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Development and evaluation of imiquimod-loaded nanoemulsion-based gel for the treatment of skin cancer

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Abstract

Background The human skin, as the body's largest organ, is particularly sensitive to many chemical mutagens and carcinogens encountered in daily life. Skin cancer has become a notable global health concern, partly due to increased exposure to environmental pollutants and UV rays. Various treatments are available to treat skin cancer. Imiquimod is approved for the treatment of actinic keratosis and basal cell carcinoma. The present investigation aimed to develop nanoemulsion-based gel with imiquimod (2.5% w/w) and carbopol ultrez 10 NF using a modified method to enhance the solubility, permeation, and therapeutic effectiveness of imiquimod to treat skin cancer. Combinations of rose oil and oleic acid, with Tween 20/Propylene glycol as Smix, were used in the formulation. The formulation underwent evaluation for parameters such as % drug content, in vitro drug diffusion studies, viscosity, skin irritation, in vitro cytotoxicity assay (MTT assay) and the DMBA/ croton oil skin cancer in vivo model.

Results The formulation showed a minimum globule size of 118 nm, a zeta potential– 56.26 mV, a PDI of 0.378 and a drug content of 99.77%. In vitro drug release exhibited 45.00% of imiquimod release within 8 h, while approximately 34.32% release was found from the commercial cream. The imiquimod-loaded nanoemulsion-based gel showed significant cytotoxicity ($p < 0.001$) against the A431 cell line compared to Imiquad cream. The IC_{50} value of the imiquimod-loaded nanoemulsion-based gel was noted to be 10.76 ± 2.54 µg/mL. In vivo results showed a significant reduction in tumor incidence (16.66%), tumor volume (140.26 ± 3.48 mm^3), tumor burden (5.50 mm^3) and tumor mass (0.66 ± 0.05 g) compared with the DMBA/croton oil carcinogen treatment control group. Histopathological finding showed the absence of keratinized pearls, epidermal hyperplasia, and acanthosis in the formulation treated group.

Conclusion The results revealed that the nanoemulsion-based gel, with half the IMQ concentration of the commercial cream and incorporating Carbopol Ultrez 10NF, is a promising method for treating skin carcinogenesis. It potentially reduces dose-dependent side effects and demonstrating enhanced efficacy.

Keywords Nanoemulsion, Imiquimod, Skin cancer, Cytotoxicity, Nanoemulsion-based gel

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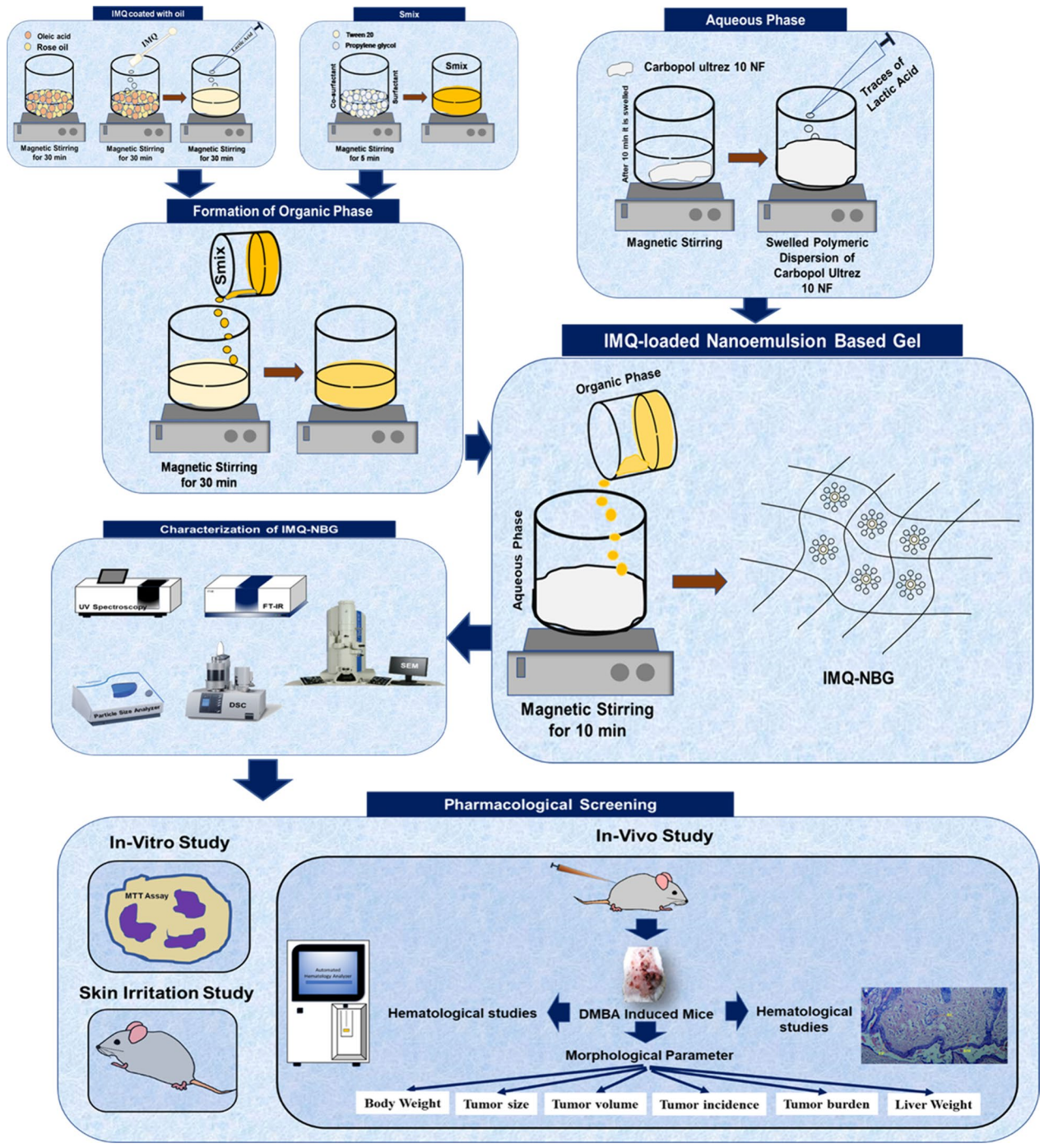
Graphical abstract



Introduction

The human skin, as the body's largest organ, is particularly vulnerable to various chemical mutagens and carcinogens encountered in everyday life [1]. Cancer is a non-communicable disease with a significant global burden. Cancer cases are on the rise due to environmental and lifestyle changes [2]. Skin cancer has become a prominent global health concern, primarily fueled by

heightened contact with environmental contaminants and ultraviolet rays. Specifically, within the demographic context of India, the prevalence of skin neoplasms constitutes a percentage ranging between 2 and 3% of the overall incidence of human cancers. In the USA, there are annually more than 2–3 million patients with non-melanoma skin cancer, exceeding the total incidence of all other cancer types combined [3].

Based on the findings gleaned from an extensive examination of existing literature, skin cancer is acknowledged as a highly prevalent ailment afflicting the global populace, constituting roughly 40% of newly identified cancer cases across the globe [4]. Topical drug delivery has gained attention for its noninvasiveness and convenience compared to parenteral and oral methods, making the skin a focal point for improving drug penetration; the skin's outer layer serves as a barrier, prompting efforts to enhance topical permeation [5, 6]. Nanoparticulate drug delivery systems are gaining prominence in topical applications due to advantages such as enhanced skin absorption, prolonged action, and protection of drug from degradation [7]. Emulgels provide a versatile and effective solution for advanced drug delivery, addressing solubility, stability, controlled release, bioavailability, and site-specific targeting, positioning them as a promising choice in targeted drug delivery systems [8]. Nanoemulsion-based gel (NBG) represents an advanced development in the realms of nanotechnology and pharmaceuticals. Its primary objective lies in augmenting the delivery and effectiveness of a broad spectrum of substances, ranging from medicinal drugs to topical products. This distinctive gel-based formulation incorporates the concept of nano-sized emulsions based gel, where minuscule droplets of one substance are uniformly dispersed within another [9]. This innovative approach imparts exceptional characteristics to NBG, including heightened absorption rates, enhanced stability, and controlled release mechanisms. As a result, NBG has demonstrated its utility across a wide array of disciplines, offering novel solutions for precise drug administration, the formulation of skincare items, and various other applications where optimized performance and meticulous precision are indispensable [10]. This introduction serves as a gateway to the exploration of the multifaceted realm of NBG, which has been investigated as promising carriers for topical delivery systems due to their capability to penetrate into the deeper layers of the skin [11].

In 1997, the USFDA approved imiquimod (IMQ) for therapy of external genital warts, and it was subsequently granted approval for actinic keratosis and basal cell carcinoma [12]. IMQ is distinguished by its characteristic as immune response modifier and has demonstrated potential antiviral and antitumor effects in preclinical studies [10]. IMQ, an imidazoquinoline moiety, acts as an immune modifier with a low molecular mass which possessing the ability to locally enhance various cytokines, including tumor necrosis factor-alpha, interferon-alpha, interleukin-1, interleukin-8, interleukin-6, interleukin-12 and many more. It augments the innate and adaptive immune responses, fostering an upregulation of endogenous defenses, thereby enhancing the body's intrinsic antitumor cell and antiviral responses [13]. IMQ activates TLR7 and TLR8, prompting proinflammatory cytokine production via NF-κB-driven transcription [14].

Additionally, IMQ is classified as a biological classification system (BCS) class IV pharmacotherapeutic agent, featuring a low molecular weight of 240.3 g/mol. It demonstrates limited penetrability attributed to its very low solubility in both hydrophilic and lipophilic solvents [15]. Consequently, formulating topical delivery methods for IMQ poses a significant challenge. The available IMQ cream has been associated with unwanted effects such as redness, erythema at the application site and itching, often leading to treatment discontinuation [16, 17]. Numerous researchers have delved into the preparation of nanoemulsions and microemulsions. Petrová et al. [18] pioneered the fabrication of four distinct nanoparticle types—lipid nanocapsules, liposomes, nanoemulsion, and nanocrystals—as carriers for IMQ. Their investigation showcased the efficient delivery of IMQ to skin tissue by these nanoparticles while mitigating undesired transdermal permeation, marking a notable advancement over conventional formulations. Panoutsopoulou et al. [19] specifically formulated microemulsions loaded with IMQ using phospholipids and oleic acid to enhance IMQ penetration into the epidermis. Additionally, Algahtani et al. [17] demonstrated the efficacy of IMQ in combination with curcumin within a NBG delivery system, achieving a drug release rate of approximately 78.00% within 8 h for the optimized formulation. Conversely, Kaur et al. [20] prepared nanoemulsions through the aqueous phase titration method, optimizing them via response surface methodology (RSM) using mixture design, Scheffe model. Their study reported a drug permeation rate of 73.67% within 6 h for the optimized formulation across the diffusion membrane. The studies mentioned above have indicated unsatisfactory results in terms of drug permeation or drug release. These challenges underscore the importance of conducting thorough research and development efforts to investigate alternative formulations and improve the therapeutic potential of IMQ. This is especially crucial in addressing the limitations linked to its limited dosage forms and penetration characteristics. Thus, formulating topical delivery methods for IMQ poses a significant challenge. Therefore, the current study focuses on the development of a nanoemulsion-based gel

of IMQ to enhance its solubility, permeation while minimize side effects, ultimately improving its therapeutic effectiveness against cancerous cells with an augmenting anticancer effect.

Experimental methods

Materials

IMQ was acquired as a complimentary material from Ferror Health Tech, Interquim S A, Spain. Propylene glycol, oleic acid, propyl paraben, lactic acid (98%), and phosphotungstic acid and sodium hydroxide were procured from Loba Chemie, Mumbai. Moreover, the rose oil was obtained from Research Laboratory Fine Chem Mumbai. Acetone obtained from Merck Life Sciences Pvt. Ltd, DMBA from Sigma-Aldrich, croton oil from Alvika Chemicals, Mumbai, formalin from Shree Chemical Industries, Pune, hematoxylin from Pathozyme, Kagal and eosin from SRL Pvt Ltd Mumbai. Carbopol ultrez 10 NF was gifted by Lubrizol Advanced Material India Pvt Ltd, Gujrat, and Tween 20 was received from Intas Pharmaceuticals Limited, Gujarat, as a gift sample. All other chemicals and reagents used were of analytical grade.

Cell culture and media

The A431 (epidermoid carcinoma) human cell line was procured from the National Centre for Cell Science (NCCS) in Pune. Antibiotic–Antimycotic 100X and Eagle's Minimum Essential Medium solution were sourced from Thermo fisher Scientific, India Pvt Ltd. Gibco Invitrogen USA provided the sample of Earle's Balanced Salt Solution (BSS), and Fetal Bovine Serum was sourced from Gibco Invitrogen USA.

Animals

The in-house facility of Crystal Biological Solutions, Pune, provided the male Swiss albino adult mice. All selected animals were accommodated in polypropylene cages and underwent a 1-week acclimatization period before the commencement of the study. The mice were fed commercial pelleted food provided by Nutrivet Pvt Ltd, Pune, and were given RO unit-passed potable water ad libitium during acclimatization and study. The environmental condition maintained at 22 ± 3 °C, with relative humidity at 55 ± 6°Cfor a 12:12 light/ dark cycle. The present study protocol was executed as per the norms approved by Institutional Animal Ethical Committee (IAEC) of Crystal Biological Solutions Pune (CRY/2223/086).

Preliminary screening of process parameters

The impact of the concentration of the gelling agent and stirring speed was investigated on viscosity and globule size, respectively. The concentration of the gelling agent affects the viscosity and appearance of the nanoemulsion gel. Simultaneously, stirring speed can affect the globule size of the nanoemulsion gel. To achieve the desired globule size with the required viscosity, the process parameter of stirring speed was assessed from 650 to 1050 rpm, while the concentration of the gelling agent, Carbopol Ultrez was changed from 0.5 to 2% w/w. Details are provided in Table 1.

Formulation of IMQ nanoemulsion-based gel

For the development of nanoemulsion oils, surfactant and co-surfactants were selected based on previous work [21]. Details of procedure is given below.

***Stage* 1:** The oils (rose oil and oleic acid) were mixed in concentrations as per Table 2 together in a glass beaker tightly covered with aluminum foil on magnetic stirrer for 5 min.

***Stage* 2:** IMQ was completely dissolved in this mixture of oil phase from stage 1 with stirring for 30 min on magnetic stirrer.

Table 1 Preliminary screening of process parameters

Parameters	Variable	
Concentration of gelling agent (%)	**Visual observation**	**Viscosity in poise**
0.5	Liquid	5.85 ± 0.092
1.0	Semisolid	17.36 ± 0.60
1.5	Semisolid	20.75 ± 0.18
2.0	Semisolid	31.37 ± 0.69
Stirring speed (rpm)	**Visual observation**	**Globule size in nm**
650	Improper mixing	194.73 ± 3.98
850	Homogenous formulation	118.46 ± 4.04
1050	Homogenous formulation	133.7 ± 2.52

Table 2 Formula composition of imiquimod nanoemulsion-based gel (IMQ-NBG)

Composition (%w/w)	IMQ-NBG1	IMQ-NBG 2	IMQ-NBG 3
IMQ	2.5	2.5	2.5
Oleic acid	10.71	10.71	10.71
Rose oil	2.14	2.14	2.14
Tween 20	19.28	19.28	19.28
Propylene glycol	19.28	19.28	19.28
Carbopol ultrez 10	1.0	1.5	2.0
Lactic acid	0.95	0.95	0.95
Methyl paraben	0.03	0.03	0.03
Sodium hydroxide solution (18%)	q.s	q.s	q.s
Purified water(qs)	100	100	100

***Stage* 3:** Lactic acid was added to stage 2.

***Stage* 4:** Smix was prepared by mixing Tween 20 and propylene glycol in 1:1 proportion. The ratio was selected from our previous study [21].

***Stage* 5:** Smix was added to stage 3 to constitute as an organic phase.

***Stage* 6:** The aqueous phase was prepared with aid of previously soaked carbopol ultrez 10 NF polymer (Table 2) with traces of lactic acid. Lactic acid is generally added to aqueous phase.

***Stage* 7:** Organic phase prepared in stage 5 added to aqueous phase prepared in stage 6 with magnetic stirring.

***Stage* 8:** The formulation was kept for an overnight, and pH was adjusted to 5.2 with traces of 18% NaOH as viscosity modifier.

Characterization of IMQ-NBG

IMQ content

The drug content of IMQ-NBG was assessed by dissolving 120 mg of IMQ-NBG into 100 mL of 0.1 M HCl solution and further diluted it with the same solution. The resultant aliquot of the sample was assessed by UV–visible spectrophotometer (Schimadzu 1800 Japan) at 243.50 nm.

$$\%\text{Drug content} = [\text{Sample absorbance/Standard absorbance}] \times [\text{Std dilution/Sample dilution}] \times \text{Avg Wt/label claim} \times \text{Standard potency} \quad (1)$$

Physical appearance, viscosity and pH analysis

The consistency, clarity and homogeneity of IMQ-NBG were assessed by visual inspection. Viscosity measurements of samples were done using a Brookfield viscometer with spindle C75, 100 rpm maintained at 25 ± 2 °C. The pH of IMQ-NBG was assessed using a digital pH meter (Hanna Instruments). Standard buffer solutions of pH 4, pH 9 were used to calibrate the digital pH meter prior to use. About 1 g of IMQ-NBG was dissolved in 100 mL of double distilled water and analyzed by the pH meter.

Spreadability studies

For the spreadability evaluation, about 2 g of IMQ-NBG was deposited onto a ground slide, which was then positioned between this slide and an identical glass slide featuring a hook [22]. A predetermined weight connected to the hook was employed for measurement using a pulley system. The duration taken for the slides to undergo the transition within the IMQ-NBG substance and stabilize under a defined load was documented in sec. The spreadability (S) was determined using the following equation:

$$S = ML/T \quad (2)$$

where M represents the weight applied to the pan, L is the distance from the hook to the center of the pan, and T denotes the duration of the slide transition [23].

Fourier Transform Infrared Spectroscopy (FTIR) analysis

The functional groups of IMQ and its interaction with excipients were assessed by FTIR in the range of 470–4100 cm^{-1}. All specimens were positioned on a sample holder and examined using an FTIR spectrophotometer (Bruker Alpha II, Japan) [21].

Differential Scanning Calorimetry (DSC)

The DSC thermogram of IMQ-NBG was conducted using Mettler Toledo (Star SW10). The heating process was carried at a fixed rate of 20 °C/min over a temperature range spanning from 40 to 340 °C. Simultaneously, a nitrogen purge was maintained at a selected flow rate of 100 mL/min. The identification of transition temperatures involved consideration the minimum values associated with the endothermic peaks noted in DSC curves [21].

Thermogravimetric analysis (TGA)

Thermogravimetric analysis was conducted using TGA 400 (Perkin Elmer, USA). Samples weighing 38.510 mg were heated at a rate of 10 °C/min from 0 to 350 °C. The protective gas utilized was nitrogen, flowing at a rate of 18 mL/min [24].

Size distribution and globule size

The globule size and size distribution of IMQ-NBG were performed based on dynamic laser scattering using a particle size/zeta potential analyzer (HORIBA Scientific SZ-100, Japan). IMQ-NBG was diluted 1:100 with

deionized water prior to analysis, and thereafter, size examination was conducted for 100 s at a selected angle of 90° [25].

Zeta potential

The zeta potential of IMQ-NBG was determined by using HORIBA-scientific SZ-100 [26]. IMQ-NBG was diluted 1:100 with deionized water prior to analysis. The prepared sample was filled into a transparent disposable zeta cell and analyzed by keeping sample for 60 s.

Transmission Electron Microscopy (TEM)

The surface morphological characterization of IMQ-NBG was determined using transmission electron microscopy (JEM-2100F, JEOL Ltd) at 200 kV. The IMQ-NBG was kept in a carbon coated copper metal grid followed by staining with 1% w/v phosphotungstic acid. Then, the air-dried IMQ-NBG 2 sample was exposed under HR-TEM [27].

In vitro drug release

The in vitro release of IMQ from the prepared nanoemulsion-based gel was carried out using a Franz diffusion cell (25 mL, 4.95 cm^2, Dolphin). The acceptor compartment was filled with 0.1 M HCl and allowed to stir at 400 rpm [21]. Commercial cream Imiquad 5% and IMQ-NBG 2.5% were applied to the donor compartment, separated by a dialysis membrane (Molecular cut off 12,000) previously soaked with release media. At each predetermined intervals, 1 mL of aliquots was withdrawn and replaced with the same amount of release media to maintain sink condition. Further sample dilutions were done appropriately and absorbances were measured by UV/vis spectrophotometer at the selected wavelength at 243.50 nm.

In vitro MTT assay

A431 cancerous cell lines were used to test the cytotoxicity of the developed formulations in vitro using the MTT technique [28–32]. Briefly, a constant strength of 1×10^4 cells/mL in a prepared medium was maintained, and the cells were incubated for a full day at 37 ± 2 °C with five percent CO_2. Subsequently, cells were seeded at 70 µL (1×10^4 cells) per well and 100 µL of IMQ-NBG samples were added to separate wells of a 96-well microplate. Control wells included a DMSO solution (0.2% in PBS) along with the cell line to determine cell viability and the percentage of live cells post-culture. The selected samples were incubated in triplicate for an additional full day under CO_2 incubation conditions (Thermo Scientific BB150). Afterward, the medium was aspirated, and 20 µL of 5 mg/mL MTT reagent solution in PBS was introduced into each well. Subsequently, the cells were re-incubated with the same solution for a 4 h at 37 ± 2 °C in the CO_2 incubator, during which formazan crystals formed, indicating viable cells through intense color formation. Following this, the medium was aspirated, and 200 µL of DMSO solution was added in every well, followed by 10 min of incubation wrapped with aluminum foil. The absorbance of IMQ-NBG samples was quantified at a wavelength of 550 nm using a microplate reader (Benesphera E21) to assess their impact and viability.

Equation (3) is used to calculate the percentage of viable cells:

$$\%Cell\ \text{viability} = (\text{Mean OD of test compound} / \text{Mean OD of negative control}) \times 100. \quad (3)$$

where OD is the optical density. The IC50 value was calculated using GraphPad Prism version 10.1.

Table 3 Scoring criteria for skin irritation study

Score	Description
0	No reaction
1	Slight, patchy erythema (light pink)
2	Moderate but patchy erythema (dark pink)
3	Moderate erythema (light red)
4	Severe erythema with or without edema (extreme red)

The interpretation of scoring; < 2 = slight irritation, 2–5 = moderately irritation and > 5 = severely irritation

Skin irritation studies

The selected group of mice, weighting between 20 and 25 g, were used in this study. Prior to the application of the formulations, the dorsal portion hairs of the selected mice were clean shaved using clipping and removed with the assistance of depilatory cream. The targeted areas were identified and left untreated for the next complete one day. Following this, a daily application of a topical gel (200 mg/mice) on the backs of the mice was carried out for a consecutive week.

Skin reactions were carefully observed and scored according to the criteria outlined (Table 3) [3]. Animals were categorized into three groups viz., Group I (Control) was also housed in normal condition without application, Group II was applied with blank gel (without IMQ), and Group–III was applied with IMQ-NBG 2.5%. Observation was conducted after a week.

In vivo anticancer study

In this animal study, Swiss albino mice were categorized into four distinct groups, each comprising six mice. Group I, the normal control group, served as the baseline, with mice receiving water and standard food pellets throughout the experiment, representing the healthy

control treated group. Group II (the carcinogen control group) was treated a single dose of DMBA (25 µg/100 µL of acetone) for the first 2 months, followed by the application of croton oil solution (1% in 100 µL of acetone) as a promoter three times per week for remaining 12 weeks [1]. Group III (Positive Control/Standard group/ Commercial Imiquad cream) received DMBA for the initial 2 months and then underwent treatment with a standard drug, Commercial Imiquad cream, applied after tumor formation on the selected animals for five times a week upto 6 weeks. Group IV received DMBA for the first 2 months and subsequently received the test formulation, IMQ-NBG 2.5% after tumor formation. The formulations were applied as decided dose for 6 weeks with a frequency of five times per week. Throughout the 14th weeks study, animals were closely monitored, with daily observations and weekly weight measurements. Various parameters were assessed during the experiment. At the conclusion of the 14th weeks, all animals were sacrificed, and serum and skin samples were collected for further analysis.

Morphological parameters

The study systematically evaluated various tumor parameters to comprehensively assess the impact of treatments on the Swiss albino mice [2]. Tumor incidence, denoting the proportion of mice that have at a minimum one tumor, was a key metric to gauge the overall susceptibility of the groups to tumor development. Tumor yield, distinct as the total average value of tumors per mouse, provided insights into the extent of tumor formation within each group. Tumor burden, calculated as the total average value of tumors/tumor-bearing mouse, offered a nuanced perspective on the severity of tumorigenesis. Tumor diameter and volume were measured to quantify the physical dimensions of the tumors, with the latter computed using a formula incorporating the three diameters. Additionally, tumor weight or mass, recorded at the study's endpoint, served as a crucial measure of the overall tumor load. Concurrently, the study monitored the body weight of treated animals weekly, providing a dynamic parameter to track potential treatment-related effects on the general health and well-being of the mice throughout the experimental period. These comprehensive evaluations aimed to elucidate the multifaceted impact of the experimental treatments on tumor development and overall physiological status. The following tumor parameters were screened [2] namely Tumor yield: an average numbers of tumors per animal; Tumor incidence: the number of animals carrying at minimum one tumor expressed as % incidence; Tumor burden: an average number of tumors per tumor-bearing animal; Tumor diameter: the average diameter of tumors; Tumor volume [4]: The tumor volume was quantified by using following Equation:

$$\text{Tumor volume} = (4/3)\pi[D1/2][D2/2][D3/2] \quad (4)$$

where $D1$, $D2$ and $D3$ are the 3 mm in diameters of the tumors; Tumor weight/mass: an average weight of tumors quantified at the end of the experiment and Body weight: an average body weight of selected animals measured weekly.

Hematological parameters

Blood samples were drawn from the treated animals into a vacutainer sterile container coated with EDTA as an anticoagulant, and the whole blood count was determined using an automated hematology analyzer.

Histopathological studies

The experimental animals' skin tumor regions were immediately excised and cleansed with a saline solution, and a section of skin was then fixed in formalin at a strength of 10% in strength. The skin sample were embedded in paraffin. Thin sections with a thickness ranging from 3 to 5 µm were prepared using a microtome. Subsequently, they were stained with hematoxylin and eosin dye [33]. Observation of the stained skin sections was conducted using a light binocular microscope (Meswox Cmos), and photomicrographs were captured for both group (control and experimental).

Stability studies

The optimized IMQ-NBG 2 was packed in glass vials and subjected to stability studies at 25 °C/60% RH, 40 °C/75% RH and 30 °C/65% RH for 6 months. Samples were evaluated for physical appearance, % drug content, pH, viscosity, globule size and zeta potential.

Statistical analysis

The present study for the optimization of nanoemulsion gel was determined by applying two-way ANOVA with Bonferroni's multiplying comparison test and accomplished using Graph Pad Prism 10.2 software. The findings were verified as statistically significant with a p value below 0.05.

Results

Preliminary screening of process parameters

An increase in the concentration of carbopol ultrez, from 0.5 to 2%, increased viscosity from 5.85 to 31.37 poise (Table 1). A concentration of 0.5% Carbopol Ultrez was found to be insufficient for preparing a nanoemulsion-based gel. Hence, the gelling agent was used at concentrations of 1–2% for further analysis and formulation. An increase in the stirring speed decreased the globule size [34], as shown in Table 1. The stirring speed of 850 rpm showed an optimum globule size of 118.46 nm, whereas

it was 194.73 nm at 650 rpm. A homogeneous formulation was observed at a stirring speed of 850–1050 rpm. The optimum speed was selected as 850 rpm for the formulation of IMQ-NBG.

Characterization of IMQ-NBG

Drug content

The IMQ content of IMQ-NBG 1–3 was determined to fall within the range of 98.42–99.77% (Fig. 1A). IMQ-NBG 2 showed highest drug content of 99.77%. No significant difference was observed between NBG 1–3.

Physical appearance, viscosity, pH analysis and spreadability studies

White IMQ-NBG 1–3 a viscous gel was observed. pH of formulations was found in range of 5.2–5.6.

Spreadability studies

Spreadability was found to be in the range of 10.31–15.72 g cm/s as shown in Table 4. The IMQ-NBG 2 showed the spreadability12.61 g cm/s.

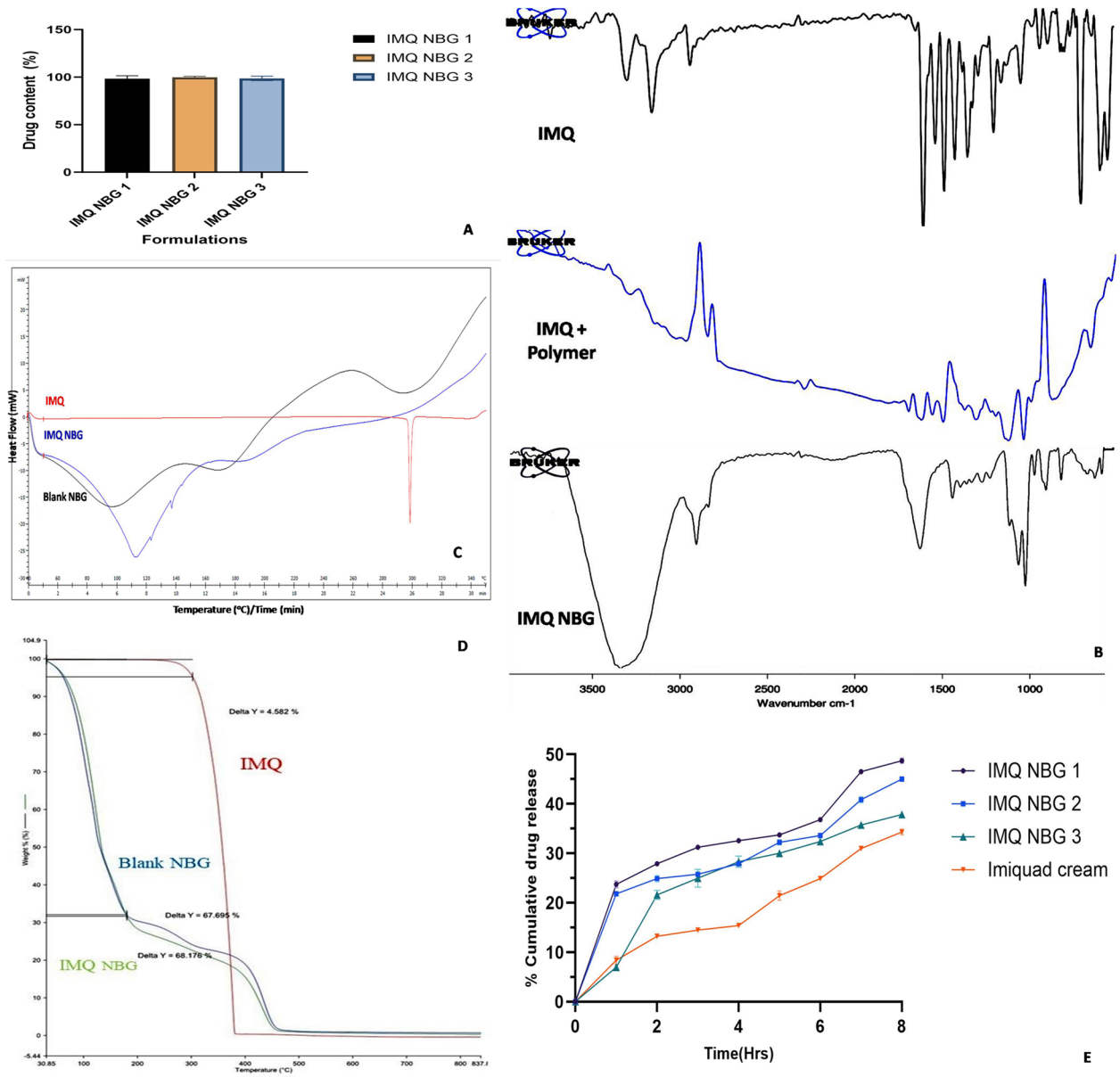


Fig. 1 Drug content of IMQ-NBG 1–3 (**A**), FTIR spectra of physical mixture and IMQ-NBG (**B**), DSC analysis (**C**), TGA analysis (**D**) and In vitro release of IMQ-NBG and commercial cream (**E**)

Table 4 Physical appearance, drug content, viscosity, pH analysis and spreadability studies

Formulation	Physical appearance	Viscosity (poise)	pH	Spreadability (g cm/s)
IMQ-NBG1	White semi liquid	17.36 ± 0.60	5.60 ± 0.20	15.72 ± 1.15
IMQ-NBG 2	White viscous gel	20.75 ± 0.18	5.65 ± 0.07	12.61 ± 0.46
IMQ-NBG 3	White viscous gel	31.37 ± 0.69	5.23 ± 0.25	10.31 ± 0.09
Imiquad cream	Yellowish semiliquid	0.45 ± 0.07	5.30 ± 0.10	17.32 ± 0.28
Voveran gel	White gel	47.87 ± 1.85	7.03 ± 0.15	8.58 ± 0.52

IR spectrum analysis

The FTIR spectra (depicted in Fig. 1B) of IMQ exhibited distinct peaks associated with different functional groups. It encompassed $N–H_{stretch}$ (1° amine), $N–H_{bend}$, $C=N_{stretch}$ (imine), $CH_{2stretchi}$ (alkane), CH_3, aromatic $C–H_{stretch}$, and aliphatic $C–H_{stretch}$ with characteristic wavenumbers at 3171, 1466, 1644, 1395, 1248, 2956, and 2929 cm^{-1}, respectively [21]. Physical combination of the polymer and IMQ exhibited distinctive peaks associated with different functional groups. These comprised $N–H_{stretch}$ (1° amine), $N–H_{bend}$, $C=N_{stretch}$ (imine), alkane $CH_{2stretch}$, CH_3, aromatic $C–H_{stretch}$, and aliphatic $C–H_{stretch}$ at specific wavenumbers, namely 3304.69, 1459.53, 1637.73, 1400.59, 1218.14, 2880.31, and 2999.86 cm^{-1}, respectively. However, the IMQ-loaded formulation also displayed comparable functional groups, such as $N–H_{bend}$, $C=N_{stretch}$ (imine), $CH_{2stretch}$ (alkane), CH_3, aromatic $C–H_{stretch}$, aliphatic $C–H_{stretch}$, and aromatic $C=C_{stretch}$, at wavenumbers 1459.44, 1645.03, 1414.30, 1244.79, 2925.19, and 2856.61 cm^{-1}, respectively, when contrasted with standard peaks. Particularly, the $N–H_{stretch}$ (1° amine) peak in the IMQ peaks overlapped with the formulation graph owing to the increased % transmission of the $O–Hs_{trech}$ peak induced by propylene glycol. Generally, the most of the peaks observed with their positions when comparing the IR peak values of the formulation to the standard IMQ drug graph [21].

DSC analysis

Figure 1C represents the melting pattern of IMQ, Blank NBG and IMQ-NBG 2. IMQ exhibited melting at 297.34 °C, whereas endothermic peaks at 112.5 °C and 187.11 °C were observed for IMQ-NBG 2 indicating the solubilized form of IMQ [21]. Blank NBG exhibited endothermic peaks at 95.74 °C, 174.23 °C and 291.92 °C due to surface oxidation.

Thermogravimetric analysis (TGA)

The first weight loss event (Fig. 1D) occurred up to 200 °C, with a loss of 67.695% and 68.176% for the blank nanoemulsion-based gel and the optimized nanoemulsion-based gel, respectively. This first weight loss is due to evaporation of volatile constituents.

The second stage weight loss of 21.92% at 350 °C for IMQ-NBG was observed due to the decomposition of IMQ after melting point, whereas the blank gel showed a 25.03% loss due to the degradation of components. However, a 27.939% weight loss was observed for blank NBG. The final weight change of 25.890% was observed at 450 °C in case of IMQ-NBG, attributed to multistage decomposition due to heating at a faster rate.

In vitro IMQ release

The IMQ release rate was used to assess the gel formulation's capacity to deliver IMQ [2]. The cumulative % release of imiquimod from IMQ-NBG and commercial cream at different sample intervals is shown in Fig. 1E. IMQ was released in a regulated way from IMQ-NBG 2, with 45% of IMQ released within 8 h, compared to 34.32% released from the commercial cream.

A significant increase in drug release was observed. IMQ-NBG1 showed 48.00% release, whereas IMQ-NBG 3 showed a 38.00% release of IMQ at the end of 8 h. No significant difference in drug release was observed with different concentrations of polymers (Table 2). The in vitro IMQ release data were fitted into zero-order and Higuchi models to describe the drug release patterns from the commercial cream and IMQ-NBG formulation, respectively (Table 5).

Optimization of IMQ-NBG

The optimization of IMQ-NBG was carried out with consideration of % drug content and in vitro IMQ release; hence, the IMQ-NBG 2 was selected as optimized nanoemulsion-based gel formulation.

Table 5 In vitro IMQ release kinetics from nanoemulsion-based gel and commercial product

Formulations	Zero order	First order	Korsmeyer Peppas		Higuchi model
	r^2	r^2	r^2	N	r^2
IMQ-NBG 1	0.826	0.880	0.878	0.32	0.943
IMQ-NBG 2	0.847	0.893	0.857	0.32	0.946
IMQ-NBG 3	0.872	0.941	0.872	0.72	0.961
Imiquad cream	0.969	0.967	0.936	0.655	0.928

Globule size and size distribution

A small droplet size is prerequisite for topical drug delivery. Optimized formulation IMQ-NBG 2 showed globule size of 118 nm with a PI value of 0.378 (Fig. 2A).

Zeta potential

Zeta potential indicates the stability of formulation. IMQ-NBG 2 showed (Fig. 2B) zeta potential of − 56.26 mV, illustrating the stability of the formulation.

TEM

The spherical shape of globules was confirmed with TEM analysis (Fig. 3). It was observed under different magnifications as small size with smooth surface.

In vitro cytotoxicity assay

The optimized IMQ-loaded nanoemulsion-based gel was evaluated for its anticancer potential through the MTT approach against A431 cell lines. IMQ-NBG demonstrated an IC50 value of 10.76 ± 2.54 µg/mL, signifying a significant difference compared to the commercial cream (IC50 = 17.31 ± 1.08 µg/mL) [21]. Figure 4A shows optimized IMQ-NBG 2 is more cytotoxic than Imiquad cream.

Skin irritation studies

Throughout the entire testing duration, Fig. 5 visually depicts the consistent absence of irritation on the skin of the control area. Consequently, considering the above observations, the irritation score remained below 1 until day 2, thus indicating that the current investigation identifies IMQ-NBG 2 as non-irritating to the skin of mice. Slight erythema with edema was observed in blank formulation and IMQ-NBG 2 but score was below 1. No discernible clinical toxicity was observed in any of the animals subjected to testing.

In vivo anticancer studies

Morphological parameters

In normal-treated control group (Group I), the gradual increased body weight was observed. Carcinogen-treated Group II mice showed significant reduced body weight ($p < 0.0001$) compared to Group I (Fig. 4B). Topical application of IMQ-NBG 2 significantly improved body weight ($p < 0.0001$) compared to Group II. Body weight measurement during the treatment period from week 1 to 6 showed no significant difference between standard commercial Imiquad cream-treated Group III mice and IMQ-NBG 2-treated Group IV mice. A significant decrease in total body weight was noted in the disease control group ($p < 0.0001$). The application of commercial Imiquad cream (Group III) and IMQ-NBG 2 (Group IV) demonstrated a significant increase in body weight ($p < 0.0001$) when compared to the carcinogen control group.

The liver weight significantly ($p = 0.0047$) reduced in carcinogen-treated Group II mice as compared to normal control (Fig. 4C). Test group IMQ-NBG 2 (Group IV) significantly ($p = 0.0247$) elevated liver weight as compared to carcinogen control. No significant difference ($p = 0.9964$) was obtained between standard treated mice (Group III) and IMQ-NBG 2-treated Group IV mice. No

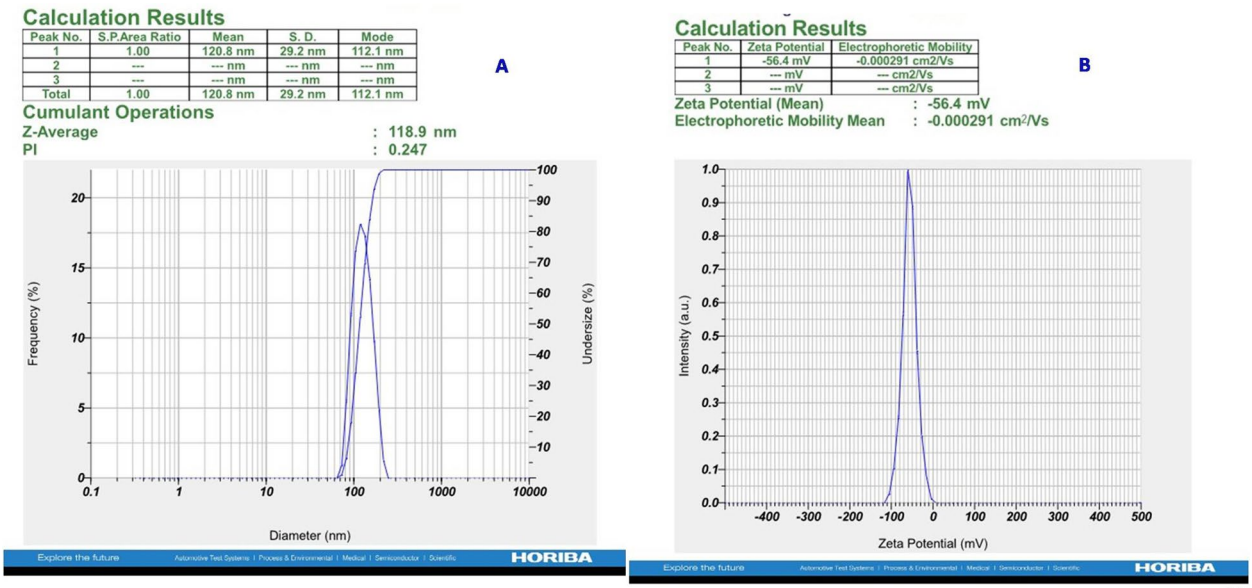


Fig. 2 Globule size (**A**) and zeta potential (**B**) of an optimized formulation IMQ-NBG 2

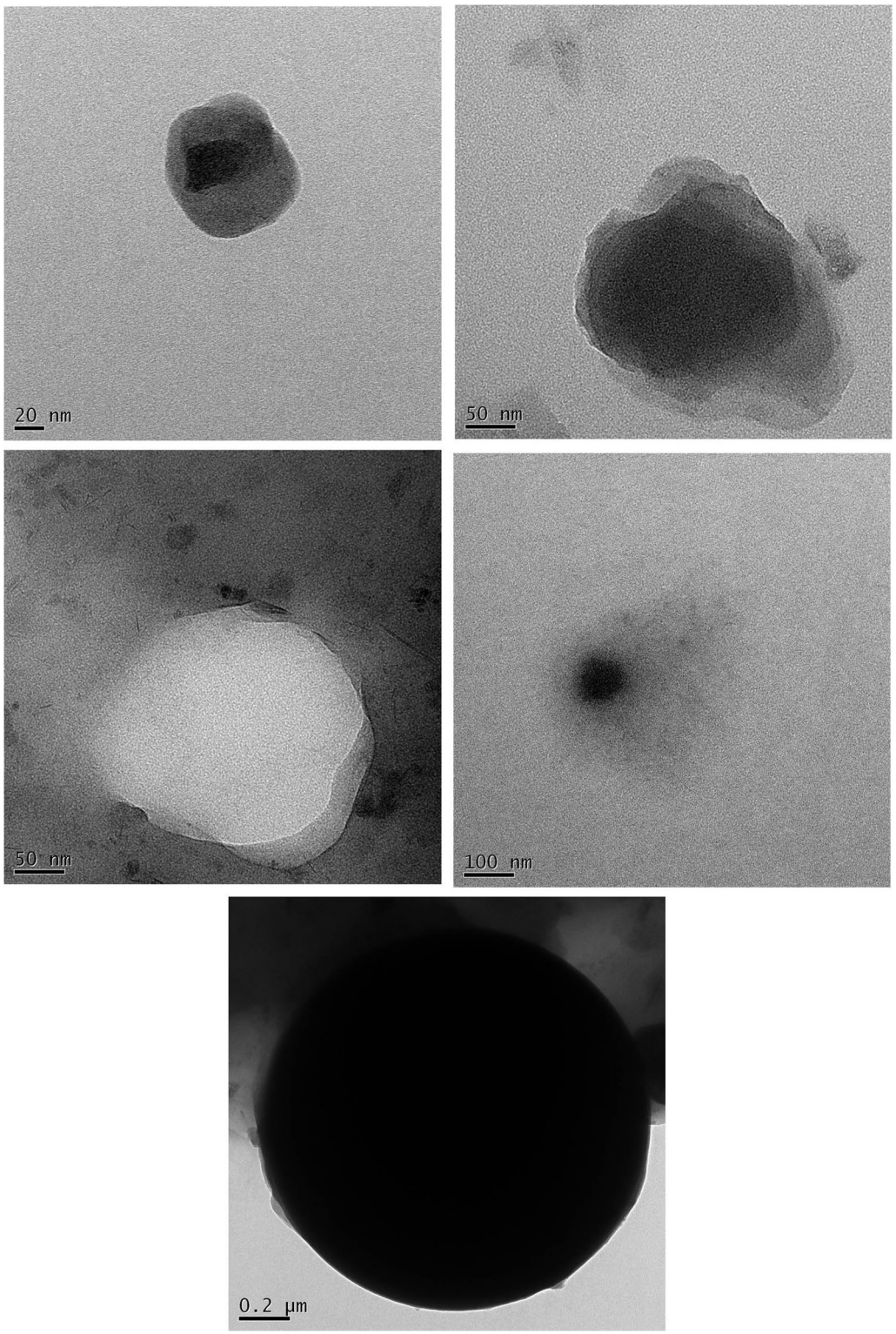


Fig. 3 TEM analysis of IMQ-NBG 2

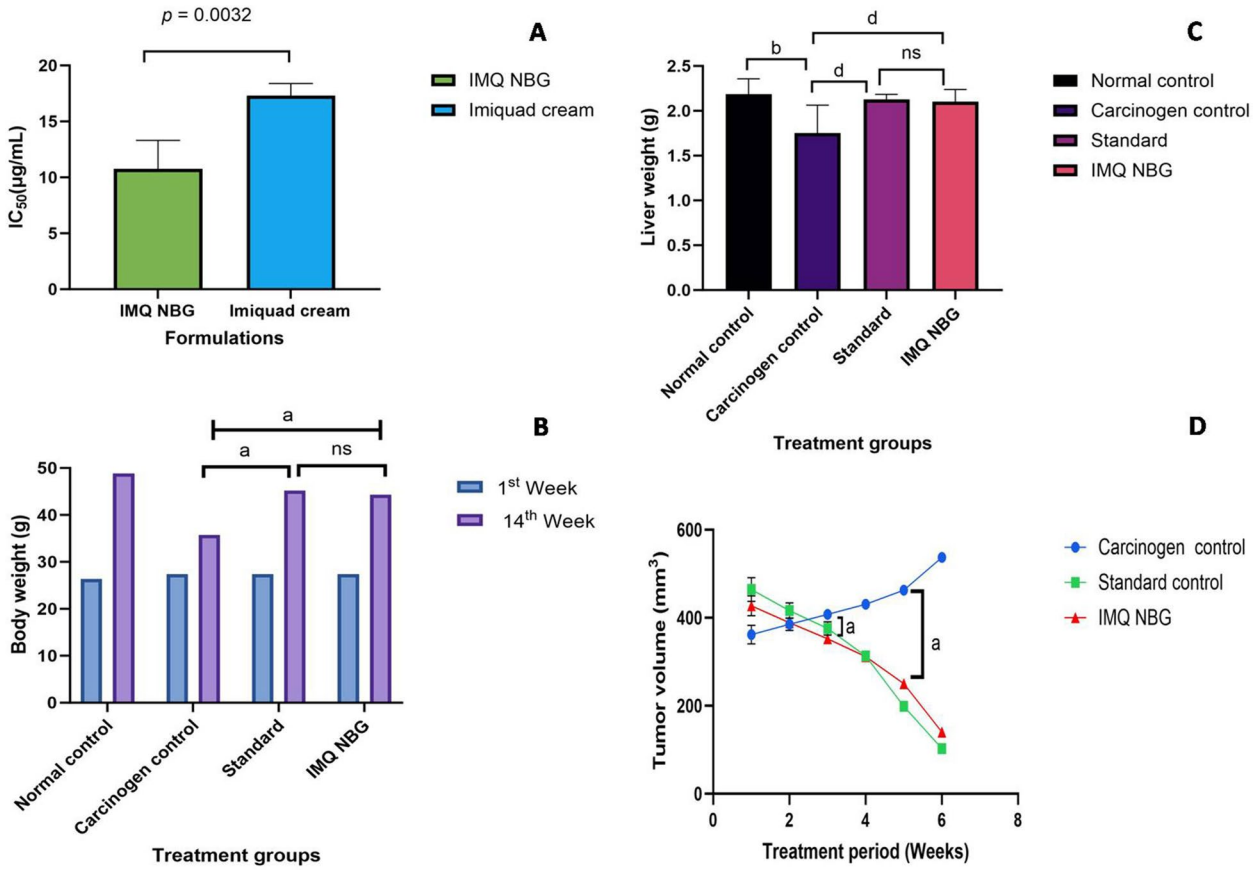


Fig.4 In vitro cytotoxicity assay result – IC50 value (**A**); In vivo anticancer studies Morphological Parameters—Body weight (**B**), Liver weight (**C**), and Tumor volume (**D**). *Note*: **a** $p < 0.0001$, **b** $p < 0.001$, **c** $p < 0.01$, **d** $p < 0.05$, ns $p > 0.05$

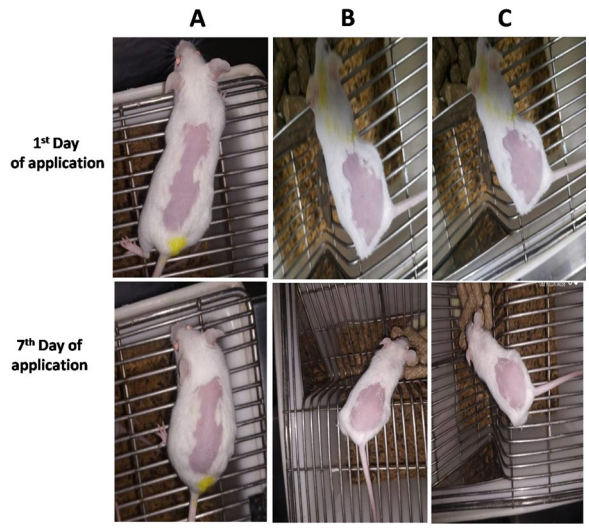


Fig. 5 Skin irritation studies of IMQ-NBG 2

significant difference was obtained when normal control-treated Group I mice compared with standard treated Group III mice and IMQ-NBG 2-treated Group IV mice.

The tumor volume, tumor incidence, and tumor burden of both groups (control and experimental) were computed for each respective group (Table 6). In carcinogen-treated Group II animal, 100% tumor formation with an actual mean tumor volume of 537.34 mm^3 was observed, whereas Group IV showed a tumor incidence and tumor volume was 16.66% and 140.26 mm^3, respectively.

Tumor volume was significantly ($p < 0.0001$) reduced after the application of the test formulation IMQ-NBG 2. After the 1st week of treatment with IMQ-NBG 2, a significant ($p < 0.0001$) lower in tumor volume was noted compared to the standard treatment group (Group III) (Fig. 4D). By the 4th week of treatment, no significant

Table 6 Morphological parameters at 14th week for in vivo studies

Groups	Tumor incidence (%)	Tumor mass (g)	Tumor volume (mm^3)	Tumor burden	Tumor yield
Normal control (Group I)	0	0	0	0	0
Disease control (Group II)	100.00	1.92 ± 0.23	537.34 ± 6.64	6.17 ± 0.98	6.17 ± 0.98
Standard (commercial cream) (Group III)	16.66	0.41 ± 0.15[a]	102.75 ± 4.68[a]	5.33 ± 0.51	5.33 ± 0.51
IMQ-NBG (Group IV)	16.66	0.66 ± 0.05[a]	140.26 ± 3.48[a]	5.50 ± 0.83	5.50 ± 0.83

Values are expressed as mean ± SD, a $p < 0.0001$,

difference between Group III and Group IV, indicating a probable similar anticancer potential. Topical application of IMQ-NBG 2 standard Imiquad cream totally prevented tumor occurrence in carcinogen treated mice. From the 5th week to the 6th week, a significant difference between was between the carcinogen treatment group (Group II) and standard group (Group III). Tumor mass and tumor burden were significantly ($p < 0.0001$) reduced to 0.66 ± 0.05 g (Fig. 6A) and 5.50, respectively, after treatment with IMQ-NBG 2, compared to 1.92 ± 0.23 g and 6.17 for Group II.

A statistically significant ($p < 0.0001$) decrease in tumor size was noted in Group III and IV when compared with disease control group (II) (Fig. 6B). In the last week of treatment, a significant difference ($p = 0.0338$) between III and IV was observed showcasing the comparable effectiveness of IMQ-NBG 2 with the commercial cream with in terms of tumor size. Tumor size measurement showed no any significant difference, maintaining a p value below 0.05 between the standard commercial Imiquad cream-treated Group III mice and the IMQ-NBG

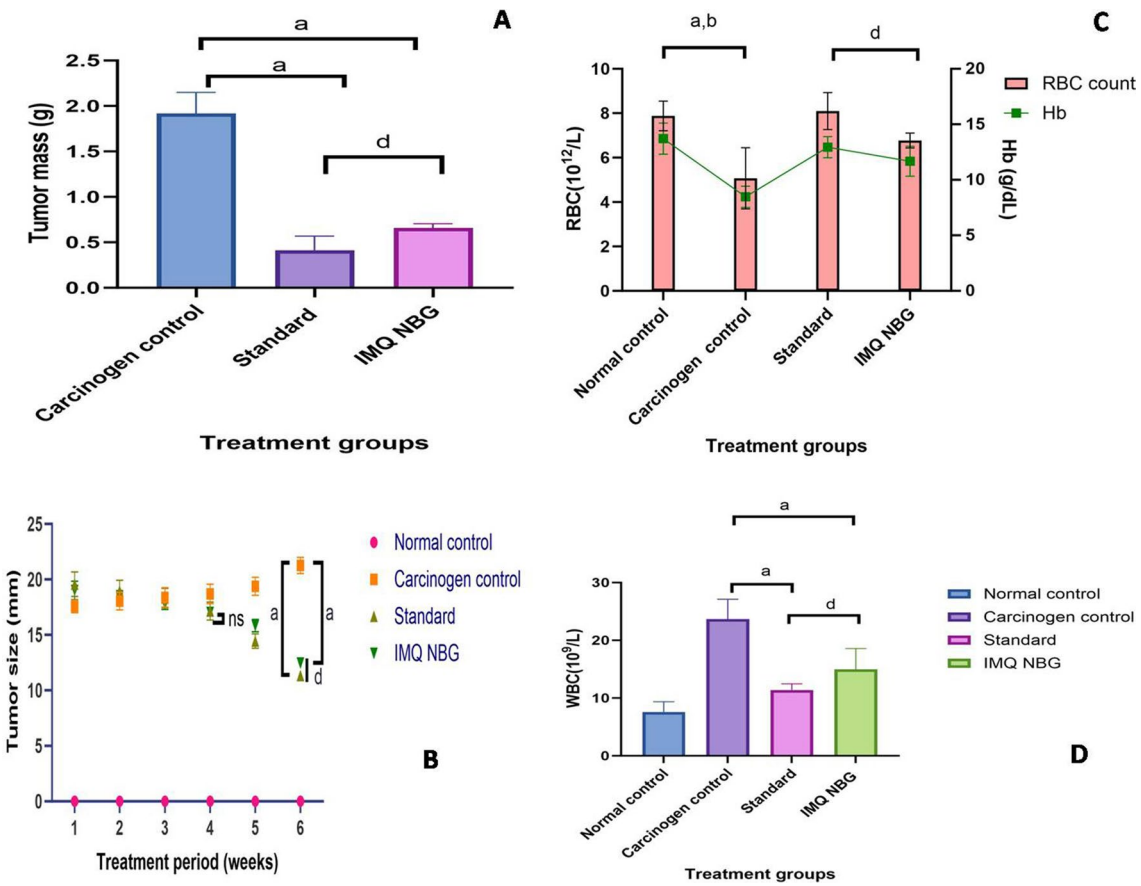


Fig. 6 In vivo anticancer study result—Tumor Mass (**A**) Tumor Size (**B**) RBC Count (**C**) and WBC Count (**D**)

2-treated Group IV mice at the end of 4th week of application of formulations.

Hematological studies

Figure 6C and D shows a significant decrease ($p < 0.0001$) in RBC and Hb content in the carcinogen-treated group when compared to control group (normal). However, a significant increase in RBC ($p = 0.0165$) and Hb ($p = 0.0008$) was observed when comparing Group IV with Group II. No statistically significant difference was observed between the standard group and the IMQ-NBG 2 test group, indicating the comparable effectiveness of IMQ-NBG 2 with Imiquad cream. A significant ($p < 0.0001$) decrease in WBC count was observed in standard-treated Group III mice and IMQ-NBG 2-treated Group IV mice compared to carcinogen-treated Group II mice. No statistically significant ($p = 0.1325$) difference in WBC count was observed in the case of animals in standard and IMQ-NBG 2 test group.

Table 7 depicts the increase in neutrophils, monocyte, eosinophils and basophils to 2.4 ± 0.75 10^9/L in case of carcinogen-treated group compared to normal control group (0.4 ± 0.15 10^9/L), whereas Group III and IV showed the values 8.1 ± 0.83 10^9/L and 6.8 ± 0.34 10^9/L, respectively, lesser than group II, indicating the healing behavior of the formulation against the cancerous cell line. Lymphocyte count was reduced in the case of Group II [35], whereas increase in count was observed in group IV.

In the removal of pathogens and the healing of wounds, neutrophils and polymorphonuclear leukocytes (PNL) are crucial [36]. Neutrophils and PNL are frequently seen at the site of inflammation when the body is experiencing inflammation for whatever reason (tumor differentiation, infection, or trauma). Activated neutrophils release many proteins into the area of inflammation. These proteins significantly impact the way the immune system forms [36]. According to clinical research, having neutrophilia may indicate a poor prognosis in individuals with cancer [37] confirming the results obtained in study.

Figure 6C and Table 7 shows the significant increase in Hb count of IMQ-NBG-treated group compared to the carcinogen control, whereas no significant effect was observed when Group III compared to group I. Platelet functions in maintaining hemostasis, platelets are also involved in inflammation, cancer invasion, and metastasis due to their abundance of bioactive chemicals.

Through a process known as tumor cell-induced platelet aggregation (TCIPA), tumor cells interact with platelets to help them evade the immune system [38]. The platelet count was about 499.50 (10^9/L) in the normal control group (Group I), whereas it significantly increased in standard (Group II) as 693.2 10^9/L. Treatment of standard and IMQ-NBG showed the platelet

Table 7 Hematological studies of IMQ-NBG 2

Parameter (unit)	Normal control Group I	Carcinogen control Group II	Standard Group III (commercial cream)	IMQ-NBG Group IV
WBC (10^9/L)	7.6 ± 1.78	23.7 ± 3.43	11.4 ± 1.07	16.0 ± 3.57
LYM (%)	82.4 ± 3.86	74.1 ± 10.81	72.0 ± 9.19	81.3 ± 5.44
MID (%)	9.2 ± 1.27	10.4 ± 1.42	9.0 ± 1.19	11.4 ± 1.75
GRAN (%)	5.3 ± 0.85	13.0 ± 2.52	9.9 ± 3.58	11.4 ± 2.31
LYM (10^9/L)	6.5 ± 1.62	18.3 ± 2.08	8.5 ± 1.59	14.1 ± 1.41
MID (10^9/L)	0.7 ± 0.12	2.9 ± 1.14	1.3 ± 0.58	3.3 ± 0.79
GRAN (10^9/L)	0.4 ± 0.15	2.4 ± 0.75	1.6 ± 1.03	3.1 ± 1.17
RBC (10^{12}/L)	7.9 ± 0.67	5.1 ± 1.38	8.1 ± 0.83	6.8 ± 0.34
HGB (g/dL)	13.7 ± 1.40	8.5 ± 0.96	12.9 ± 0.95	11.7 ± 1.37
HCT (%)	42.7 ± 1.90	30.2 ± 5.68	44.4 ± 3.59	41.0 ± 4.25
MCV (fL)	41.3 ± 4.34	56.9 ± 4.42	49.5 ± 3.12	49.2 ± 1.36
MCH (Pg)	16.9 ± 1.29	22.3 ± 2.19	16.8 ± 0.89	19.9 ± 1.71
MCHC (g/dL)	34.6 ± 1.54	25.2 ± 3.38	33.0 ± 1.39	31.3 ± 1.82
RDWCV (%)	13.2 ± 1.15	24.7 ± 3.34	15.2 ± 2.64	18.0 ± 2.75
PLT (10^9/L)	499.50 ± 50.91	693.20 ± 61.2	374.00 ± 60.54	424.20 ± 31.15
MPV (fL)	4.5 ± 0.26	7.1 ± 1.06	5.5 ± 0.80	7.3 ± 0.50
PDW (fL)	6.4 ± 0.16	10.8 ± 0.83	8.6 ± 0.65	9.5 ± 0.57
PCT (%)	0.3 ± 0.08	0.4 ± 0.05	0.3 ± 0.06	0.3 ± 0.03
PLCR (%)	0.61 ± 0.14	1.4 ± 0.21	1.1 ± 0.23	1.3 ± 0.34
PLCC (10^9/L)	49.2 ± 4.07	85.3 ± 5.72	53.0 ± 11.1	58.3 ± 2.80

count 374 (10^9/L) and 424 (10^9/L), respectively. It showed improvement in the cancerous cell state compared to the carcinogen control (Group II).

Histopathological findings

The skin layers of the mice in the normal control group, including the basal lamina, dermis, and epidermis, were normal (Fig. 7A). In the normal control Group I, no histological alterations were noted, the subcutaneous tissue was found to be normal. After receiving promoter and carcinogen therapy, differentiated squamous cell carcinoma was observed. The slides of Group II animal (Fig. 7B) demonstrated the formation of keratin pearls, invasion of the dermis by epidermal cells, and thickening of the epidermis (acanthosis). Group II exhibited a significant infiltration of cancerous cells into the underlying dermis. The standard treatment group III showed decreased hyperplasic papillomatous lesions (Fig. 7C). In Fig. 7D, the IMQ-NBG 2 treatment group IV displayed minimal hyperplasia, no pearl formation, and no signs of infiltration.

Stability studies

The physical appearance of the optimized IMQ-NBG 2 was assessed at 25 °C/60% RH, 40 °C/75% RH and 30 °C/65% RH with intervals of 1, 3 and 6 months (Table 8). The formulation was found to be stable at above listed conditions except at 40 °C (at the 6-month time point). Samples at 40 °C showed no significant change in parameters such as % drug content and pH. However, at 6 months (40 °C/75% RH), there was a significant change in globule size, zeta potential, and viscosity.

Discussion

IMQ is currently formulated exclusively as a cream [39], limiting its available dosage forms. Additionally, it is classified as a BCS Class IV drug, implying challenges in terms of bioavailability and permeation. Notably, there is a lack of in vitro permeation studies for imiquimod, which further complicates the understanding of its penetration characteristics. These factors collectively underscore the need for comprehensive research and development efforts to explore alternative formulations and enhance the therapeutic potential of imiquimod, especially in addressing challenges associated with its limited dosage forms and penetration characteristics as a BCS Class IV drug. IMQ presents a dual challenge in its formulation and development. The drug's limited water solubility necessitates exploration by formulation scientists into solubility enhancers and other techniques to enhance drug dissolution in aqueous solutions or suspensions. Simultaneously, stability issues, such as potential degradation over time, introduce concerns about the product's shelf life. Essential stability studies are therefore

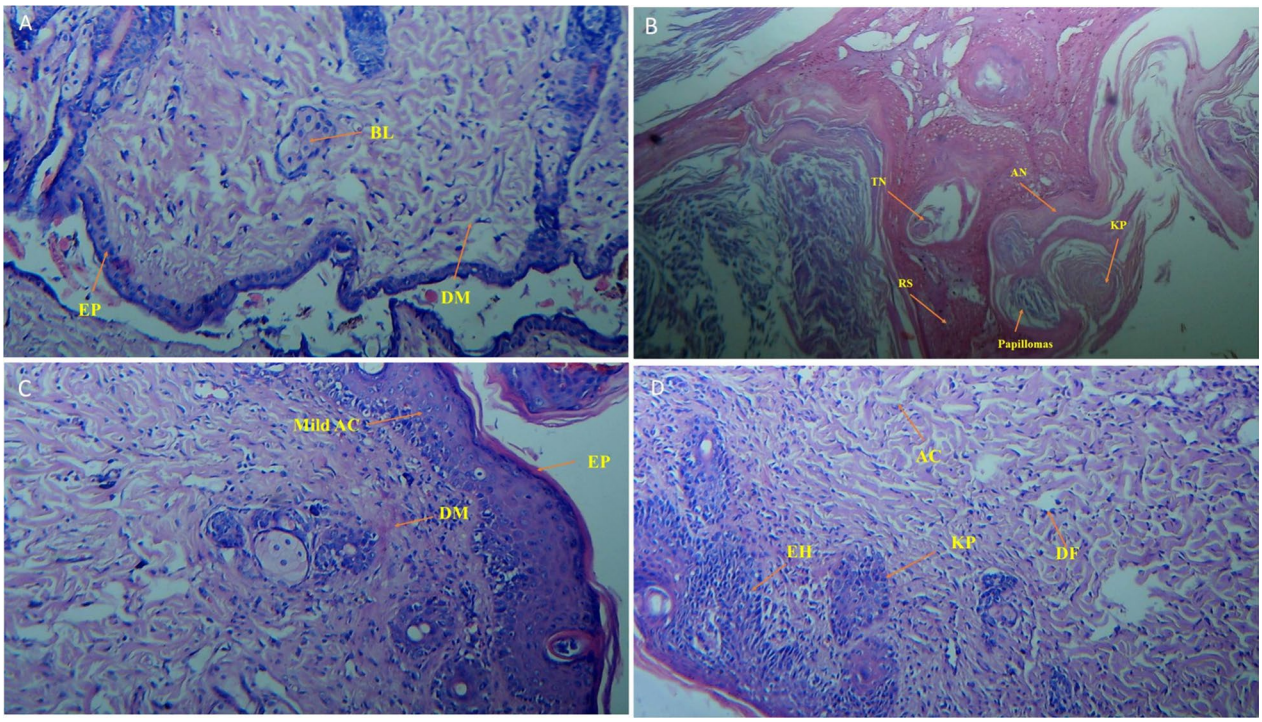


Fig. 7 Histopathological study of treated mice (**A**) Group I (**B**) Group II (**C**) Group III and (**D**) Group IV

Table 8 Stability studies of IMQ-NBG 2

Stability condition	Parameters	Time period (months)			
		0	1	3	6
25 °C/60%RH	Globule size (nm)	118.46 ± 4.04	120 ± 1.00	121.4 ± 1.50	125 ± 3.00
	Zeta potential (mv)	56.266 ± 1.00	52.933 ± 1.40	49.667 ± 1.89	43.366 ± 2.91
	Drug content %	99.77 ± 1.18	99.35 ± 0.35	98.15 ± 0.06	98.31 ± 0.37
	Viscosity (Poise)	20.753 ± 0.189	20.053 ± 0.976	19.866 ± 0.11	19.89 ± 0.078
	pH	5.65 ± 0.07	5.53 ± 0.06	5.51 ± 0.09	5.50 ± 0.10
	Physical appearance	White viscous gel	White viscous gel	White viscous gel	White viscous gel
40 °C/75%RH	Globule size (nm)	118.46 ± 4.04	122 ± 1.00	126 ± 1.5	133.7 ± 2.52
	Zeta potential (mv)	56.266 ± 1.00	49.39 ± 1.211	48.946 ± 2.46	46.896 ± 1.53
	Drug content %	99.77 ± 1.18	99.09 ± 1.48	98.733 ± 0.515	98.526 ± 0.11
	Viscosity (Poise)	20.753 ± 0.189	20.053 ± 0.950	19.87 ± 0.105	17.45 ± 0.332
	pH	5.65 ± 0.07	5.57 ± 0.06	5.5 ± 0.17	5.4 ± 0.20
	Physical appearance	White gel	White viscous gel	White viscous gel	Slight changes in appearance
30 °C/65%RH	Globule size (nm)	118.46 ± 4.04	121.67 ± 1.52	126.33 ± 0.57	129.66 ± 0.50
	Zeta potential (mv)	56.266 ± 1.00	49.25 ± 1.21	48.95 ± 2.46	43.7 ± 3.25
	Drug content %	99.77 ± 1.18	99.266 ± 0.72	98.66 ± 0.49	98.52 ± 0.19
	Viscosity (Poise)	20.753 ± 0.189	20.19 ± 0.840	20.233 ± 0.125	19.77 ± 0.026
	pH	5.65 ± 0.07	5.50 ± 0.10	5.41 ± 0.10	5.47 ± 0.15
	Physical appearance	White viscous gel	White viscous gel	White viscous gel	White viscous gel

crucial to ensuring the drug's sustained efficacy throughout its intended duration.

As imiquimod is frequently formulated as a topical cream, the next hurdle lies in achieving an optimal equilibrium between drug release and skin permeation, requiring formulators to experiment with various excipients for enhanced skin penetration while maintaining controlled release. Compounding this, imiquimod's tendency to cause skin irritation [40] underscores the need for formulations designed to minimize irritation while preserving therapeutic effectiveness—calling for careful selection of excipients or exploration of alternative drug delivery methods. Moreover, dosage form optimization becomes imperative, with formulators tasked with determining the ideal concentration of imiquimod to strike a delicate balance between achieving the desired therapeutic effect and avoiding adverse reactions. This intricate interplay of solubility, stability, topical delivery, irritation mitigation, and dosage optimization encapsulates the nuanced challenges inherent in imiquimod's formulation and development.

Moreover, in our formulation strategy, we have incorporated the use of rose oil and oleic acid to introduce a targeted emollient effect at the application site. This deliberate choice aims to leverage the emollient properties inherent in rose oil and oleic acid, intending to enhance the formulation and tactile attributes of the product upon application. Rose oil brings several advantages over medium-chain triglyceride (MCT) oil when utilized in formulation. Firstly, its pleasant floral fragrance enhances the sensory appeal of the formulation, a quality lacking in odorless MCT oil. Moreover, rose oil is renowned for its therapeutic properties, including anti-inflammatory and antioxidant effects, which can provide added benefits to skin care or cosmetic formulations [41]. Additionally, the inclusion of rose oil adds a touch of luxury and elegance to the formulation, making it more attractive to consumers, especially those interested in natural or botanical-based products. Lactic acid is an ingredient used due to its antioxidant potential, smooth effect on rough area and moisturize the skin. [42]. Lactic acid is added in traces to aqueous phase with gelling agent carbopol ultrez 10NF for probable penetration enhancement of formulation.

The crucial innovation in our work is centered on a distinctive combination of two separate oils within a nanoemulsion-based gel. This novel approach represents a departure from conventional formulations and encapsulates a multifaceted strategy to enhance the overall performance and therapeutic attributes of the product. By strategically blending different oils and incorporating them into a nanoemulsion gel matrix, we aim to achieve a harmonized and optimized formulation that combines the unique benefits of each component. This innovative combination is designed to impart enhanced stability, improved bioavailability, and potentially significant effects, contributing to a more

efficacious and versatile product. This strategic integration of oils within a nanoemulsion framework represents a pioneering advancement in formulation science, with the potential to offer novel solutions and applications across various domains. Our primary objective was to formulate a gel with a release rate significantly slower than that of an emulsion. This deliberate modulation of drug release kinetics addresses concerns related to the accelerated release associated with emulsions, which could potentially exacerbate symptomatic side effects. By incorporating a gelling agent, we aimed to entrap the volatile constituents of rose oil within the gel matrix. The strategic use of a gel formulation not only enables controlled release over an extended period but also improves patient acceptability, providing a more favorable and adherent alternative compared to traditional Imiquad cream. This broader formulation strategy seeks to strike a balance between therapeutic efficacy and patient comfort, showcasing the potential for improved outcomes and user satisfaction.

Hence, the principal aim of this investigation was to formulate and assess the IMQ-NBG utilizing combinations of oils facilitating solubilization of IMQ with minimal drug side effects. An attempt has been made to formulate IMQ-loaded NBG having drug strength 2.5% to minimize the drug induced side effects with improvised drug release. From our previous study conducted on nanoemulsion system [21], we selected the combination of rose oil and oleic acid as a phase of oil, Tween 20: Propylene glycol as Smix. DSC thermogram of IMQ nanoemulsion-based gel showed solubilization of IMQ in the prepared formulation with indication of no any peak observation at melting point of IMQ. The detection of an initial endothermic peak within the IMQ-NBG 2 was observed due to evaporation of water from gel base. Water takes time to come out of gel base hence peak observed 111.52 °C and 187.11 °C.

Significantly, an interesting observation in the IR spectra was the merging of the N–H stretch peak (characteristic of 1° amine) in the drug spectra with the corresponding peak in the formulation graph. This occurrence can be attributed to the increased percentage of transmission in the O–H peak, a result of the occurrence of propylene glycol in the IMQ-NBG. It is important to highlight that this merging of peaks emphasizes the impact of the constituents of the formulation on the resulting infrared (IR) spectrum.

Upon detailed scrutiny, it becomes apparent that, overall, most of the observed peaks remained in their anticipated positions when matching the IR peak values of the formulation with those in the standard drug peak. This consistency in peak positions suggests the overall structural integrity and chemical similarity between the formulation and the reference drug, notwithstanding the observed interaction between the N–H and O–H peaks.

Carbopol was chosen as the gelling agent for NBG based on its capacity to produce a homogeneous dispersion attributable to its elevated water solubility [43]. Furthermore, it demonstrates the capability to yield a transparent gel upon introduction of an alkaline reagent [44, 45]. Gelling agent carbopol ultrez 10 NF might have assisted to maintain intact shape of globules. Preliminary screening of process parameters and stirring speed showed a significant change in the globule size of the nanoemulsion-based gel. Stirring speeds up to 850 rpm resulted in decreased globule size, and a further increase in stirring speed had no impact on globule size reduction. The higher the concentration of carbopol, the greater the viscosity [46].

Globule size below 300 nm exhibited the nanodrug delivery system of formulation. The small globule size facilitates the higher in vitro release. Low polydispersity indicates uniformity of droplet in formulation [47]. The image unequivocally depicted uniform nanoscale globules, each measuring less than 200 nm, displaying a homogeneous distribution, as illustrated in Fig. 6B. The spherical configuration of these globules was duly confirmed. The surface potential of the resuspended droplets can be well described by the zeta potential. Therefore, by determining the zeta potential values, the electrical characteristics of the IMQ-NBE were measured [48, 49]. Stability is ensured by a high zeta potential value because electrostatic repulsion between the droplets can stop them from aggregating if the charge is large enough [48]. Zeta potential value more than − 30 mV indicate stability [50]. The obtained zeta potential value − 56.26 mV is confirming the stability of formulation.

Drug content obtained in the range of 98.42–99.77%, which indicating the homogeneity of formulation. No significant impact of gelling agent was observed on drug content. There is no any significant difference noted between formulation and commercial cream. For semisolid dosage forms like creams, gels and implants that deliver pharmaceuticals over the specified period of time spans from hours to days, the release of drugs is a significant importance [51, 52]. IMQ-loaded nanoemulsion-based gel (IMQ 2.5%) showed 1.3fold drug release compared to commercial cream (IMQ 5%) at the end of 8 h. Small globule size (118 nm) of IMQ-NBG 2 facilitate the high drug release. Use of appropriate combinations of oil, Smix and gelling agent may imparted the significant % CDR compared to Imiquad cream.

So as to elucidate the mechanism of IMQ release, a comprehensive investigation was conducted by plotting the cumulative drug release using several kinetic models. These models characterize release rates in terms of time,

strength, square root of time, and diffusion coefficient. This approach provides a comprehensive understanding of the intricate dynamics, offering insights into temporal, concentration-dependent aspects, and diffusion characteristics of the drug release process [53, 54]. Prepared formulations showed Higuchi models, whereas Imiquad cream illustrated zero-order release. In the context of IMQ-NBG 2, drug particles exhibit significantly reduced dimensions compared to the thickness of the system. This configuration facilitates unidirectional drug diffusion, with the drug diffusivity remaining constant [55]. The formulations IMQ-NBG1 and 2 depicted the fast release at in initial 1 h attributed to lower concentration of gelling agent and probable minimum particle size. However, the IMQ-NBG 3 demonstrated the slow release compared to IMQ-NBG 1 and 2 (Fig. 4). In vitro release profile showed no significant difference between observed IMQ-NBG 1 and 3, IMQ-NBG 2 and 3, whereas at 4 h, release study revealed no significant difference between NBG 1 and 2. After 4 h a significant impact of different concentration of gelling agent was observed. However, a significant difference in commercial cream and formulations.

Statistical analysis showed no significant difference between IMQ-NBG 1–3 with respect to in vitro drug release, whereas significant difference obtained between IMQ-NBG 2 and commercial cream reveal the higher drug release. IMQ-NBG 2 demonstrated substantially increased cytotoxicity on A431 cell line compared to commercial cream. Formulation having high in vitro drug release, drug content, desired viscosity and pH was selected as desired batch for further screening its In vitro and in vivo anticancer potential.

The in vivo study investigated the effects of topical application of IMQ-NBG 2 on DMBA/croton oil-induced carcinogenesis. The observed results demonstrated a significant suppression of carcinogenesis in the treated groups (Fig. 8). The study characterized skin cancer development into three stages: initiation, propagation (dysplasia, hyperplasia), and progression. IMQ-NBG 2 showed potential anticancer effects as evidenced by a decrease in tumor size and volume. Body weight measurements indicated a normal increase in body weight in the treated groups, contrasting with a decrease observed in the disease control group. The study also compared IMQ-NBG 2 with a standard commercial Imiquad cream, showing comparable effectiveness in terms of body weight and tumor size. Tumor incidence and volume significantly decreased in the sample applied groups compared to the control group (carcinogen). These findings suggest the anticancer potential of IMQ-NBG 2 throughout the treatment period. Figure 7A shows no histopathological changes were observed in the normal control. Normal subcutaneous tissues were observed in the control group normal epidermal tissue, underlying subcutaneous tissue, dermis and basal lamina. Disease control group showed proliferating neoplastic cells (Fig. 7B). In an advanced manifestation of squamous cell carcinoma, distinct features include the development of keratin pearls and substantial infiltration of cancerous cells into the underlying dermal layers. Standard group exhibited reduced the hyperplasic papillomatous lesions (Fig. 7C). On application of IMQ-NBG resulted in hydration of stratum corneum which may facilitate penetration of IMQ to deeper layer of skin. The observed phenomenon could be linked to a reduction in the density of corneocytes and an expansion of the intercorneocyte spaces [3]. The experimental group subjected to IMQ-NBG 2 treatment manifested mild hyperplasic papillomatous lesions characterized by cellular atypia, and notably, there was an absence of evidence indicating infiltration (Fig. 7D). Stability study

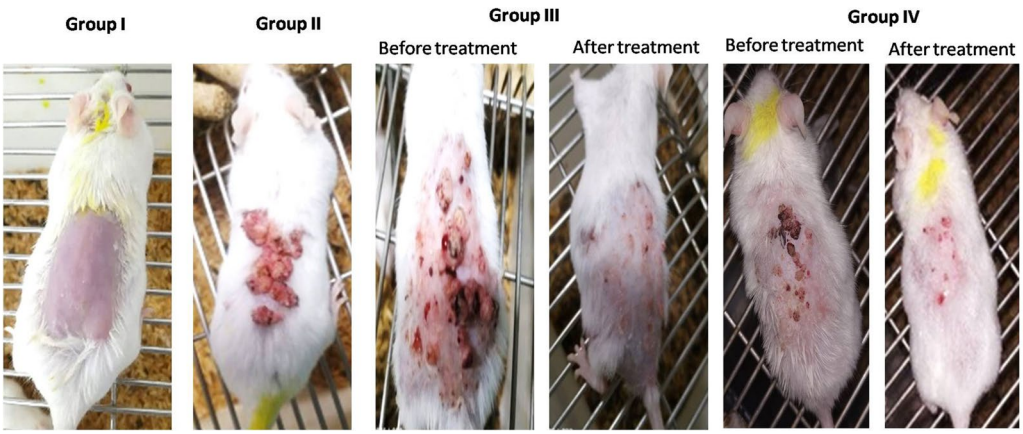


Fig. 8 Recovery of the animals after the treatment

showed no significant change in % drug content, pH, globule size, zeta potential and physical appearance of formulation at 25 °C, 30 °C.

Moreover, the present work extends the findings of Jadhav et al. [18], who initially developed the IMQ nanoemulsion, by advancing to the preparation of IMQ-NBG, thereby offering further insights into their comparative performance and potential applications. The comparison between IMQ nanoemulsion and IMQ-NBG formulations reveals significant differences in their physical and functional properties. IMQ nanoemulsion has a larger globule size of 154.2 nm compared to the 118 nm of IMQ-NBG. Both formulations exhibit similar zeta potentials, with IMQ nanoemulsion at − 59.0 mV and IMQ-NBG at − 56.26 mV, indicating comparable stability profiles. The IC50 values, which measure the efficacy of the formulations, show that IMQ-NBG has a slightly lower value (10.76 ± 2.54 µg/mL) compared to IMQ nanoemulsion (13.20 ± 1.80 µg/mL), suggesting that IMQ-NBG might be more effective at inhibiting cell growth. In terms of in vitro drug release, IMQ nanoemulsion releases 69.92% of its content within 8 h, significantly higher than the 45.00% release observed for IMQ-NBG. Additionally, the release kinetics differ between the two; IMQ nanoemulsion follows a zero-order release model, indicating a constant release rate, whereas IMQ-NBG follows the Higuchi model, which is characteristic of a diffusion-controlled release.

Conclusion

The topical IMQ-NBG formulation was successfully developed with half the concentration of IMQ compared to the commercial cream and the incorporation of Carbopol Ultrez 10NF, which had the property of swelling at a low pH. The formulation was characterized for selected parameters such as % drug content, in vitro release, MTT assay and in vivo anticancer studies. IMQ-NBG (2.5% w/w IMQ) significantly improved the in vitro IMQ release and cytotoxicity potential compared to Imiquad cream (5% w/w IMQ). The prepared formulation IMQ-NBG has a pH near to the skin. The formulation was non-irritant to skin. In vitro release study validated the significantly increased IMQ release compared to commercial Imiquad cream. In vivo studies proved the significant decreased in tumor size, tumor mass, tumor volume when comparison was done between IMQ-NBG and carcinogen control. The results of present investigation suggest that topical IMQ-based nanoemulsion-based gel has anticancer potential in DMBA/ croton oil-induced skin cancer. The stability study reveal that IMQ-NBG 2 is stable at 25 °C, 30 °C. Our study concludes that IMQ-NBG might be a promising moiety to treat skin carcinogenesis.

Abbreviations

IMQ	Imiquimod
Smix	Surfactant and cosurfactant mixture
NBG	Nanoemulsion-based gel
DMSO	Dimethyl sulfoxide
UV	Ultraviolet
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared spectroscopy
TEM	Transmission electron microscopy
BCS	Biopharmaceutics classification system
PBS	Phosphate buffer saline
SD	Standard deviation
ANOVA	Analysis of variance
nm	Nanometer
hrs	Hours
Rpm	Rotations per minute
Min	Minutes
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
CDR	Cumulative drug release
US	United States
FDA	Food and Drug Administration
NCCS	National Centre for Cell Science
IIT	Indian Institute of Technology
EP	Epidermis
KP	Keratin pearl
AC	Acanthosis
DM	Dermis
EH	Epidermal hyperplasia
DF	Dermal fibroblast
BL	Basal lamina
RS	Residual stroma
AN	Atypical nuclei
TN	Tumor Necrosis
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
RDW	Red blood distribution width
PLT	Platelet count
MPV	Mean platelet volume
PDW	Platelet distribution width
LYM	Lymphocytes
GRAN	Granulocytes
MID	Rare blood cells
PCT	Plateletcrit
PLCR	Platelet–large cell ratio
HGB	Hemoglobin

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Author contributions

All authors have contributed to the conception and design of the study. The initial draft of the manuscript was authored by Shital Jadhav, with subsequent input and revisions provided by all authors on prior iterations of the manuscript. All authors have thoroughly reviewed and endorsed the final version of the manuscript.

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Declarations

Ethics approval and consent to participate
Animal study was approved by institutional animal ethical committee (IAEC) of Crystal Biological Solutions Pune (CRY/2223/086) following standard protocols for animal handling and care.

Consent for publication
Not applicable.

Competing interest
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