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Investigation of Tretinoin-Loaded Cubosomal Hydrogel for Actinic Keratosis: Enhanced Skin Permeation, Confocal Microscopy Visualization, and Stability Assessment

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Abstract:
Tretinoin is a well-established topical agent for dermatological conditions such as actinic keratosis but faces challenges including poor skin permeation, irritation, and photo-instability. This study aimed to develop a tretinoin-loaded cubosomal hydrogel (cubogel) to enhance skin permeation, prolong drug release, and improve safety. Cubosomes were formulated using glyceryl monooleate and poloxamer 407 and incorporated into hydrogels containing different concentrations of Carbopol 940. The optimized formulation (CG2) was characterized for particle size, entrapment efficiency, pH, viscosity, spreadability, drug release kinetics, and stability under ICH guidelines. Skin permeation was visualized using Confocal Laser Scanning Microscopy (CLSM) with Rhodamine B as a fluorescent marker, while histopathological studies evaluated dermal safety in animal models. CLSM confirmed enhanced skin penetration compared to conventional formulations, while histopathology showed no irritation or structural damage. In vitro drug release kinetics followed Higuchi kinetics (R^2 = 0.9988), indicating diffusion-controlled drug release. Stability studies demonstrated formulation integrity under refrigeration for 3 months. Tretinoin-loaded cubogels significantly improved skin permeation, sustained drug release, and dermatological safety over conventional creams. This formulation shows strong potential for topical management of actinic keratosis and warrants further clinical investigation.
Keywords: *Tretinoin, Cubogels, Actinic Keratosis, Skin Permeation, Confocal Microscopy, Histopathology, Stability, Controlled Release.*

1. INTRODUCTION

Cubosomes are lipid-based nanoparticles at the nanoscale that, when hydrated, self-assemble into cubic-phase liquid crystalline formations that are thermodynamically stable. Cubosomes are well suited for the encapsulation of both hydrophilic and lipophilic medicinal drugs, improving drug loading efficiency and bioavailability. They are distinguished by their large interior surface area and bicontinuous lipid-water channels. (Schäfer-Korting, 2007). Cubosomes have received a lot of attention in advanced drug delivery applications because of their structural plasticity, especially for enhancing transdermal penetration, controlled release, and drug stability (Manconi et al., 2011). The resultant device, known as a cubogel, has further benefits for topical medication administration when distributed inside a hydrogel matrix. By making application easier and decreasing formulation run-off, the hydrogel base's enhanced viscosity improves patient compliance while also enhancing the encapsulated active agent's chemical and physical stability (Schwarz, 2012). Actives like tretinoin, which have significant difficulties with cutaneous penetration and photochemical degradation, benefit greatly from this combination. An essential method for observing the distribution and penetration of formulations based on nanocarriers within biological tissues is confocal laser scanning microscopy (CLSM). High-resolution imaging of drug-loaded nanocarriers as they move through the stratum corneum and settle in deeper skin layers, such as the viable epidermis and dermis, is made possible by CLSM in the context of cubosomal systems. This ability is essential for confirming cubosomes increased capacity for penetration, which can boost the therapeutic effects of tretinoin and other dermatological medicines used to treat disorders including actinic keratosis and acne (Ourique et al., 2011). Furthermore, determining the dermal safety and tolerability of innovative topical formulations still requires histological examination. Microscopic analysis of treated skin samples enables the identification of any histological changes that may occur after topical administration, such as inflammation, oedema, or structural disturbance (Pierard, 2014). Histopathological investigation ensures

that the formulation of tretinoin-loaded cubogels reduces recognised side effects of topical retinoids, such erythema and epidermal thinning, indicating its safety for extended therapeutic usage. Another crucial factor in the creation of topical formulations is stability testing, particularly for chemically labile substances like tretinoin. Because tretinoin is known to degrade when exposed to light, oxygen, and high temperatures, stabilisation techniques are necessary to preserve its therapeutic effectiveness over time. In order to guarantee product safety, consistency, and performance for the duration of its intended use, stability studies for cubogels include assessments of physicochemical, microbiological, and structural integrity under regulated storage settings. (Shiva, 2012) (Gerhardt 2008).

The present study was designed to develop and comprehensively evaluate tretinoin-loaded cubosomal hydrogel formulations with the objective of enhancing topical therapeutic efficacy for the management of actinic keratosis. The formulations were systematically characterized for their physicochemical attributes and storage stability over a three-month period. Furthermore, skin permeation and deposition were investigated using confocal laser scanning microscopy, while dermal biocompatibility was assessed through histopathological examination of treated skin tissues. This integrated evaluation aimed to improve the cutaneous delivery of tretinoin, investigate irritation potential, and ensure formulation stability for optimized clinical performance

2. MATERIALS AND METHODS

2.1 Materials:

Tretinoin was graciously supplied as complementary sample by Akum Drugs and Pharmaceuticals Ltd., Haridwar, India. Poloxamer 407, Glyceryl Monooleate (GMO), methanol, triethanolamine, and Carbopol 940 were procured from the laboratory facilities of Baba Mastnath University, Rohtak, Haryana, India. All the reagents used were of analytical grade. (Shailja1 2024)

2.2 Methods

2.2.1 Development of Tretinoin-Loaded Cubogels:

Tretinoin-loaded cubosomes were formulated using a bottom-up nanoprecipitation technique, employing glyceryl monooleate (350 mg) as the lipid phase and poloxamer 407 (110 mg) as a stabilizing agent. Both components were melted together at 60°C, after which tretinoin (0.025% w/w) was uniformly incorporated into the molten lipid mixture. This lipid phase was then gradually introduced, dropwise, into preheated distilled water maintained at 60°C under continuous mechanical stirring at 1350 rpm, facilitating the spontaneous formation of cubosomes. The resulting dispersion was subsequently characterized for particle size, polydispersity index (PDI), and drug entrapment efficiency to evaluate its physicochemical properties. (Shailja1 2024)

Cubogels were formulated by incorporating Carbopol 940 at concentrations of 0.5%, 1%, and 1.5% w/v. The hydrated Carbopol was thoroughly blended with the prepared cubosomal dispersion in a 1:2 ratio. The pH of the mixture was carefully adjusted to a range of 5.5–6.5 using triethanolamine to ensure dermal compatibility. To enhance consistency and texture, polyethylene glycol (0.1% v/v) was incorporated into the formulation. The resulting cubogels were then subjected to ultrasonication to remove entrapped air bubbles, following the procedure outlined in our previous study. (Shailja S 2025)

3. Evaluation Parameters

3.1 Confocal Laser Scanning Microscopy (CLSM)

To visualize and assess the skin permeation behavior of the optimized cubosomal hydrogel formulation, a Confocal Laser Scanning Microscopy (CLSM) study was conducted using Rhodamine B as a fluorescent probe. Rhodamine B was incorporated into the cubosomal formulation in place of the active drug at a concentration of 0.1% w/w to enable visualization of the formulation's diffusion through skin layers. Freshly excised rat abdominal skin was carefully cleaned, trimmed of subcutaneous fat, and hydrated with phosphate-buffered saline (pH 7.4) for 30 minutes prior to the experiment. The skin was then mounted between the donor and receptor compartments of a Franz diffusion cell, with the stratum corneum facing the donor compartment and the dermis toward the receptor. Phosphate-buffered saline was added to the receptor compartment, which was kept at 37 ± 0.5°C while being constantly stirred. A precisely measured amount of the cubogel formulation containing rhodamine B was applied to the donor compartment's

skin surface. Following an 8-hour permeation time, the skin was taken off, gently washed with distilled water to get rid of extra formulation, and then carefully blotted dry. A cryomicrotome was then used to vertically cut the treated skin, creating uniformly thin slices. A Confocal Laser Scanning Microscope (CLSM) fitted with a 543 nm excitation laser appropriate for Rhodamine B was used to analyse the skin slices that had been placed onto glass slides. Z-stack images were captured to assess the depth of fluorescence penetration, and the images were analyzed using compatible imaging software. The extent of dye diffusion through the different skin layers for the cubogel formulation was compared to that of a control hydrogel containing Rhodamine B. (Sureka S 2018)

3.2 Histopathology Studies

To assess the dermal safety and potential irritancy of the optimized Tretinoin-loaded cubosomal hydrogel (1%), histopathological analysis was performed on excised rat abdominal skin following topical application. The study was conducted in accordance with CPCSEA ethical guidelines under approved protocol. Healthy Wistar rats were divided into three groups (n = 3): Group I (untreated control), Group II (treated with conventional tretinoin cream), and Group III (treated with 1% Tretinoin-loaded cubosomal hydrogel). Each animal's dorsal side was shaved, washed, and treated topically with the appropriate formulation once a day for seven days in a row. Following the animals' sacrifice at the conclusion of the treatment period, the treated skin slices were carefully removed, washed with regular saline, and fixed right away in 10% neutral buffered formalin for a full day. Following dehydration in a succession of alcohol grades, the fixed skin tissues were cleaned in xylene and embedded in paraffin wax. A rotary microtome was used to segment paraffin blocks at a thickness of 5 μm. Following deparaffinization and mounting on glass slides, the resulting slices were stained with haematoxylin and eosin (H&E). The stained sections were examined under a light microscope (at 40× magnification) for evaluation of epidermal thickness, dermal integrity, inflammatory cell infiltration, edema, necrosis, and other signs of irritation or histological alteration. (Ahmad Nasrollahi 2013) (Ananda K C 2021)

3.3 Drug Release Kinetics for Optimized Cubosomal Gel

Drug release kinetics to investigate the mechanism of drug release from cubogel was performed. The release data was analyzed with the following mathematical models.

Zero-order equation

$Q_t = Q_o + k_o t$

where ko is the zero-order release rate, Qt is the quantity of drug release in time t, and Qo is the starting amount of drug in the solution (usually, Qo = 0). Data from in vitro drug release tests were shown as the cumulative amount of drug released vs time in order to examine the release kinetics.

First-order equation

$\ln Q_t = \ln Q_o + k_1 t$

where k1 is the first-order release rate constant, Qt is the amount of drug released in time t, and Qo is the starting amount of drug in the solution. The gathered data are shown against time as a log cumulative percentage of medication remaining.

Higuchi equation

$Q = kH \times t^{½}$

where kH is the Higuchi diffusion rate constant and Q is the quantity of drug release at time t. The acquired data were displayed against the square root of time as a cumulative percentage of medication release.

Korsmeyer-Peppas equation

Korsmeyer used an equation for a polymeric system to develop a straightforward connection that explained drug release. At time t, $Qt/Q\infty = Kkp.tn$ $Qt/Q\infty$ = proportion of drug released Kkp = Korsmeyer-Peppas rate constant jeopardises the device's geometric and structural properties. Plotting data from in-vitro drug release experiments as log cumulative percentage drug release vs log time allowed researchers to examine the release kinetics.

3.4 Stability Studies

The stability study of the optimized cubosomal hydrogel formulation was conducted in accordance with ICH guideline Q1C to evaluate its physicochemical stability under various environmental conditions.

The objective of this testing was to assess how the formulation's quality attributes, including appearance, viscosity, and drug content, change over time when exposed to factors such as temperature, humidity, and light thereby facilitating the determination of appropriate storage conditions, retest periods, and shelf-life.

The optimized formulation was stored for a period of three months under two conditions: accelerated stability at 40 ± 2°C / 75% ± 5% RH and refrigerated conditions at 4 ± 0.5°C, as recommended by ICH guidelines. At the end of the storage period, the samples were carefully evaluated for any alterations in color, texture, viscosity, and percentage drug content uniformity to determine the formulation's stability and suitability for long-term topical use. (Castro 2009) (Kar 2010) (Joshi 2015)

4. RESULTS AND DISCUSSION

The cubosomal hydrogel formulations loaded with tretinoin that were optimised showed good physicochemical characteristics. The observed entrapment efficiency, polydispersity index (PDI), and particle size were 82.13% ± 1.02, 0.312, and 69.5 ± 2.34 nm, respectively. With pH levels ranging from 5.35 to 5.87, all three formulations maintained high homogeneity, demonstrating their suitability for topical application. Viscosity ranged from 21,331 cps in CG1 to 29,681 cps in CG3, increasing proportionately with Carbopol content. With the highest drug content uniformity (91.54% ± 0.43%) and the best spreadability (47.3 g/cm2), CG2 stood out among the others as the best formulation. As such, it is the most appropriate candidate for additional testing and therapeutic use.

4.1 Confocal Laser Scanning Microscopy (CLSM)

The depth-wise skin permeation of the optimized Tretinoin-loaded cubosomal hydrogel was assessed using Confocal Laser Scanning Microscopy (CLSM). By acting as a fluorescent probe, rhodamine B made it possible to see how the formulation was distributed across the layers of the skin. The CLSM image (Figure 2) revealed intense red fluorescence within the viable epidermis and dermis following treatment with the cubogel formulation. The fluorescence was notably more profound and uniform in the deeper layers when compared to the control hydrogel, in which the fluorescence remained largely restricted to the stratum corneum.

The distribution of the red fluorescence within the deeper epidermal layers and possibly into the dermis indicates that the formulation successfully enhanced drug penetration beyond the stratum corneum (outermost layer) which is desirable in topical/transdermal drug delivery studies. These findings confirm the enhanced permeation capability of the cubosomal hydrogel system, consistent with the results of the in vitro skin permeation and skin retention studies. The nanostructured lipid carriers facilitated improved transport of the lipophilic drug through the skin barrier, supporting their potential application for effective topical therapy of actinic keratosis.

In this study, we have observed about 61.451% reduction in atypical honeycomb patterns and a 64.013 % decrease in architectural disarray. The confocal microscopy images in Figure 3 depict the epidermal architecture characteristic of actinic keratosis and its response to treatment. Image (A) reveals the classic features of actinic keratosis with marked architectural disarray and an atypical honeycomb pattern, indicating significant keratinocyte dysplasia and epidermal disruption. In contrast, Image (B), captured after being treated with tretinoin-loaded cubosomal hydrogel (1%), demonstrated a noticeable improvement in epidermal organization, with only focal areas of atypical honeycomb remaining. This suggests partial restoration of normal keratinocyte arrangement and epidermal integrity, reflecting the therapeutic potential of the cubosomal gel in reducing dysplasia and promoting epidermal repair in actinic keratosis.

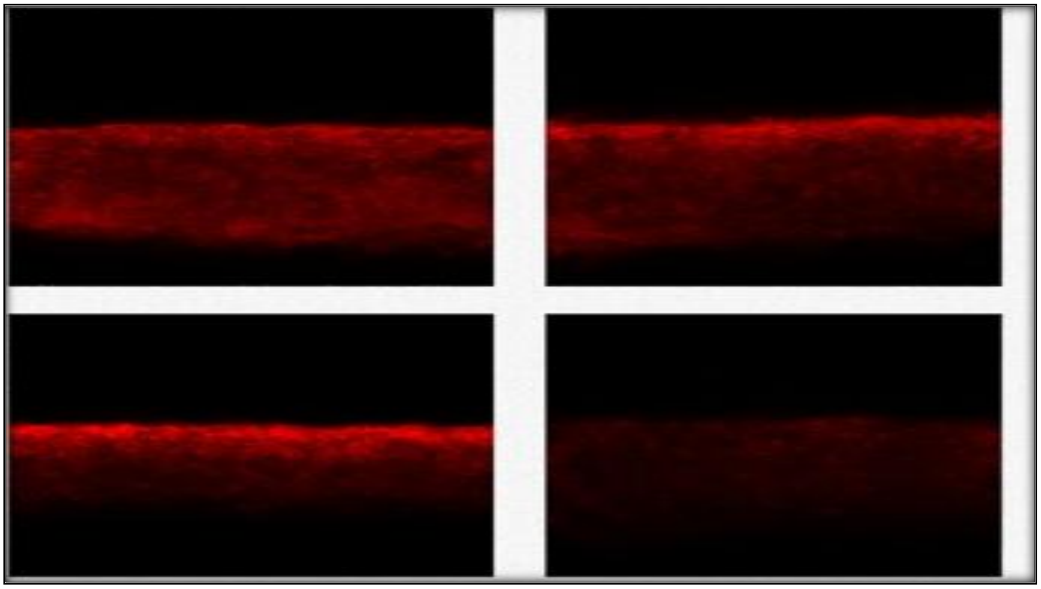

Figure 1. CLSM image showing the skin permeation profile of Rhodamine B-loaded cubosomal hydrogel formulation. Significant penetration through the epidermis and into the dermal layers is observed.

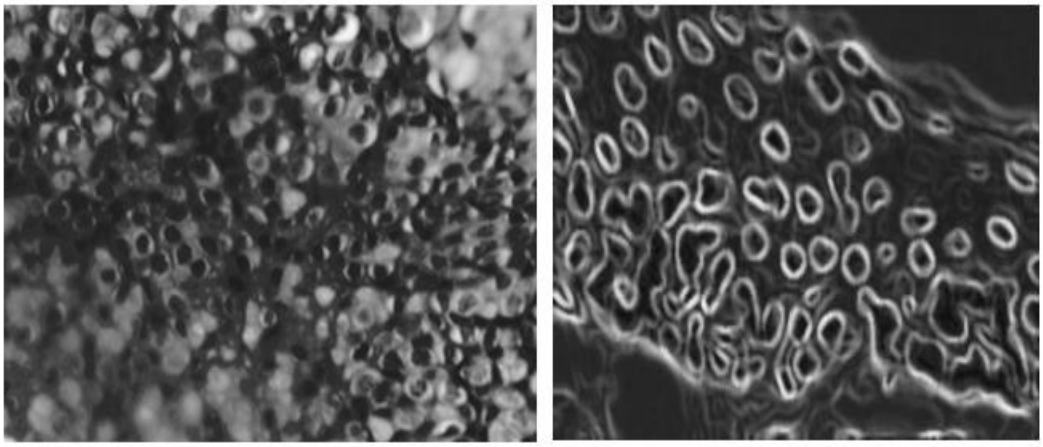

Figure 2(A) symbolises the actinic keratosis confocal microscopy displaying architectural disorder (atypical honeycomb); (B) shows the focal region of an atypical honeycomb in confocal imaging of actinic keratosis following treatment with tretinoin-loaded cubosomal hydrogel (1%)

4.2 Histopathological Safety

Histopathological examination of skin tissues treated with the 1% Tretinoin-loaded cubosomal hydrogel revealed preserved epidermal and dermal architecture, with no evidence of edema, necrosis, or inflammatory cell infiltration. The epidermis remained intact without signs of hyperplasia, thinning, or irritation, and the dermal collagen fibers appeared well-organized and unaltered. In contrast, skin samples treated with conventional tretinoin cream exhibited noticeable epidermal thinning, mild dermal edema, and inflammatory infiltrates, characteristic of retinoid-induced irritation. Untreated control skin sections showed normal histological features. These findings confirm the dermal safety and biocompatibility of the cubogel formulation, suggesting that the cubosomal delivery system mitigates tretinoin-associated irritation while maintaining therapeutic potential for actinic keratosis as presented in table 1. The results confirmed that CG2 did not cause any significant skin irritation. The epidermis and dermis maintained their normal structure, with no indication of inflammation, erosion, or other signs of toxicity. This suggests that the tretinoin-loaded cubogels are safe for topical application, as no adverse reactions were observed during the histopathological evaluation. The results from this study provide valuable insights into the biocompatibility of the formulation and suggest that CG2 can be safely used for dermatological treatments.

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Table 1: Histopathological Analysis of Tretinoin Cubogels

Formulation	Epidermis Structure	Dermis Structure	Signs of Inflammation	Other Findings
CG1 (0.5% Carbopol)	Intact, no damage	Normal, no damage	None	No adverse reactions
CG2 (1% Carbopol)	Normal, no irritation	Normal, no damage	None	No epidermal damage
CG3 (1.5% Carbopol)	Intact, no damage	Normal, no damage	None	No toxicity observed

The histopathological images presented in Figure 3 illustrate the comparative effects of different treatments on skin tissue architecture. Image (A), representing the negative control group (untreated actinic keratosis skin), exhibits distinct pathological features, including loss of basal cell polarity, nuclear pleomorphism, and early dysplastic alterations, characteristic of actinic keratosis progression. In contrast, Meanwhile, Image (B), depicting the positive control group treated with 0.025% conventional tretinoin cream, reveals marked epidermal damage with thinning, nuclear atypia, and notable inflammatory infiltration in the dermis, confirming the well-documented irritation potential of conventional tretinoin-based formulations. Image (C), corresponding to skin treated with the 1% Tretinoin-loaded cubosomal hydrogel, displays well-preserved epidermal and dermal structures, with no signs of morphological disruption, indicating excellent dermal compatibility and safety of the nanoformulation.

Table 2. Histopathological Evaluation of Inflammation and Tissue Integrity

Group / Treatment	Signs of Inflammation	Effect on Epidermis	Effect on Dermis
A: Tretinoin Cubosomal Hydrogel (1%)	Absent	Intact, no cytological damage	Normal connective tissue, no edema
B: Negative Control (Untreated AK skin)	Mild	Loss of polarity, nuclear pleomorphism	Normal to mild inflammatory infiltration
C: Positive Control (0.025% Tretinoin Cream)	Present	Epidermal thinning, damage, nuclear changes	Inflammatory infiltrates, mild edema

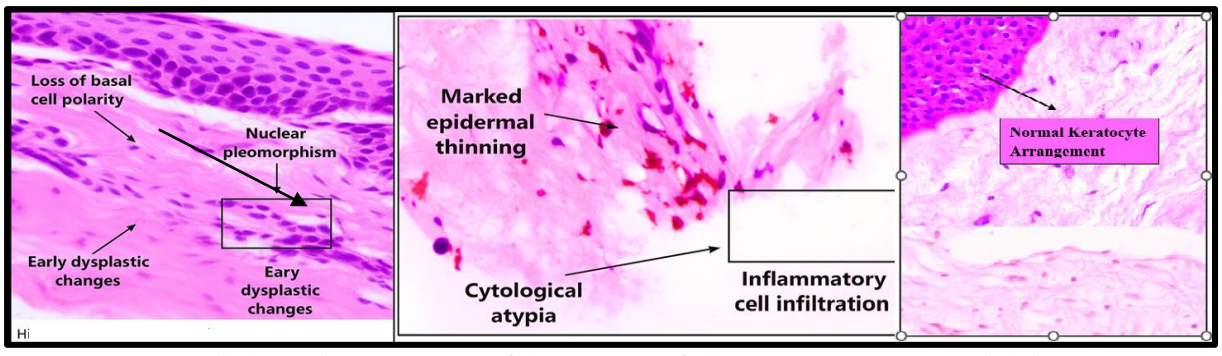

Figure 3: Histopathological examination of skin sections following treatment revealed distinct morphological differences across the study groups.
(A) The negative control group (untreated actinic keratosis skin) exhibited typical pathological features, including loss of basal cell polarity, nuclear pleomorphism, and early dysplastic changes, consistent with actinic keratosis progression.
(B) The positive control group (0.025% tretinoin cream-treated skin) showed pronounced epidermal thinning, cytological atypia, and marked inflammatory cell infiltration within the dermis, indicative of tissue irritation and retinoid-associated adverse effects.

(C)The section from skin treated with 1% tretinoin-loaded cubosomal hydrogel displayed a well-preserved epidermal and dermal structure, with no evidence of histopathological alterations, inflammation, or tissue disruption.

4.3 Drug Release Kinetics for Tretinoin Cubogel (1%)

The in vitro release data were fitted to a number of kinetic models, including Zero-order, First-order, Higuchi, and Korsmeyer-Peppas models, in order to clarify the drug release mechanism from the optimised nanogel formulation. Plotting the cumulative drug release data against the corresponding model equations allowed for the analysis of the release patterns. These graphical analyses were carried out in order to better understand the underlying release mechanism and choose the most suitable kinetic model for characterising the formulation's release behaviour.

1. Drug release percentage cumulatively vs time
2. First-order model log of cumulative percentage of medication released vs time
3. Higuchi model: cumulative percentage of medication released vs SQRT of time.
4. The Korsmeyer-Peppas equation, which compares the log of time to the cumulative percentage of drugs released.

The 1% tretinoin-loaded cubosomal hydrogel's in vitro drug release kinetics were assessed by fitting the release data into a number of mathematical models, such as the Zero-order, First-order, Higuchi, and Korsmeyer-Peppas models. (Figure 4, Table 3). With the highest correlation coefficient (R2 = 0.9988) among them, the Higuchi model suggested that the drug release from the cubogel mostly proceeded via a diffusion-controlled process via the cubosomal matrix. With a release exponent (n) value indicating a non-Fickian, anomalous diffusion process comprising a mix of drug diffusion and polymeric matrix degradation, the Korsmeyer-Peppas model likewise demonstrated a strong match (R2 = 0.9964). The drug release was not solely reliant on concentration or time, as demonstrated by the lower correlation values of the Zero-order (R2 = 0.9275) and First-order (R2 = 0.9195) models. Collectively, these findings indicate that the optimized cubogel provided a sustained and controlled drug release profile, making it a promising and effective delivery system for topical management of actinic keratosis.

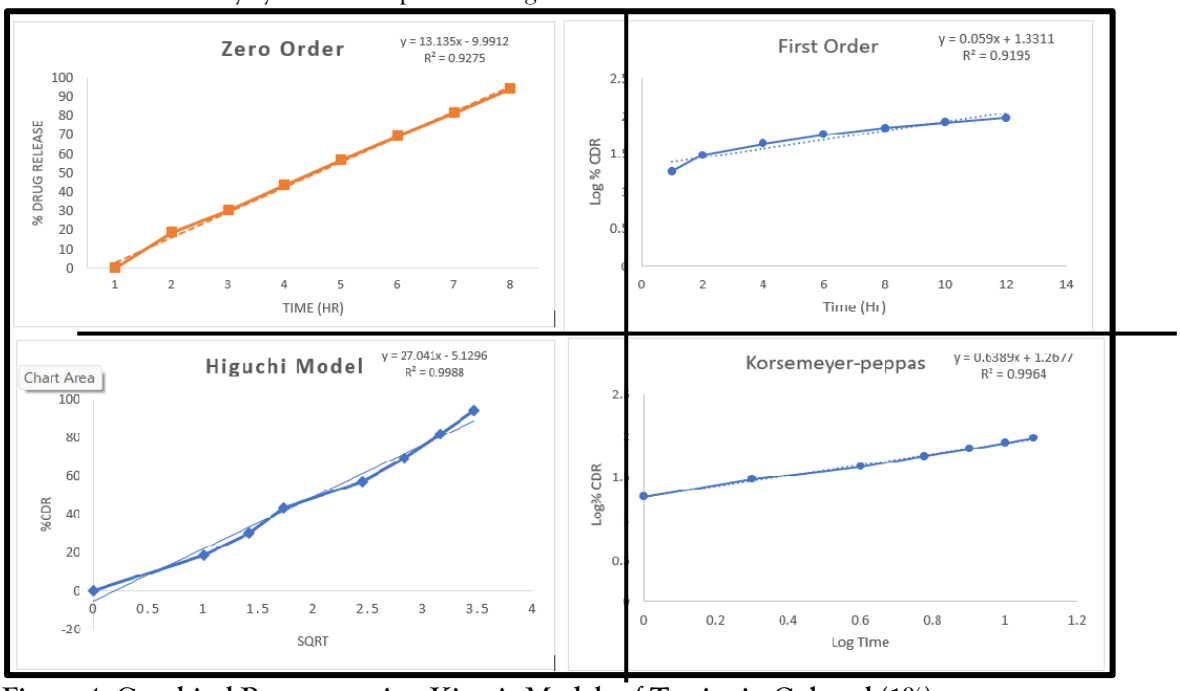

Figure 4. Graphical Representation Kinetic Models of Tretinoin Cubogel (1%)

Table 3: Drug Release Kinetics for Tretinoin Cubogel (1%)

S.No.	Model	Regression Coefficient (R^2)
1.	Zero Order	0.9275
2.	First Order	0.9195

3.	Higuchi Model	0.9988
4.	Korsmeyer-Peppas	0.9964

4.4 Stability Studies

The stability study of the optimized cubosomal hydrogel was conducted in accordance with ICH Q1 guidelines. Following a three-month storage period, the formulation was assessed for color, texture, viscosity, and drug content uniformity. As illustrated in Table 4, the findings showed that the formulation remained physically stable under refrigeration temperatures (4 ± 0.05°C), with only a little decrease in viscosity and drug content homogeneity and no discernible changes in colour or texture. The formulation, on the other hand, showed obvious precipitation and deterioration when kept under accelerated circumstances (40 ± 2°C / 75% ± 5% RH), jeopardising its chemical and physical stability. These results led to the conclusion that the optimised cubogel is inappropriate for storage at high temperatures but retains sufficient stability when refrigerated.

Table 4: Stability Studies of Tretinoin Cubogel (1%)

Parameter	Temperature	Time (Months)		
		1st	2nd	3rd
Colour and Texture	4±0.05°C	White to light pale yellow in colour.	White to light pale yellow in colour.	White to light pale yellow in colour.
	40 ± 2°C	White to light pale yellow in colour.	White to light pale yellow in colour.	White to light pale yellow in colour.
Viscosity	4±0.05°C	23540	23538	23535
	40 ± 2°C	23540	23542	23047
% Drug Content Uniformity	4±0.05°C	91.54±0.43	90.12±0.32	90.01±0.21
	40 ± 2°C	91.54±0.43	89.35±0.02	88.01±0.64

5. DISCUSSION

The current work effectively created a cubosomal hydrogel laden with tretinoin that can get beyond the drawbacks of traditional tretinoin formulations. Excellent physicochemical characteristics, such as nanosized particle diameters and high entrapment effectiveness, made the optimised formulation (CG2) appropriate for topical administration. The spreadability of CG2 was ideal for application convenience, and the pH and viscosity values were within permissible cutaneous limits. With fluorescence seen beyond the stratum corneum into deeper epidermal and dermal layers, the CLSM investigation demonstrated strong evidence of the cubosomal system's improved dermal penetration. This performance was noticeably better than that of control formulations. The increased surface area and nanostructured structure of cubosomes, which promote better drug diffusion, are responsible for this higher penetration. According to histopathological study, the cubogels were well tolerated and did not cause any cytotoxicity or irritation while maintaining the structure of the skin.

The cubogel formulation showed improved biocompatibility, which is important for topical retinoids with irritant potential, in contrast to traditional tretinoin cream, which showed obvious epidermal thinning and inflammatory infiltration. Higuchi kinetics were followed by the in vitro drug release profile, suggesting a diffusion-controlled mechanism that is advantageous for long-term topical treatment. The formulation's appropriateness for long-term cutaneous usage was further supported by stability testing, which confirmed its integrity under chilled settings. These results are consistent with other studies on

nanocarrier technologies that enhance retinoid delivery and lessen skin irritation. Therefore, the cubosomal hydrogel offers a viable method for increasing tretinoin's topical effectiveness while reducing side effects.

6. CONCLUSION

To conclude, we developed a cubosomal hydrogel loaded with tretinoin that is stable, efficient, and biocompatible for the topical treatment of actinic keratosis. Superior skin penetration, prolonged release kinetics, low cutaneous toxicity, and outstanding physicochemical stability were all displayed by the optimised formulation (CG2). Significant drug penetration into deeper skin layers was proven by the results of confocal microscopy, and the formulation's safety was validated by histopathology. All things considered, this cubogel administration method presents a viable, patient-friendly substitute for traditional tretinoin creams and merits more in vivo and clinical testing for dermatological uses.

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