC gel is shown in Figure 3. A biphasic release pattern was observed for CP, with an initial burst release followed by a sustained release, while PH showed a sustained release pattern. 33% of CP and 3% of PH were released within the first hour; by the end of 24h, 84% of CP and 64% of PH were released. The initial burst release of CP may be due to the localization of CP in the outer shell of the NLC, and the unentrapped drug in the NLC dispersion could have been dispersed in the gel matrix, which could have caused the initial burst release [49]. A sustained-release pattern was observed in the case of PH, which may be due to the localization of PH in the core of the NLC. The release kinetics of drugs from the formulation was determined by fitting the *in vitro* drug release data into zero order, first order, Higuchi, and Korsmeyer Peppas models. The release of CP can be best fitted to first-order kinetics, while the release of PH can be best fitted to zero-order kinetics; the Korsmeyer-Peppas model was used to determine the release mechanism, and it was seen that CP followed Fickian diffusion, while PH followed super case II.

3.6 *Ex vivo* drug permeation studies

Ex vivo skin permeation studies were carried out to determine the amount of drug permeated across the skin from the DNLC gel, and the results were compared with those of the marketed CP cream

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and drug solution. The results (Figure 4) revealed that drugs from DNLC gel showed better skin permeation when compared with individual drug solutions, and permeation of CP from DNLC gel was enhanced 1.42 times when compared with permeation from the marketed CP cream. Table 3 lists the steady-state flux, enhancement ratio, and permeability coefficient of the formulations. CP from DNLC showed a permeability flux of 5.88 µg/cm^2/h and an enhancement ratio of 1.92 when compared with the CP drug solution, whereas the PH from DNLC gel showed a permeability flux of 9.52 µg/cm^2/h and an enhancement ratio of 1.62 when compared with the pH drug solution. This enhancement of drug permeation from DNLC gel may be due to the incorporation of drugs into NLC particles in the nanometric size range that can penetrate the skin, forming micro drug reservoirs in dermal layers by enhancing the permeation, which is seen from the increase in the enhancement ratio. The steady-state flux (*Jss*) was obtained from the slope of the plot using a linear regression analysis. The permeability coefficient (*Kp*) of the drug through the membrane was calculated using the following equation

$$Kp = \frac{Jss}{C}$$

Where C is the Initial concentration of the drug in the donor compartment.

The penetration-enhancing effect was calculated in terms of the enhancement ratio (ER)

$$ER = \frac{Jss\ F}{Jss\ C}$$

Where, Jss F is Jss of formulation & Jss C is Jss of control

3.7 *Ex vivo* drug retention studies

Ex vivo drug retention studies were carried out to determine the amount of drug retained in different layers of skin at the end of the 24th hour. The amount of CP retained at the end of 24h (Figure 5) in stratum corneum, epidermis and dermis were 4.25 ± 0.02µg, 75.77 ± 0.01µg and 32.04± 0.012µg respectively while the amount of PH retained at the end of 24h in stratum corneum, epidermis and dermis were 11.82 ± 0.003µg, 344.0 ± 0.05µg and 172.85 ± 0.040µg respectively. It is plausible that increased PH retention would prolong its numbing effect, thereby reducing itch and improving patient comfort. It can be seen that a greater amount of CP is retained from the DNLC gel compared to the marketed CP cream, which could be due to the incorporation of CP into the Nano carrier, enabling drug retention. More of the drug is retained in the epidermal and dermal region that constitutes the dermoepidermal junction.

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3.7 Dermal pharmacokinetic parameters

The data obtained from *ex vivo* porcine skin studies were used to determine various dermal pharmacokinetic parameters, including Cmax, Tmax, AUC, AUMC, and MRT, which are presented in Table 4.

3.8 Rhodamine-tagged DNLC gel skin retention study

Skin retention study using rhodamine-tagged DNLC gel was carried out to confirm DNLC's skin penetration and retentive properties. The investigation was conducted over 24 hours using a Franz diffusion cell apparatus; throughout the experiment, the system was kept out of direct sunlight to prevent photolytic breakdown of rhodamine. Figure 6 depicts fluorescence pictures, which show that the rhodamine-tagged DNLC-treated skin sample fluoresced; however, the rhodamine-tagged commercial CP cream and medication solution did not fluoresce in the epidermis or dermal region. This experiment further demonstrates DNLC's capacity to penetrate and persist in dermal layers.

3.9 *In vivo* animal studies

3.9.1 Induction of atopic dermatitis in an animal model

Atopic dermatitis-type skin lesions were induced on mice back skin by the topical application of 2, 4 DNCB. Many investigators have used murine models to carry out *in-vivo* studies on atopic dermatitis, these models are of two types (a) spontaneous mutants and genetically engineered mutants such as NC/Nga mice and IL-4/18-overexpressing mice, (b) sensitizer induced models (using ovalbumin, microbial antigen, chemical reagents like picryl chloride, thinitrochlorobenzene or 2,4 dinitrochlorobenzene) [50,51]. Among these, the murine models with repeated sensitizer application possess benefits in reproducibility in atopic dermatitis-like features [53]. Upon repeated topical application of DNCB on the skin, an immune response conversion from Th_1 to Th_2 mediated reaction, along with high serum IgE levels, epidermal hyperplasia, and infiltration of mast cells in dermis was seen, which are all regarded as particular features of human atopic dermatitis [53, 54]. Hence, for the induction of an animal model, sensitizer induced atopic dermatitis was induced in animals with 2, 4-dinitrochlorobenzene as a sensitizer.

3.9.2 Atopic dermatitis animal studies

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The animals were divided into seven groups (n=6). Group I was the untreated control in which the animals were induced with disease but were not treated; group II was combinational therapy in which after the induction of disease the animals were treated with CP and PH loaded NLC gel; group III was conventional therapy in which after the induction of disease, the animals were treated with marketed CP cream (0.05% CP); group IV was plain gel treatment in which the animals were treated with plain NLC gel after the induction of disease; group V, anti-inflammatory therapy in which the animals after disease induction were treated with CP-loaded NLC gel (0.05%); group VI was anti-pruritic therapy in which the animals were treated with PH NLC gel; and group VII was the normal control in which the animals were not induced with disease. Figure 7(A) shows the images of animal studies at days 14 and 28, Figure 7(B) shows the atopic dermatitis score of all the groups, and Figure 7(C) shows the area of disease induced. From the *in vivo* animal studies, it was seen that the prepared CP and PH-loaded NLC gels decreased the induced disease on par with that of the marketed CP cream, which can be seen from the Figures; the IGAD score and area decreased after the beginning of the treatment and attained 0 by the end of the 28th day, whereas the untreated control group retained the disease throughout the study period. Group IV, which involved plain gel therapy, showed no reduction in disease due to the absence of a drug, whereas Group V, which employed anti-inflammatory therapy, demonstrated a reduction in disease, albeit slightly less than that achieved with conventional therapy. Group VI also showed a reduction in IGAD score, but the area of disease induced was not decreased sufficiently, which could be due to the absence of CP, which mainly reduces inflammation and atopic dermatitis-like lesions.

3.9.3 Serum IgE and AEC count

The blood collected during the *in vivo* animal studies was evaluated for the levels of serum IgE and AEC. Generally, IgE and AEC levels are elevated in the blood of patients with atopic dermatitis; hence, it is essential to determine the levels of IgE and AEC. Blood samples were collected from the retro orbital plexus of rats at 3 time points in the study, which are on the 0th day which is the day before the topical application of DNCB which gives the value of IgE and AEC

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before induction of disease, on 14th day which is the day before beginning of treatment which gives the value of IgE and AEC after the induction of disease and on 28th day which is the day of euthanasia giving the values of IgE and AEC after the treatment of disease. Figure 8(A) represents the values of IgE, and Figure 8(B) represents the values of AEC. It can be seen that the values of IgE and AEC are low for the normal control animal; in the disease control group, the values of IgE and AEC are significantly elevated, among the treatment groups. The DNLC gel treatment group showed the best reduction in IgE and AEC values, followed by the marketed CP cream. This could be due to the presence of CP in both formulations, which helps reduce inflammation. CPNLC gel treatment and PHNLC gel treatment groups also showed a reduction in IgE and AEC values, whereas bare gel, i.e., plain NLC gel treated group, showed no reduction in the values of IgE and AEC.

3.9.4 Histological analysis

After euthanasia, the skin samples were removed, and histological sections were evaluated (Figure 9). From the histological sections, the thickness of the epidermis was measured using ImageJ software (Figure 10). The skin histological studies revealed the increased epidermal thickness (acanthosis) along with focally fused pitted ridges, spongiosis, neutrophilic abscess, and areas of ulceration in the untreated control group, showing an epidermal thickness of 354.32 ± 63.71 µm. The normal control group showed an epidermal thickness of 54.13 ± 3.34 µm with normal epidermis, dermis, and dermal appendages. DNLC therapy animal group showed normal epidermis, overlying dermis and dermal appendages with an epidermal thickness of 60.49 ± 10.24 µm which was close to normal control group indicating that treatment with DNLC gel has significantly reduced the induced disease, while the conventional therapy (marketed) group showed similar histological features the epidermal thickness was found to be 86.4 ± 12.66 µm. The anti-inflammatory group showed an epidermal thickness of 74.69 ± 14.73 µm. The plain gel treatment showed epidermis with irregular acanthosis and ulcerations with an epidermal thickness of 316.50 ± 55.61 µm, in which the disease was not reduced. Treatment with DNLC gel retained the normal dermal features of the skin, which resemble those of the normal control group. Disease control and bare gel (NLC gel without drugs) showed ruptured and inflamed epidermis, and the epidermal thickness was also high.

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Conclusion

DNLCs were prepared using the optimized formula from our previous work, and DLS and TEM analyses confirmed the nanoparticle size range, consistent with prior findings. This study primarily focused on loading DNLCs into a topical gel system to evaluate their effectiveness in mitigating disease by improving skin permeation and retention. The DNLC gel was successfully prepared; rheological evaluation confirmed it exhibited a shear-thinning behavior. In vitro drug release studies showed an initial burst followed by a sustained release pattern. Ex vivo skin studies using porcine inner ear skin, chosen for its morphological similarity to human skin, demonstrated that drugs from DNLC gels permeated the skin better than commercial creams, with enhanced drug retention when loaded into NLCs. Rhodamine-tagged DNLC gel retention fluorescence studies suggested that NLCs can form microdrug reservoirs in the skin, enabling controlled drug release. In vivo animal studies confirmed that DNLC gel is more effective in treating atopic dermatitis than commercial creams or individual drug-loaded DNLC gels, attributed to the sequential and distinct pharmacological actions of CP and PH. Further studies will include the pharmacodynamic interaction assays to understand the nature of drug combinations within the DNLC system, and clinical studies in humans to confirm its efficacy. In conclusion, clobetasol propionate- and pramoxine-hydrochloride-loaded NLC gels demonstrate improved skin retention and permeation, supporting their potential in effectively managing atopic dermatitis.

Conflict of Interest

The authors have no conflict of interest.

Ethical Statement

All animal procedures were approved by the Institutional Animal Ethical Committee of Amrita Institute of Medical Sciences (AIMS) and conducted in accordance with the guidelines of the

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Committee for the Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

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Figure	Title
1	TEM and SEM analysis (A) TEM image (B) SEM image.
2	Rheological evaluation (A) Elastic modulus (EM) vs. Viscous modulus (VM) and Phase angle (PA) vs. frequency of plain gel (PG) and drug-loaded NLC gel (DNLCG), (B) Viscosity vs. Shear rate of PG and DNLCG
3	*In vitro* drug release profile
4	*Ex vivo* skin permeation studies
5	Drug retention
6	Rhodamine-tagged fluorescent skin permeation study
7	In-vivo animal studies (A) 0th day, 14th day, and 28th day images of animal studies (B) IGAD score (C) Area of disease induced
8	IgE and AEC serum levels at 0th day, 14th day, and 28th day. (A) IgE (B) AEC
9	Histological evaluation of skin after the 28th day
10	Epidermal thickness calculated from histological sections

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Table	Title
1	Design for preparing NLC gel and DNLC gel formulation
2	Homogeneity, grittiness, color, spreadability, and pH of NLC gel and DNLC gel formulations
3	Permeation parameters from *ex vivo* porcine skin permeation study.
4	Pharmacokinetic parameters

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Table 1. Design for preparing NLC gel and DNLC gel formulation

Sl. No	Ingredients			
	Carbopol 934(% w/w)	Propyl paraben (% w/w)	DNLC (mL)	Distilled water(mL)
1	1	0.002	50	-
2	1.5	0.002	50	-
3	1	0.002	-	50
4	1.5	0.002	-	50

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Table 2. Homogeneity, grittiness, color, spreadability, and pH of NLC gel and DNLC gel formulations

Sl. No	Gel Type	Homogeneity	Grittiness	Color	Spreadability (mm^2)	pH
1	1% w/w NLC gel	+++	-	Transparent	2109.1	5.81 ± 0.31
2	1.5% w/w NLC gel	+++	-	Transparent	1950.0	6.12 ± 0.12
3	1% w/w DNLC Gel	+++	-	Color	2446.1	5.92 ± 0.04
4	1.5% w/w DNLC Gel	+++	-	Color	2359.5	6.01 ± 0.14

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Table 3. Permeation parameters from *ex vivo* porcine skin permeation study.

Sample	Steady state flux (µg/cm²/h)	Enhancement ratio	Permeability constant (cm²/h)
Clobetasol Drug solution	3.0713	-	0.01288 ± 0.003
Pramoxine Drug solution	5.9001	-	0.00118 ± 0.001
Clobetasol from DNLC Gel	5.8862	1.92	0.024 ± 0.002
Pramoxine from DNLC Gel	9.5295	1.62	0.002 ± 0.001
Clobetasol from the marketed ointment	4.1213		0.0169

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Table 4. Pharmacokinetic parameters

Pharmacokinetic parameters	Pramoxine hydrochloride			Clobetasol propionate		
	SC	Epidermis	Dermis	SC	Epidermis	Dermis
T max (hr)	2	24	24	2	24	24
C max (µg)	99.86	344.01	172.85	17.77	75.77	32.04
AUC last (hr. µg/L)	141.84	4128	2074.2	51	909.24	384.48
MRT last	7.31	17.08	13.99	8.41	15.73	15.03

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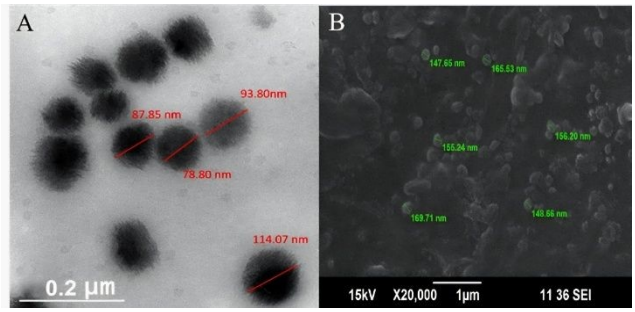

Figure 1. TEM and SEM analysis (A) TEM image (B) SEM image.

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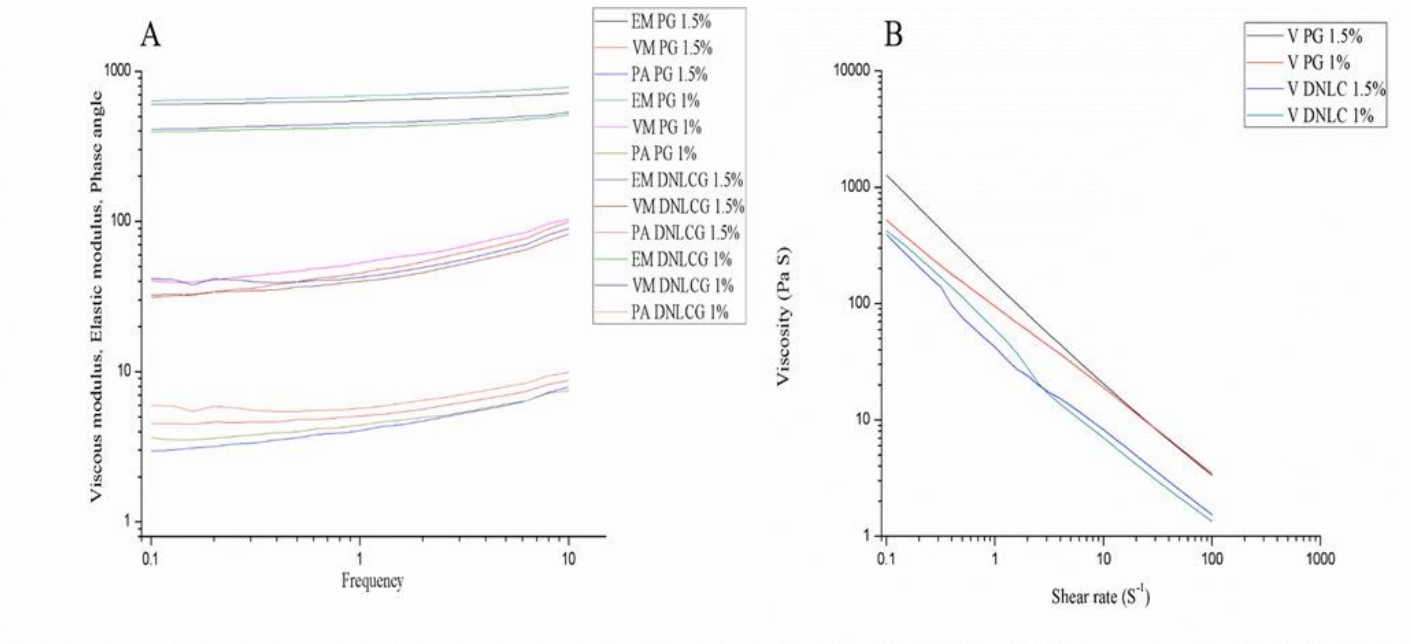

Figure 2 (A) Elastic modulus (EM) vs. Viscous modulus (VM) and Phase angle (PA) vs. frequency of plain gel (PG) and drug-loaded NLC gel (DNLCG).

Figure 2 (B) Viscosity (V) vs. Shear rate of PG and DNLCG

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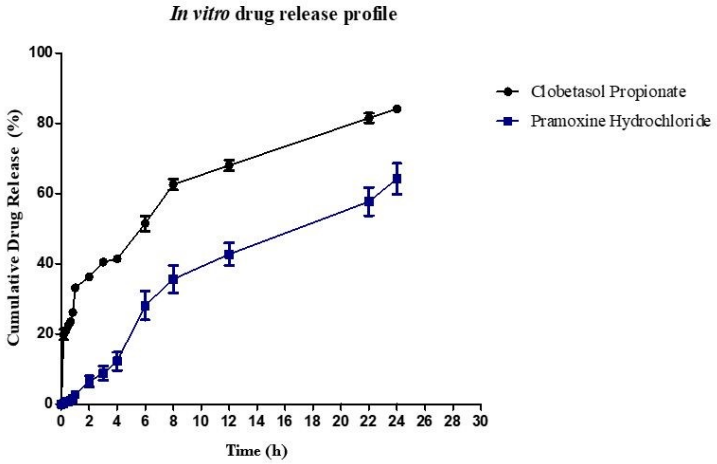

Figure 3. *In vitro* drug release profile (n=3)

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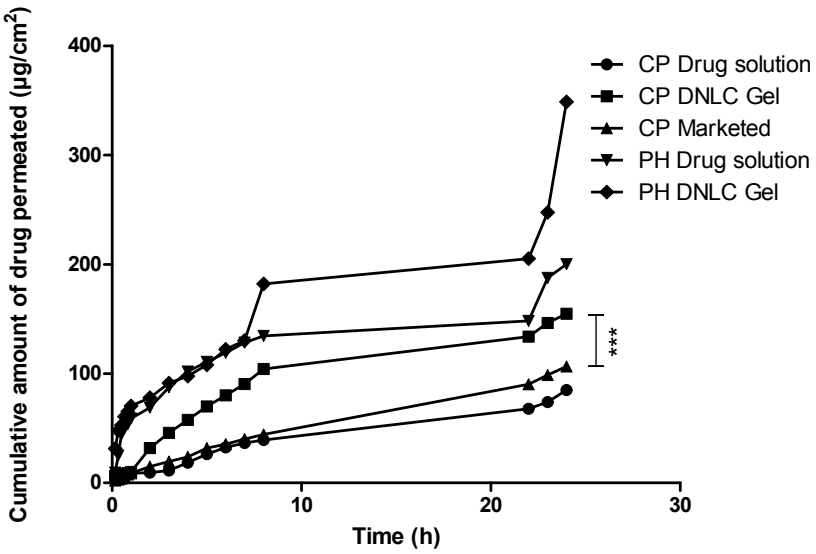

Figure 4. *Ex vivo* skin permeation studies (n=3)

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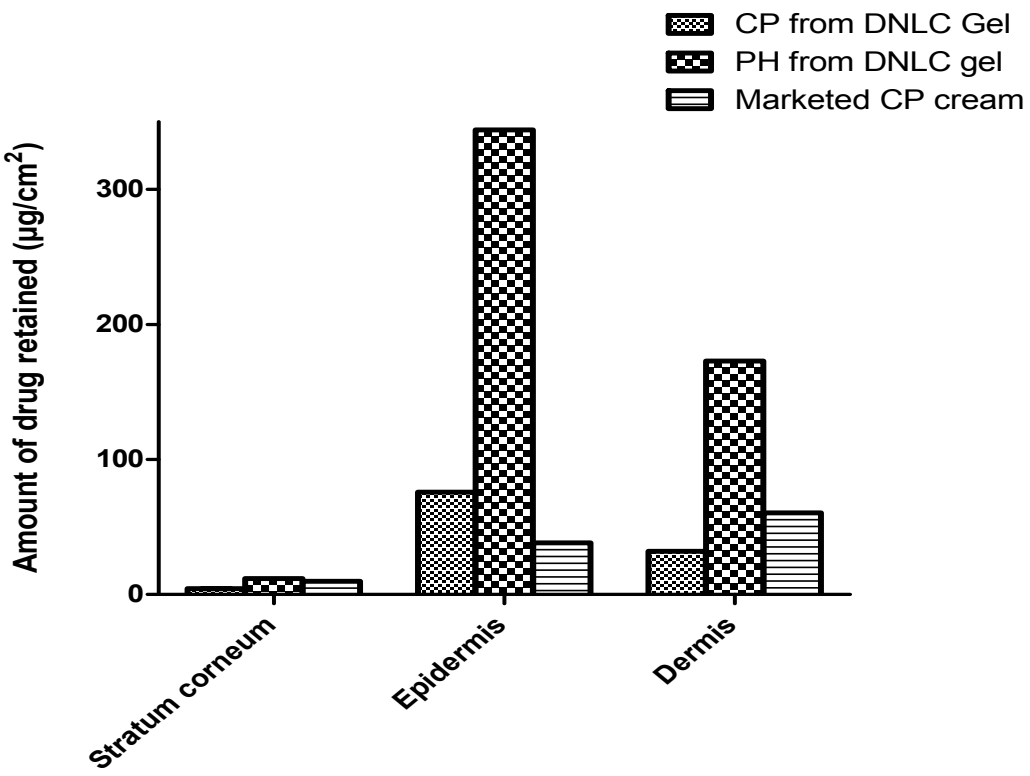

Figure 5. Drug retention study at the end of 24h in the stratum corneum, epidermis, and dermis

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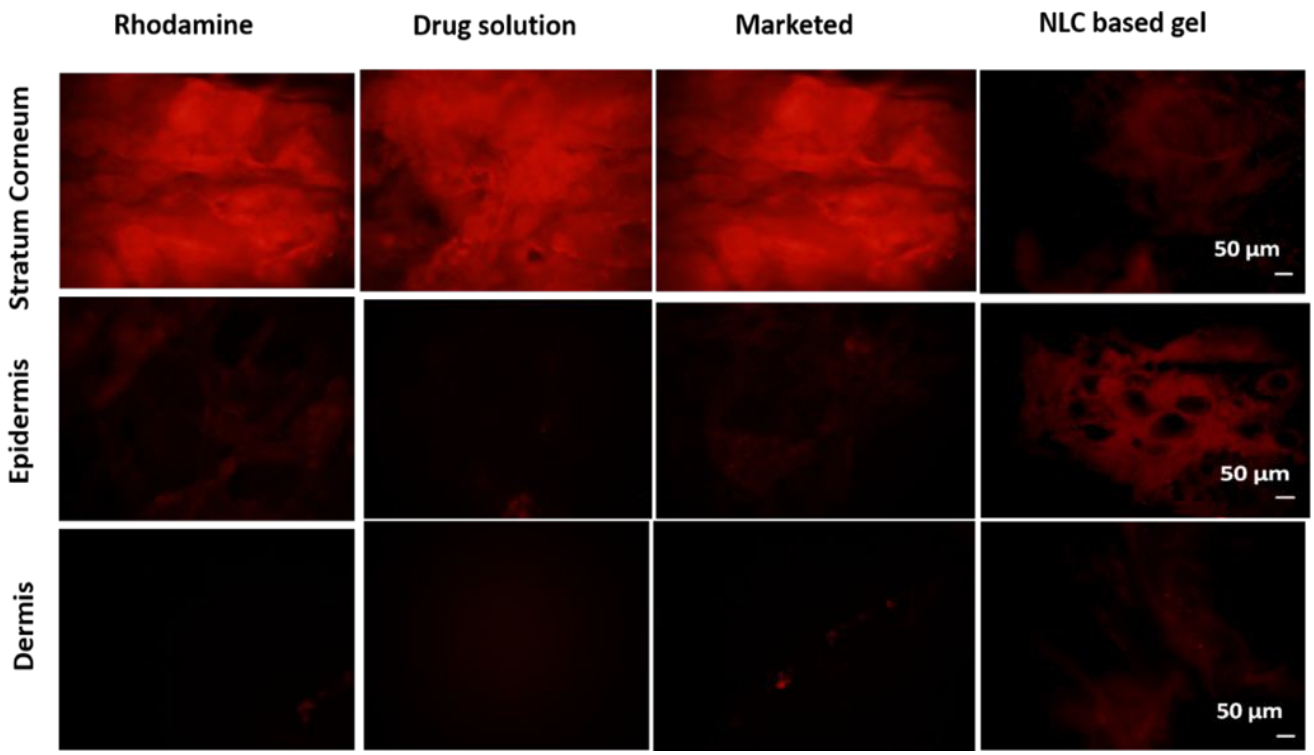

Figure 6. Rhodamine tagged fluorescence images

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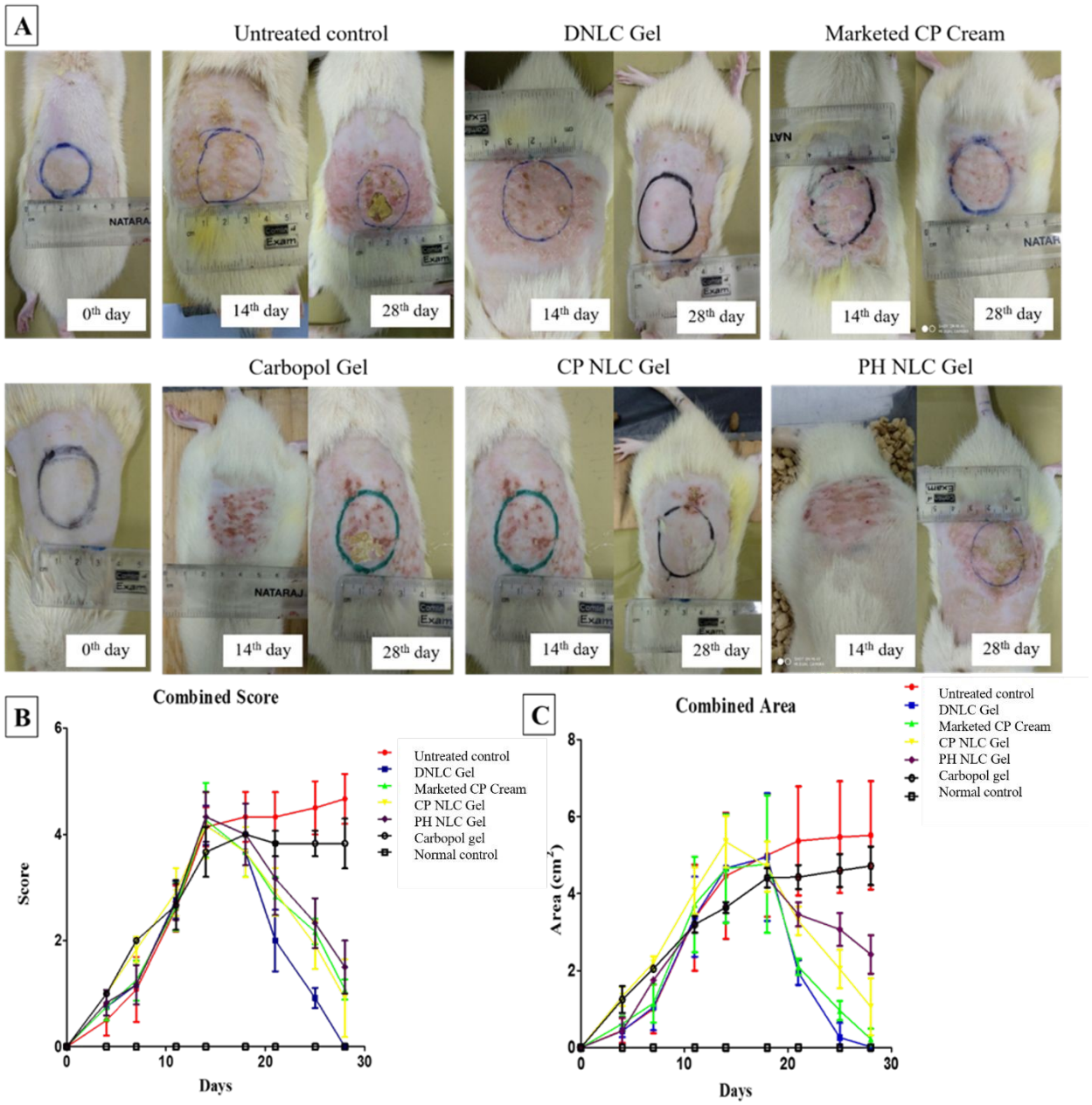

Figure 7. *In vivo* animal studies (n=6)

(A) 0th day, 14th day, and 28th day images of animal

(B) IGAD score

(C) Area of disease induced

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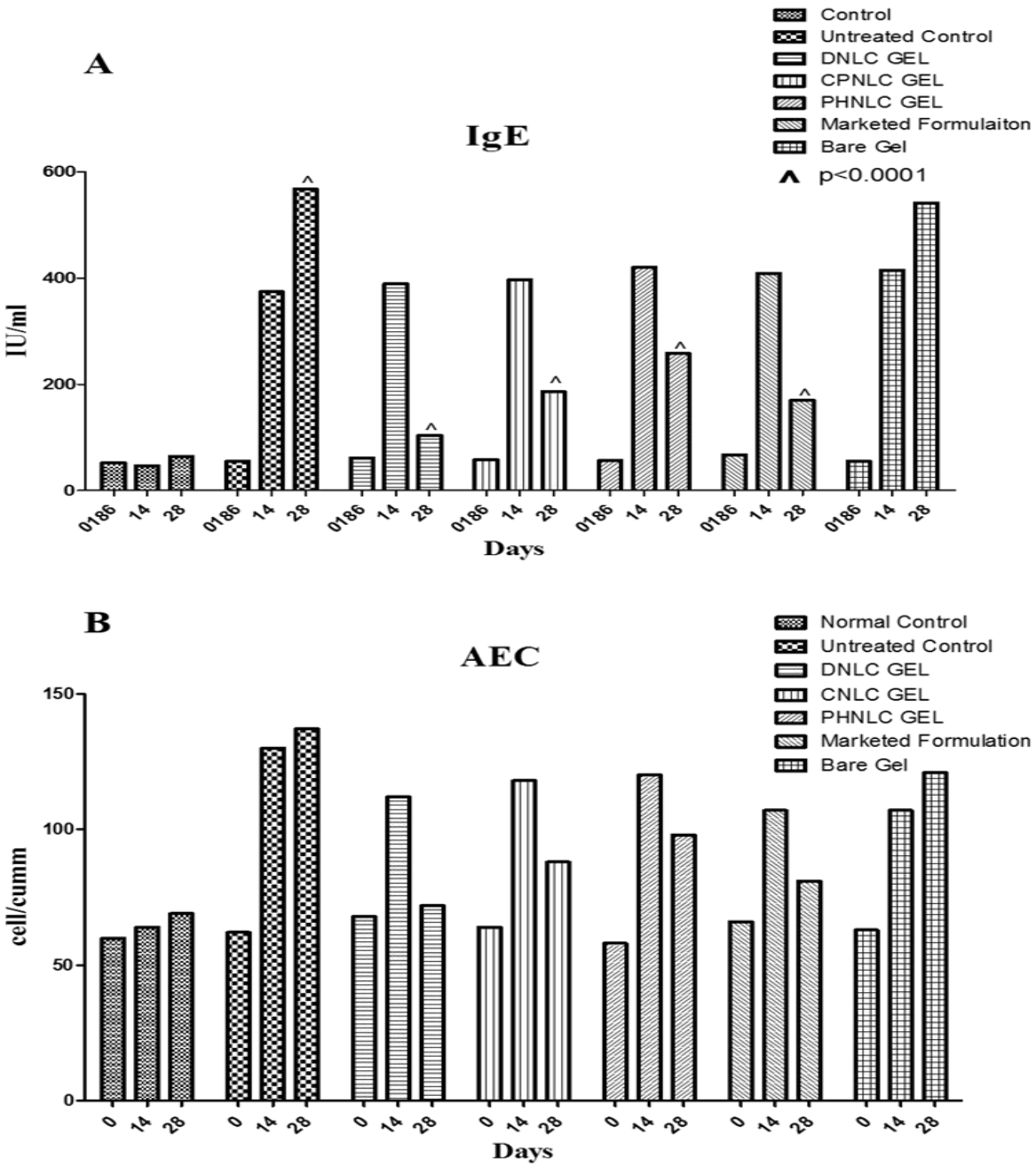

Figure 8. IgE and AEC serum levels at 0th day, 14th day, and 28th day. (A) IgE ($p<0.0001$), (B) AEC

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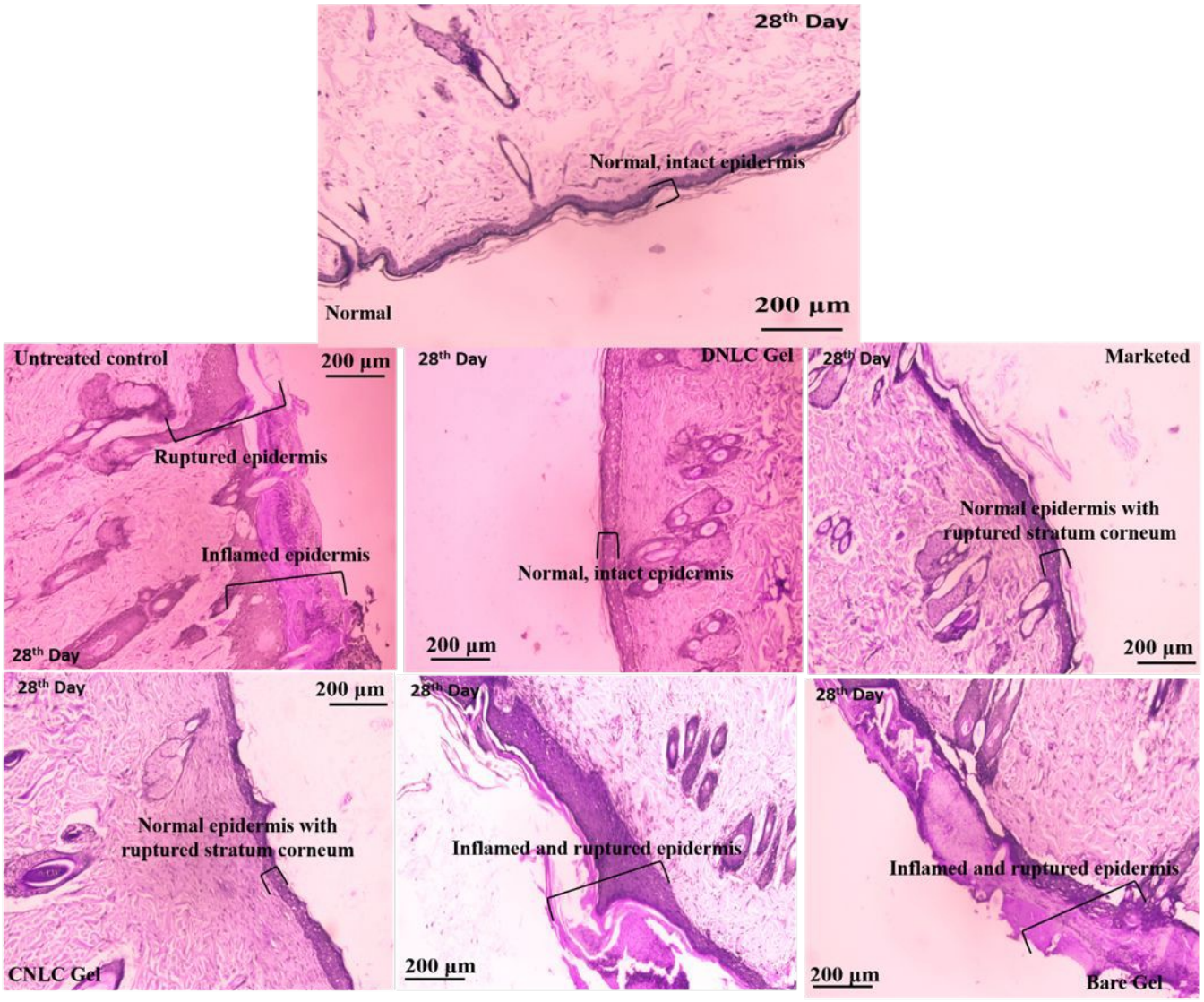

Figure 9. Histological evaluation of skin after the 28th day

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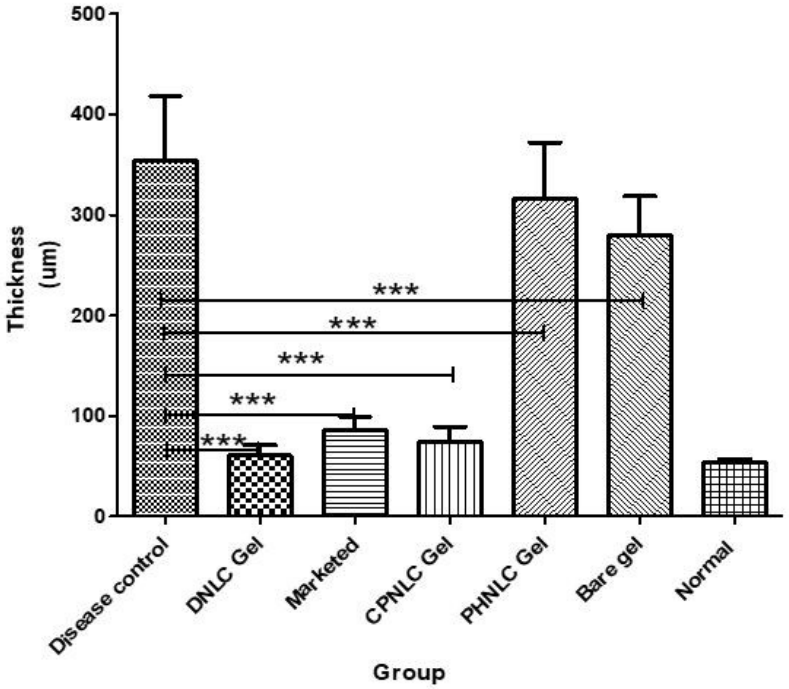

Figure 10. Epidermal thickness calculated from histological sections (n=6)

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The data supporting this article have been included as part of the Supplementary Information.