

Article

Exploring the Potential of Transdermal Nanobilosomal Gel for Magnified Anti-Inflammatory Efficacy of Thymol for Managing Rheumatoid Arthritis

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Abstract

This research aims to develop a chitosan-coated, TH-loaded nanobilosomal gel (CH-TH-BG) to magnify the transdermal delivery and anti-inflammatory efficacy of thymol (TH) for the management of rheumatoid arthritis (RA). Initially, chitosan-coated, TH-loaded bilosomes (CH-TH-BLs) were prepared and optimized by Box–Behnken design. The optimized CH-TH-BLs exhibited enhanced entrapment efficiency (83.52%) and a positive zeta potential (+36.3 mV). Further, the optimized lyophilized CH-TH-BLs were incorporated into the carbopol gel (CH-TH-BG) and characterized thoroughly. The CH-TH-BG exhibited superior pharmaceutical properties, including high drug content ($98.65 \pm 1.43\%$), optimal viscosity ($10,400 \pm 12.6$ cps), excellent spreadability (5.33 ± 0.15 cm), extrudability, and a slightly acidic pH (5.40 ± 0.10), which resembles the pH of human skin. In vitro drug release revealed that the developed gel exhibited a biphasic release pattern, with a rapid release followed by sustained release. Notably, ex vivo results revealed a ~2.0-fold increase in permeation flux and a ~2.8-fold increase in skin retention compared to the TH solution. In vivo results confirmed a significant reduction in paw edema and pro-inflammatory biomarkers (TNF- α and IL-6), alongside recovery of body weight and ankle joints. In conclusion, the CH-TH-BG is a transformative transdermal platform for effective management of RA.

Keywords: rheumatoid arthritis; bilosomes; thymol; bilosomal gel; chitosan; transdermal delivery



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1. Introduction

Rheumatoid arthritis (RA) is a systemic and chronic autoimmune inflammatory disease that has a significant global impact (0.5–1%) and is more common in Western countries. Moreover, it predominantly affects women (~three times higher), especially those over 60, due to hormonal variations [1]. RA is characterized by joint inflammation, synovial hyperplasia, immune cell infiltration, and pannus formation [2]. Additionally, patients can face a higher fatality rate due to various extra-articular complications caused by RA that affect the lungs, skin, and heart [3]. The pathogenesis of RA is complex and not fully understood. However, some studies suggest that its development is associated with an abnormal immune response, leading to the production of RA autoantibodies and the activation of various immune cells, including dendritic cells, T and B cells, neutrophils,

fibroblast-like synoviocytes (FLSs), and macrophages. Furthermore, these cells infiltrate the joint synovium, leading to increased acidity and hypoxia. Simultaneously, activated synovial cells release vascular endothelial growth factors, causing angiogenesis that promotes synovial tissue inflammation. Moreover, the intensification of synovial tissue inflammation produces various pro-inflammatory cytokines (tumor necrosis factor (TNF- α) and interleukins (IL-6, IL-1 β)), leading to joint swelling and inflammation. IL-1 β and TNF- α also stimulate osteoclast formation and activate matrix metalloproteases (MMPs), contributing to cartilage and bone degradation in affected joints, which limits mobility and causes stiffness, especially in the morning [4]. RA not only diminishes patients' quality of life but can also lead to long-term disability if neglected [4,5]. The primary goal of RA treatment is to reduce inflammation, pain, joint swelling, and discomfort and to prevent joint damage [3]. Clinically, this is achieved through various medications, including glucocorticoids (GCs), non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), and traditional Chinese medicines, either alone or in combination [1,2]. Moreover, physical therapy, lifestyle changes, and surgical interventions are also used in certain cases [3]. These treatments can effectively manage symptoms and improve patients' quality of life but cannot be used for long-term treatment due to their adverse effects [2,4,5]. Furthermore, these medications are typically administered orally, parenterally, or intramuscularly and have certain limitations. Oral administration is subject to first-pass metabolism, food interactions, and drug degradation by gastrointestinal tract (GIT) enzymes, resulting in poor bioavailability. In contrast, the parenteral route can cause non-specific organ toxicity, and the intra-articularly administered drug is rapidly cleared by lymphatic drainage [1,3,6]. Therefore, it is necessary to develop a novel formulation that incorporates a natural anti-inflammatory drug for the safe management of RA with minimal adverse effects, utilizing the transdermal route.

Thymol (TH) is an FDA-approved food supplement that is safe for animals up to 500 mg/kg (oral). This terpenoid is extracted from different thyme species, including *Thymus vulgaris*, *Coridothymus capitatus*, and *Origanum vulgare* [7]. Various studies suggest that TH exerts antimicrobial, antifungal, antioxidant, anticancer, antiviral, and anti-inflammatory effects [7–11]. Further, in vitro and in vivo studies revealed that TH inhibits the production of pro-inflammatory cytokines (TNF- α and IL-6) and downregulates pro-inflammatory transcription factors and different signaling pathways like mitogen-activated protein kinases (MAPKs), nuclear factor- κ B (NF- κ B), and Signal Transducer and Activator of Transcription 3 (STAT-3) [8,10]. Despite various therapeutic effects, its clinical application is restricted owing to high volatility, poor aqueous solubility, instability, poor bioavailability, and dose-dependent toxicity [12,13]. The literature revealed that the nanocarriers can overcome the limitations of TH owing to their distinctive properties, enabling improved bioavailability and stability [9,11,14–16].

Vesicular nanocarriers like niosomes, liposomes, transferosomes, ethosomes, phytosomes, and bilosomes (BLs) are promising carriers for phytochemical delivery due to their unique properties, including biocompatibility, superior stability, and controlled, targeted drug delivery. Moreover, these nanocarriers can be easily incorporated into gels, making them suitable for transdermal drug delivery systems (TDDS) [16]. The gel-based nano-engineered approach is promising and preferred for effective RA treatment due to the combination of the gel's viscosity and bioadhesive properties with the drug-boosting capabilities of nanocarriers, enabling specific drug delivery to the local inflammation site. This approach involves embedding a drug-loaded nanocarrier within a polymeric hydrogel and can target the site through percutaneous absorption, bypassing the adverse effects associated with oral, parenteral, or intra-articular administration. This synergistic method opens new prospects for developing advanced TDDS with increased therapeutic efficacy [3].

BLs have been used to enhance the transdermal delivery of various synthetic anti-inflammatory drugs [17–19] and natural drugs [20–22] for effective arthritis treatment. BLs are vesicular nanocarriers resembling niosomes or liposomes with incorporated bile salts, which improve their permeability, stability, deformability, and flexibility [23,24]. Moreover, BLs are cost-effective, safe, biocompatible, and can overcome biological barriers and incorporate both hydrophilic and lipophilic drugs due to the amphiphilic nature of bile salts. Furthermore, biosomes release the drug in a sustained manner, allowing for lower dosing frequencies [24]. Additionally, BLs can easily cross the stratum corneum due to their fluidizing action, allowing for deep skin penetration. Moreover, the therapeutic potential of BLs can also be enhanced by surface coating of chitosan (CH) due to its unique properties, including biocompatibility, polycationic nature, biodegradability, and solubility at low pH in aqueous medium [20]. Additionally, the CH coating also protects the BLs from harsh physiological conditions and oxidative stress, ensuring the integrity of the encapsulated drug and prolonged drug release. This superiority makes them appropriate for various routes, including buccal, oral, ocular, nasal, and transdermal delivery [22]. Few existing studies are available on TH-based nanocarriers (e.g., carbon dots [9], nanoparticles [13]), various drug-loaded BLs (e.g., triterpene [21], fluticasone propionate [18], berberine [20], flurbiprofen [19], lornoxicam [17], and simvastatin [25]), and various nanogels (catalytic nanogel [26], tacrolimus-loaded gel [27], chlorogenic acid [28], carbon dot nanogel [29], strontium ranelate [30]) for inflammatory and arthritic applications. To the best of our knowledge, no study has claimed the development of a nanobilosomal gel comprising CH-coated, TH-loaded BLs (CH-TH-BLs) for the effective management of RA. Therefore, this research aims to develop a transdermal nanobilosomal gel for magnified anti-inflammatory efficacy of TH for the safe and effective management of RA. The primary objective of this study was to optimize the CH-TH-BLs by minimizing particle size while maximizing both zeta potential and entrapment efficiency (EE). Subsequently, the formulation's performance was assessed through secondary endpoints, including *in vitro* release kinetics, *ex vivo* permeation profiles, and *in vivo* studies. To accomplish this target, initially, CH-TH-BLs were prepared and optimized using Box–Behnken (BB) design to investigate the effect of different processing variables on particle size, zeta potential, and % EE, then selecting the optimal formulation based on a high desirability index. Further, the optimized lyophilized CH-TH-BLs were incorporated into a carbopol hydrogel (CH-TH-BG) to prolong their retention time over the epidermis. CH-TH-BLs and CH-TH-BG were characterized by various microscopic and analytical techniques. Further, the therapeutic potential of CH-TH-BG for RA was evaluated by *in vitro* drug release, *ex vivo*, and *in vivo* studies.

2. Results and Discussion

2.1. Preparation and Physicochemical Characterization of CH-TH-BLs

Preliminary experiments were conducted to evaluate the impact of various independent variables, including SL and drug concentration, cholesterol concentration, sonication time, and hydration medium, on the BL formation. CH-TH-BLs were successfully prepared by the thin film hydration method using 100 mg TH (drug), cholesterol (20 mg), 4 min sonication time, 10 mL PBS (hydration medium), 1 h hydration time, varying concentrations of SL to augment the stability and EE, varying concentrations of SDC as an edge activator, and varying concentrations of CH for coating. The developed BLs were characterized for particle size, zeta potential, and EE.

TH in BLs was successfully quantified using a validated UV method. The developed method was validated for 5–25 µg/mL. The linearity of the calibration curve was validated by a correlation coefficient (R^2) of 0.997, and reproducibility was confirmed from intra-day (1.14%) and inter-day (1.36%) relative standard deviation values. The recovery studies'

results revealed that the developed method was accurate, with a mean recovery of TH from the BLs ranging from 94% to 100%, confirming the non-interference of BLs excipients with the TH signal at 275 nm. The limit of detection and limit of quantification of TH were 0.43 and 1.32 µg/mL, respectively, stipulating high sensitivity for the detection of TH in BLs.

2.2. Statistical Analysis and Optimization of CH-TH-BLs

The BB design was used for the optimization of CH-TH-BLs to maximize EE, maximum zeta potential, and minimize particle size. Table 1 presents a total of 15 experimental runs and their observed results; all the results were statistically analyzed by a quadratic model using polynomial Equations (1)–(3).

$$\text{Particle size} = 140.13 - 22.40A + 6.17B + 20.83C - 3.47AB + 2.83AC - 0.4250BC + 11.60A^2 + 5.30B^2 + 2.30C^2 \quad (1)$$

$$\text{Zeta potential} = 32.03 - 3.20A - 0.8125B + 34.19C - 0.2750AB + 0.3750AC - 0.3000BC - 0.0917A^2 - 0.266B^2 - 37.67C^2 \quad (2)$$

$$\text{EE} = 78.77 + 5.04A - 0.2113B + 3.10C + 0.7750AB + 0.8200AC + 1.12BC + 0.01113A^2 + 1.09B^2 - 0.5813C^2 \quad (3)$$

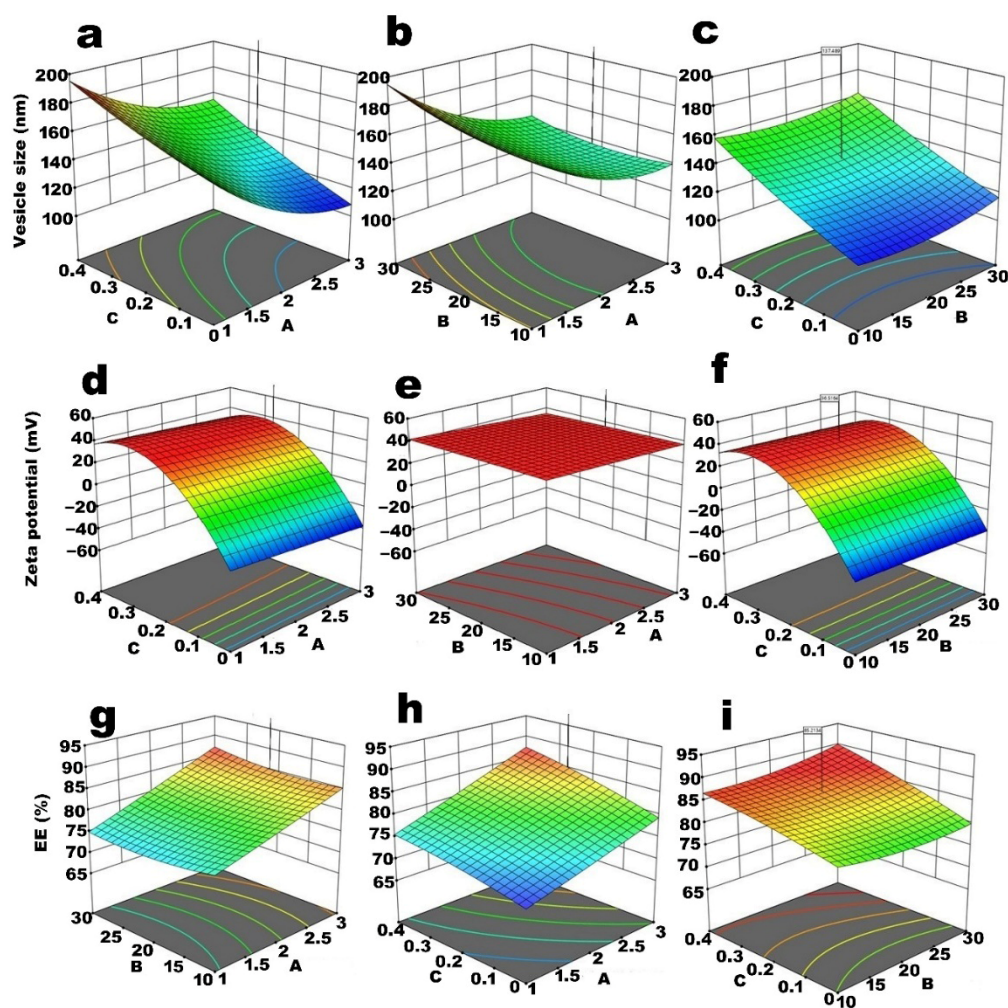
Table 1. Experimental runs with the combination of independent variables and observed responses.

Run	Independent Variables			Observed Responses		
	SL Concentration (%w/v) (A)	SDC Concentration (mg) (B)	Chitosan Concentration (%w/v) (C)	Particle Size (nm)	Zeta Potential (mV)	EE (%)
1	2	20	0.2	139.0	32.1	79.25
2	1	30	0.2	189.5	34.4	73.66
3	3	20	0.4	156.4	31.2	87.20
4	2	20	0.2	141.4	32.3	78.09
5	3	10	0.2	131.5	29.5	84.73
6	1	10	0.2	171.2	35.2	75.83
7	3	30	0.2	135.9	27.6	85.66
8	2	10	0.4	162.1	35.9	81.24
9	3	20	0	108.7	−37.2	79.10
10	1	20	0	157.3	−28.9	71.04
11	2	20	0.2	140.0	32.4	78.97
12	2	10	0	120.0	−33.8	77.54
13	1	20	0.4	193.7	36.0	75.86
14	2	30	0.4	174.6	33.4	83.25
15	2	30	0	134.2	−35.1	75.08

A, B, and C are the independent variables, i.e., SL concentration, SDC concentration, and CH concentration, respectively, at their low and high levels, which significantly influence the observed responses by variation in one variable at a time. AB, AC, BC, A², B², and C² are the interaction terms, explaining how the simultaneous variation in two independent variables affects the observed responses. The positive and negative values represent the favorable and inverse effects on the observed responses, respectively [31]. The significance of independent variables, magnitude, and suitability of the selected model were statistically determined by analysis of variance (ANOVA). A higher F value and a smaller probability $p < 0.0001$ indicate the significance of the model. The selected model was significant with a lower p -value and higher F value (Table 2). Further, the three-dimensional (3D) response surface plot presents the effect of independent processing variables on the dependent variables (Figure 1).

Table 2. Analysis of variance of the observed responses.

Source	Particle Size (nm)		Zeta Potential (mV)		Entrapment Efficiency (%)	
	F	<i>p</i> -Value	F	<i>p</i> -Value	F	<i>p</i> -Value
Model	388.53	<0.0001	2888.66	<0.0001	133.85	<0.0001
A-SL concentration	1662.60	<0.0001	161.63	0.7736	825.33	<0.0001
B-SDC concentration	126.35	<0.0001	10.42	0.0233	1.45	0.2822
C-Chitosan concentration	1437.02	<0.0001	18,448.43	<0.0001	312.30	0.0092
Model	Quadratic		Quadratic		Quadratic	
Std. Dev.	1.55		0.7119		0.4960	
R ²	0.9986		0.9998		0.9959	
C.V. %	0.4646		3.59		1.23	
Adjusted R ²	0.9960		0.9995		0.9884	
Predicted R ²	0.9819		0.9983		0.9677	
Adeq Precision	68.1416		128.6381		40.1841	

**Figure 1.** Three-dimensional response surface plots presenting the effect of independent processing variables on particle size (a–c); zeta potential (d–f); and entrapment efficiency (g–i). A, B, and C are SL concentration, SDC concentration, and chitosan concentration, respectively.

The particle size of all the experimental runs was between 108.7 and 193.7 nm (Table 1, confirming that all the BLs were in the nano range. The 3-D graphs (Figure 1a–c) revealed that particle size was negatively influenced by SL concentration and positively influenced by the SDC and CH concentrations, also supported by Equation (1). The negative effect of SL can be attributed to the accessibility of a large surface at a high level of SL, resulting in increased encapsulation of the drug into the vesicle bilayer, enabling better vesicular membrane packaging and reduced size [20,32]. The particle size was significantly increased with an increase in SDC concentration. This effect was due to the anionic nature of SDC, which generates a substantial repulsive force between the vesicle bilayer, resulting in enlargement of the interior core, enabling a large size [33]. Moreover, the positive effect of CH concentration on particle size can be attributed to an increase in the coating layer at higher concentrations of CH [20,32].

The zeta potential of all formulations was between -28.9 and 36 mV (Table 1). Equation (2) indicates that the zeta potential was negatively influenced by SL and SDC concentrations, but this effect was insignificant ($p > 0.0001$, Table 2), as shown in Figure 1e. However, zeta potential was positively influenced by CH concentration (Figure 1d,f). It has been observed that increased CH concentration changed the negative value to positive, owing to the cationic nature of CH [20]. The EE of all the formulations was between 71.04 and 87.20%, and it was positively affected by SL and CH concentrations and negatively by SDC concentration (Equation (3) and Figure 1g–i). Figure 1i and Table 2 results revealed that EE was significantly ($p < 0.0001$) influenced by SL concentration, but the effect of SDC and CH concentrations was non-significant ($p > 0.0001$). The positive effect of SL was due to a higher concentration of lipid that can saturate more hydrophobic drugs into the lipid bilayer, leading to higher EE. The optimal formulation was selected based on a high desirability index. A higher desirability index indicates that the selected set of process parameters successfully achieves specified goals. For TDDS, the key characteristics are higher EE, maximum zeta potential, and reduced particle size. Higher EE permits entrapment of enough drug concentration, maximum zeta potential ensures physical stability, and reduced particle size (< 200 nm) secures enhanced skin permeation [34]. The maximum desirability coefficient (0.832) was achieved at 3% *w/v* SL, 20.840 mg SDC, and 0.265% *w/v* CH. The reliability of selected parameters was assessed by preparing the formulation using the optimal formula and comparing the experimental results with predicted results (Table 3). The average size of optimized CH-TH-BLs was 134.2 nm with a good polydispersity index of 0.258 (Supplementary Figure S1).

Table 3. Experimental and predicted values for the selected processing variables for the optimized CH-TH-BLs.

Response Variables	Experimental Value	Predicted Value	Prediction Error (%)
Particle size (nm)	134.2	137.489	2.59
Zeta potential (mV)	36.3	36.516	0.595
EE (%)	83.52	85.213	2.027

2.3. Preparation and Characterization of CH-TH-BG

Semisolid dosage forms, such as gels and creams, are extensively used for TDDS owing to their enhanced skin permeation and increased contact time with the skin. Here, CH-TH-BG (1% *w/w* TH) was successfully prepared by incorporating 160 mg of optimized lyophilized CH-TH-BLs into 840 mg of 1% *w/v* carbopol 940. The developed gel was transparent with a smooth and glossy appearance and homogeneous in nature due to the gelling property of carbopol, which forms a 3-D gel network that facilitates the

uniform dispersion of TH. Here, 1% *w/v* carbopol 940 was used as a gelling agent because this concentration creates a viscous and strong gel (after neutralization) for transdermal delivery, offering controlled release of the drug with ideal texture and stability. Moreover, the developed gel exhibited a decrease in viscosity with increasing spindle speed, confirming the pseudoplastic behavior of the developed gel, which is ideal for topical application (Supplementary Figure S2). The CH-TH-BG exhibited an optimum viscosity of $10,400 \pm 12.6$ cps at 100 rpm, $98.65 \pm 1.43\%$ drug content, and excellent spreadability of 5.33 ± 0.15 cm, along with good extrudability, making the gel effective for TDDS.

Texture is an important parameter to evaluate the adhesiveness of the gel [35]. The texture profile of CH-TH-BG was compared with that of the conventional TH gel (Supplementary Figure S3). The texture analysis graph exhibited three regions: (i) negative force region (0–5.2 s for CH-TH-BG and 0–6 s for TH gel); (ii) the vertical spike (~5.3 s for CH-TH-BG and 6 s for TH gel); and (iii) the peak (~229.8 gm for CH-TH-BG and 204 gm for TH gel). The negative region indicates the compression phase; the vertical spike indicates the withdrawal phase, in which the machine pulls the probe upward away from the surface; and the peak indicates the maximum force required to detach the bond between the sample and the surface. The results revealed that CH-TH-BG exhibited ~1.12 times higher force than the TH gel, indicating greater adhesiveness. This increased adhesiveness may be due to the polyelectrolyte complexation between carbopol and chitosan, enhancing the gel strength [36]. Additionally, the pH of the developed gel was 5.40 ± 0.10 , which was slightly different from the experimental pH adjustment (6.0 ± 0.2). This slight difference may be due to the residual acidity of the chitosan solution even after lyophilization [37]. The pH of the developed formulation resembled the human skin pH (4.5–6.5), avoiding skin erythema or irritation [38]. In conclusion, the developed CH-TH-BG can be easily applied to the skin with excellent spreadability and extrudability due to the presence of carbopol 940 as a gel-forming agent [39].

2.4. Solid-State Characterizations

The solid-state characterization studies focused on recognizing the interaction between the formulation and its components, a key step in rational formulation development. Different analytical techniques, like FTIR and DSC, are used to assess responses to various perturbations, corroborating a thorough understanding [40]. BLs are designed to encapsulate the drugs within lipid layers through physical encapsulation, not a chemical interaction, even without any variation in the drugs chemically. Moreover, the location of the drug within the formulation is critical for its drug release, bioavailability, and stability [41].

FTIR spectroscopy was used to assess potential interactions between TH and other excipients used in the formulation. Figure 2 presents the IR spectra of TH, BL ingredients, physical mixture, optimized CH-TH-BLs, and CH-TH-BG. The FTIR spectrum of pure TH showed a broad band at 3239 cm^{-1} from hydrogen-bonded phenolic-OH, a sharp peak at 2958 cm^{-1} from alkyl C-H stretches, a peak at 1621 cm^{-1} from benzene ring C=C stretching, a peak at 1422 cm^{-1} from alkyl C-H bending, and strong peaks in the fingerprint region (805 cm^{-1}) from aromatic out-of-plane C-H bending [42]. A broad peak at 3356 cm^{-1} , a sharp peak at 2863 cm^{-1} , and a sharp peak at 1025 cm^{-1} were observed for CH, corresponding to OH/NH stretching, C-H stretching, and characteristic C-O-C/C-O stretching, respectively [43]. SL exhibited sharp peaks at 2922 cm^{-1} , 1737 cm^{-1} , and 1053 cm^{-1} , corresponding to C-H stretching from alkanes, C=O stretching, and overlapping P-O-C/C-O-C stretching, respectively [44]. Cholesterol displayed a broad peak at 3433 cm^{-1} , and sharp and strong peaks at 2929 cm^{-1} and 1052 cm^{-1} , indicating O-H stretching, aliphatic C-H stretching, and C-O stretching, respectively. SDC spectra exhibited characteristic peaks for C-H stretching at 2923 and 2859 cm^{-1} , a strong peak for COO- stretching at 1556 cm^{-1} ,

and a sharp peak for C-O stretching at 1043 cm^{-1} [33]. Physical mixture spectra exhibited a broad peak at 3285 cm^{-1} , a strong, sharp peak at 2924 cm^{-1} , and multiple sharp peaks between 700 and 1725 cm^{-1} , indicating that no chemical interaction took place between TH and bilosome components [11]. Further, the spectra of CH-TH-BLs and CH-TH-BG exhibited almost similar peaks of physical mixture, but certain peaks of TH exhibited increased width with reduced intensity, which may be due to reduced crystallinity of pure TH, supported by DSC findings. These findings indicated that the drug was successfully incorporated into the formulation through a hydrophobic interaction between TH and BLs [33].

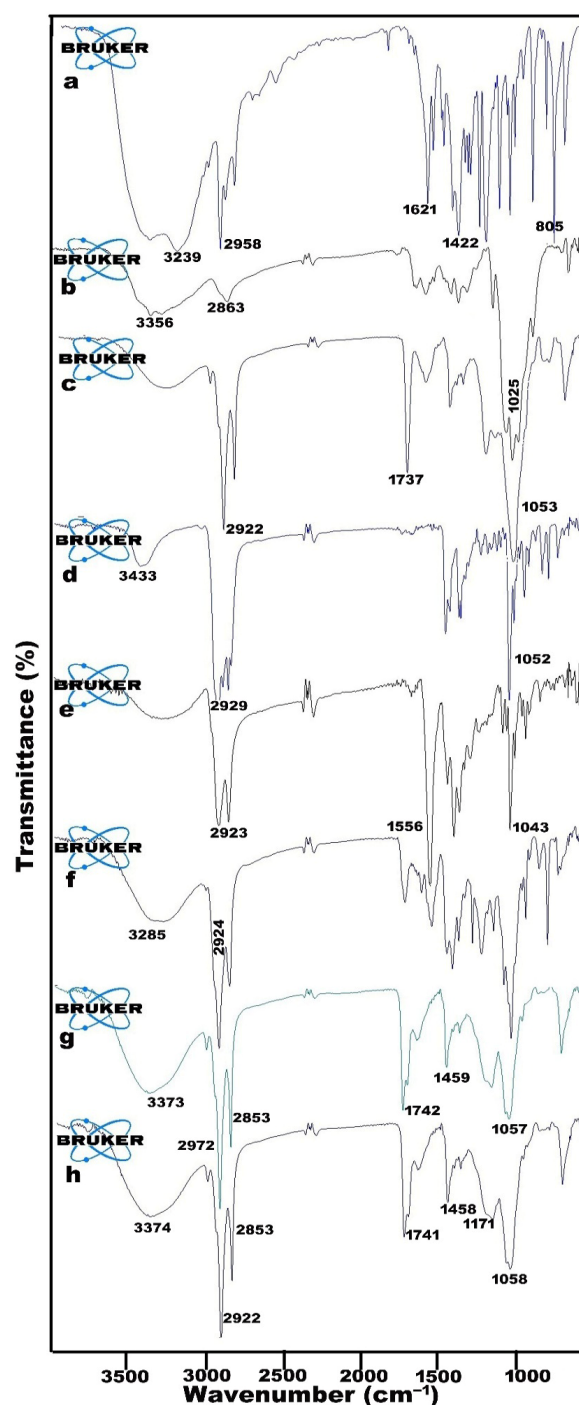


Figure 2. FTIR spectra of drug and different excipients used in formulation. (a) Thymol; (b) chitosan; (c) soya lecithin; (d) cholesterol; (e) sodium deoxycholate; (f) physical mixture; (g) CH-TH-BLs; (h) CH-TH-BG.

DSC was used to assess the thermal behavior and interaction between the drug and other excipients. A sharp peak indicates purity of the substance, a broad or shifted peak reflects chemical interaction or impurity, disappearance of the melting peak signifies chemical complexation or dispersion in other material, and emergence of a new peak presents formation of a new eutectic substance [33]. Figure 3 illustrates the DSC thermograms of TH, BL ingredients, physical mixture, optimized CH-TH-BLs, and CH-TH-BG. TH exhibited a sharp, large endothermic peak at 53.81 °C with enthalpy (ΔH) of -174.70 J/g, representing its melting point and confirming its purity [11]. The thermograms of CH exhibited a broad peak at 78.42 °C and an exothermic peak at 311.21 °C corresponding to dehydration and thermal decomposition, respectively [45]. SL and cholesterol exhibited a characteristic endothermic peak at 188.53 °C and 149.88 °C, whereas SDC exhibited a broad endothermic peak at 86.66 °C and a sharp exothermic peak at 183.88 °C [33]. The thermogram of the physical mixture exhibited the characteristic peak of TH at 54.48 °C with ΔH of -174.70 J/g and all other BL components. Further, it has been observed that the CH-TH-BL thermogram was almost similar to a physical mixture except for the TH melting endothermic peak. The complete disappearance of the TH characteristic peak indicates uniform dispersion of TH within the matrix of BLs in its amorphous form [17,18]. The TH amorphous state is favorable for increased dissolution, in vitro release, and magnified bioavailability of the drug [31]. Additionally, CH-TH-BG exhibited similar peaks to CH-TH-BLs but broader and reduced intensity. This reduced intensity may be due to the increased viscosity of the carbopol matrix. In conclusion, the proposed mechanism for magnified entrapment of TH in BLs is its hydrophobic interaction with SDC [33].

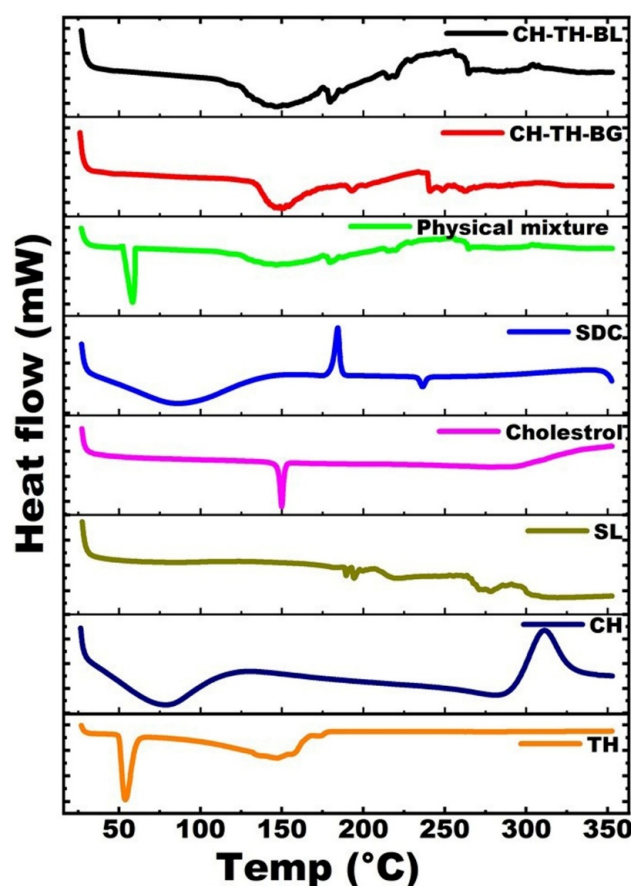


Figure 3. DSC thermogram of thymol and different excipients used in formulation.

2.5. Morphological Characterization

The surface morphology of optimized CH-TH-BLs and CH-TH-BG was characterized by TEM (Figure 4). The results revealed that both formulations were smooth, spherical in shape, and without any aggregation. Moreover, TEM micrographs of CH-TH-BLs displayed a thin layer of CH coating, in agreement with the zeta sizer results (Table 3).

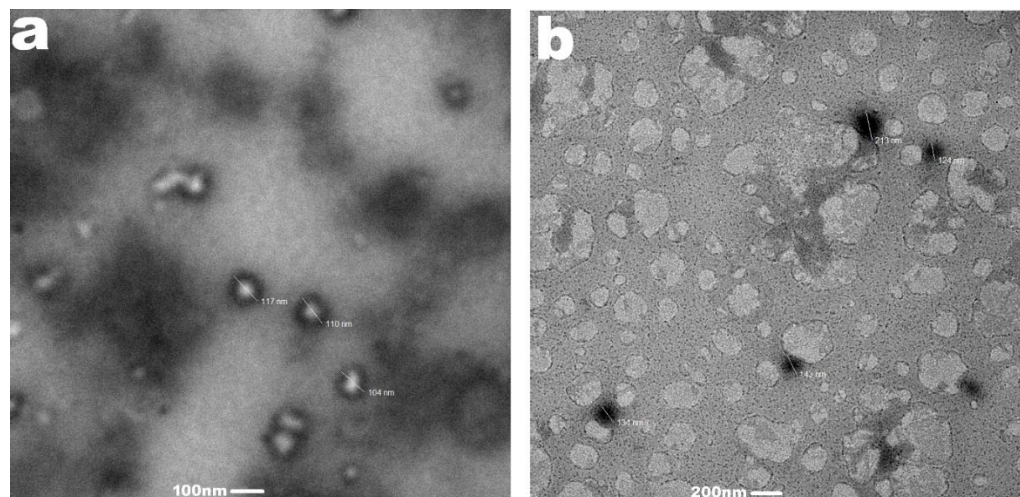


Figure 4. Transmission electron microscope image of optimized formulations. (a) CH-TH-BLs; (b) CH-TH-BG.

2.6. In Vitro Drug-Release Studies

An in vitro drug release-study is a very important parameter in the development and manufacturing of pharmaceutical products, establishing formulation efficacy, safety, and quality. This work explores the potential of CH-TH-BG to magnify the drug permeability and augment the drug concentration accessible for absorption. Figure 5a presents the in vitro drug-release profile of CH-TH-BLs, CH-TH-BG, conventional TH gel, and TH solution. The release of TH from the TH solution and conventional gel was significantly rapid, and almost 100% of the drug was released within 6 h and 8 h, respectively. This complete release was due to the presence of direct diffusion of TH without any barrier. Moreover, CH-TH-BLs and CH-TH-BG exhibited a biphasic release pattern with a rapid release of $49.45 \pm 1.7\%$ and $45.54 \pm 1.4\%$ up to 9 h, respectively, followed by sustained release of $86.23 \pm 1.8\%$ and $74.89 \pm 1.6\%$ up to 24 h, respectively. The initial rapid release may be due to the smaller size of BLs and the presence of surfactant and bile salts [11,33]. A smaller size is responsible for increased surface area, enabling magnified absorption [33]. Bile salts and surfactants were responsible for increasing fluidity [11] and reducing interfacial tension of the formulation, respectively, resulting in increased dissolution and solubility of TH in release media [33]. The sustained release behavior of the drug may be due to the progressive diffusion of the drug from the BL matrix [17]. This biphasic release pattern is effective for the management of RA because burst release is advantageous in achieving the minimum effective concentration to induce a fast therapeutic effect, while sustained release maintains drug concentration within the therapeutic window, enabling prolonged therapeutic efficacy [11,44]. Additionally, CH-TH-BG exhibited delayed release in contrast to the CH-TH-BLs due to the high viscosity of the carbopol gel matrix, which impeded the diffusion rate of the drug, facilitating localized retention and a prolonged therapeutic impact at the site of application. Moreover, the release process was governed by a dual-barrier mechanism, requiring the TH molecule to permeate through the BL layer and gel structure before reaching the release medium [33].

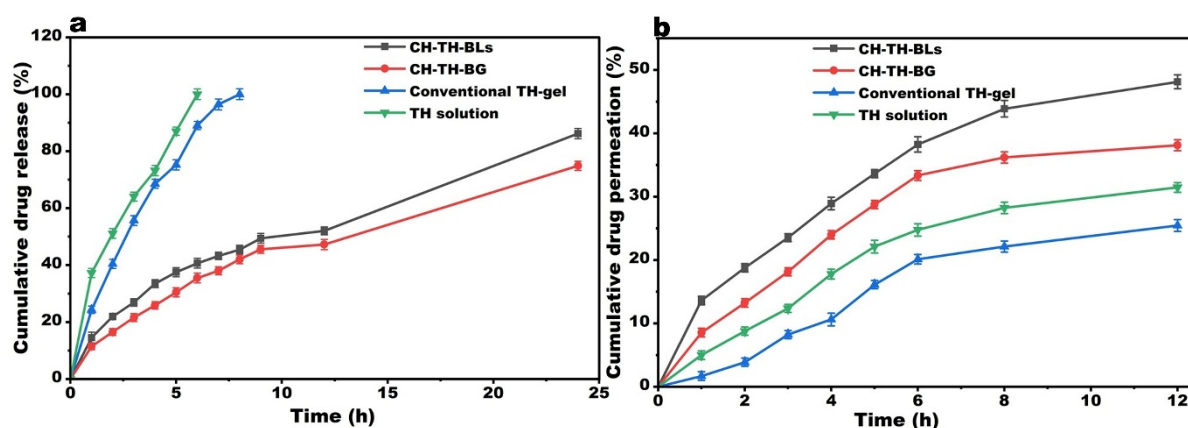


Figure 5. Comparative analysis of optimized CH-TH-BLs, CH-TH-BG, conventional TH gel, and TH solution. (a) In vitro drug-release study; (b) ex vivo permeation study.

The in vitro release data were fitted to various kinetic models, and the results revealed that TH released from the TH solution according to the Higuchi model is evident from the highest R^2 value. Whereas CH-TH-BLs, CH-TH-BG, and conventional TH gel followed the Korsmeyer–Peppas model with non-Fickian release as the value $n > 0.45$ (Supplementary Table S1) [33].

2.7. Ex Vivo Study of Optimized CH-TH-BL and CH-TH-BG

The skin penetration of TH from optimized CH-TH-BLs, CH-TH-BG, conventional TH gel, and TH solution (control) was evaluated in an ex vivo permeation study using rat skin, and various permeation parameters were calculated (Table 4). It has been observed that the CH-TH-BLs exhibited ~5 times and ~2.5 times higher permeation flux than the conventional gel and TH solution, respectively. This higher value was due to the unique properties of BLs, which overcome the skin barrier, enabling enhanced absorption and bioavailability for transdermal delivery. Moreover, CH-TH-BG also exhibited a higher in vitro permeation flux than the conventional gel and TH solution, but lower than the CH-TH-BLs. The lower value may be due to the higher viscosity of the gel network, which hinders the movement of the drug and acts as a diffusion barrier.

Table 4. Comparative analysis of the different permeation parameters and skin deposition for various TH-loaded formulations.

Parameters	CH-TH-BLs	CH-TH-BG	Conventional TH Gel	TH Solution
Permeation flux ($\mu\text{g}/\text{h}/\text{cm}^2$)	20.89 ± 0.98	16.35 ± 0.58	4.35 ± 0.45	8.11 ± 0.71
Permeation coefficient (cm/h)	18.75 ± 0.65	11.56 ± 0.35	1.24 ± 0.13	3.65 ± 0.21
Enhancement ratio	4.03	3.16	-	-
Cumulative drug permeation	$48.13 \pm 1.1\%$	38.12 ± 0.87	$25.45 \pm 0.94\%$	$31.46 \pm 0.96\%$
Skin deposition	$41.87 \pm 1.4\%$	$51.88 \pm 1.17\%$	$27.87 \pm 1.4\%$	$18.45 \pm 0.95\%$

Further, Figure 5b presents the comparative ex vivo permeation profile of all the formulations up to 12 h. The results revealed that the permeation flux of TH for CH-TH-BLs and CH-TH-BG was ~2.5-fold and 2.0-fold higher than that of the TH solution. The proposed mechanism for enhanced permeability of CH-TH-BLs may be the entry of BLs through skin appendages, the intracellular pathway (between the corneocytes), and

the intracellular pathways (within corneocytes). Further, bile salts also ameliorated the flexibility of *stratum corneum* lipids via their fluidization and disrupted the integrity of tight junctions preserved by calcium ions [11]. Additionally, CH coating also affects the transdermal transport by providing bio-adhesion and a positive charge on the surface of BLs, enabling improved interaction with the *stratum corneum*, resulting in higher permeation of the drug [20,34]. However, this study primarily quantified the TH reaching the receptor phase; the residual concentration in the donor compartment was not assessed. Therefore, a skin deposition study was conducted to ensure a complete understanding of the drug's fate within the tissue.

Skin deposition study is a critical parameter during the development of a gel-based formulation to manage RA via TDDS because it quantifies the carrier's ability to localize the drug within the skin layer, enabling its transport to the underlying diseased synovial joints [46]. It has been observed that the TH-solution exhibited higher permeability than the conventional TH gel because of the gel's higher viscosity and fair skin permeability of the TH-solution. Moreover, TH can easily disrupt the *stratum corneum* integrity owing to its high lipophilicity, leading to poor skin retention [16]. Therefore, it requires encapsulation for sustained and controlled release for improved skin retention. Thus, CH-TH-BLs were formulated, followed by incorporation into carbopol gel for magnified skin retention. The results revealed that CH-TH-BG exhibited higher skin deposition, followed by CH-TH-BLs, conventional TH gel, and TH solution (Table 4). The enhanced skin retention of CH-TH-BG may be due to the presence of carbopol and chitosan, leading to sustained release of TH in the deeper layers of skin [11,37].

2.8. In Vivo Study of CH-TH-BG

2.8.1. Arthritis Assessment and In Vivo Efficacy of CH-TH-BG

The anti-inflammatory efficacy of the developed formulation was assessed by visual inspection of FCA-induced inflammation (Figure 6). The result revealed that redness and swelling were observed within 24 h of FCA induction, and maximum swelling was observed on the 7th day. The vehicle control group (1) exhibited no signs of arthritis, whereas the arthritic control group (2) displayed arthritic signs until the 27th day of the study. On the 8th day, groups 3, 4, 5, and 6 were treated with the topical application of respective formulations. On the 28th day, the animals in the negative control and positive control exhibited a slight reduction in paw swelling. This reduction may be due to the anti-inflammatory activity of β -D-glucosamine, a key component of CH [47] and TH [48]. The animals treated with CH-TH-BG exhibited a reduction in paw swelling compared to the arthritic group and the positive control group, with results similar to those of Voltaren emulgel.

After 24 h of the FCA induction, the arthritic control group exhibited a significant reduction in body weight and continued to reduce on the 27th day compared with the vehicle control group (Figure 7a). The negative group also exhibited a significant reduction ($p < 0.0001$) in body weight, whereas the positive control group exhibited a slight regain in body weight on days 24 and 27 compared to the arthritic control group. The test control group also exhibited a significant reduction ($p < 0.0001$) in weight until the 18th day, in contrast to the vehicle control group, but further regained the body weight from day 21 to 27. The standard group also exhibited the same pattern as the test group, but the weight restoration was slightly less than that of the test group. The weight reduction is associated with a reduction in nutrient absorption, severity of inflammation, and the presence of inflammatory biomarkers. Moreover, chronic inflammation disturbs the appetite and metabolic function [49]. The restoration of the weight is an indicator of the therapeutic efficacy of the developed formulation, and CH-TH-BG restored FCA-induced body weight loss. This

indicates that CH-TH-BG was able to reduce the severity of inflammation. Regarding the paw volume, all the groups exhibited approximately similar paw volume at day 0 and maximum paw volume at day 7 (Figure 7b). It has been observed that FCA has significantly increased ($p < 0.05$) in paw volume from day 7 to 28 compared with the vehicle control group, indicating arthritis development. Further, the test control group and standard group exhibited a reduction in paw volume from day 7 to 28 compared with the arthritic group. At day 28, the test group and control group exhibited paw volume almost similar to the vehicle control group, confirming the antiarthritic potential of TH using CH-TH-BG. These findings suggested that the FCA was able to induce arthritis significantly in all groups except the vehicle control, and CH-TH-BG exhibited anti-inflammatory efficacy of TH through enhanced permeation, skin retention, and controlled release of TH, supporting the ex vivo findings.

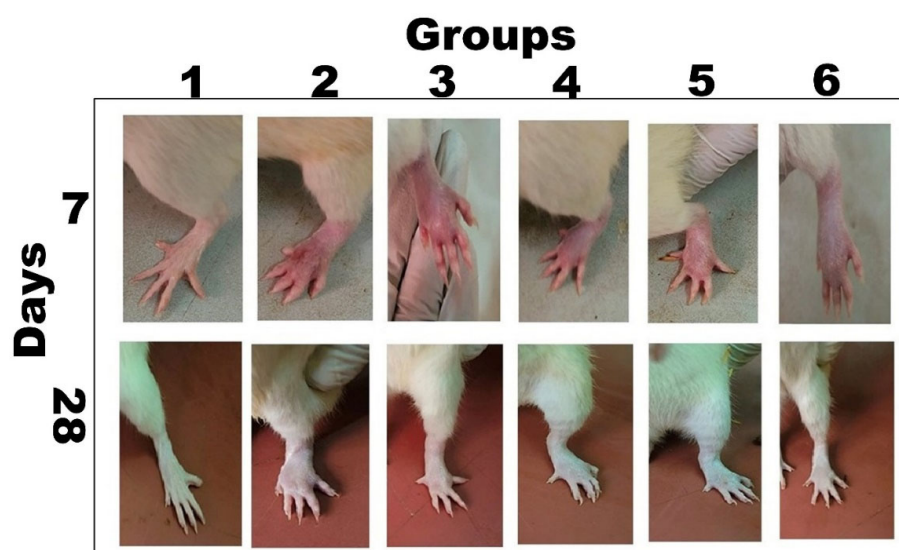


Figure 6. Comparative visual analysis of paw for assessment of arthritis and in vivo efficacy of CH-TH-BG at Day 7 and 28. Group 1 (vehicle control), Group 2 (arthritic control), Group 3 (negative control: CH-BLs without TH), Group 4 (positive control: conventional TH gel), Group 5 (test control: CH-TH-BG), and Group 6 (standard control: Voltaren emulgel).

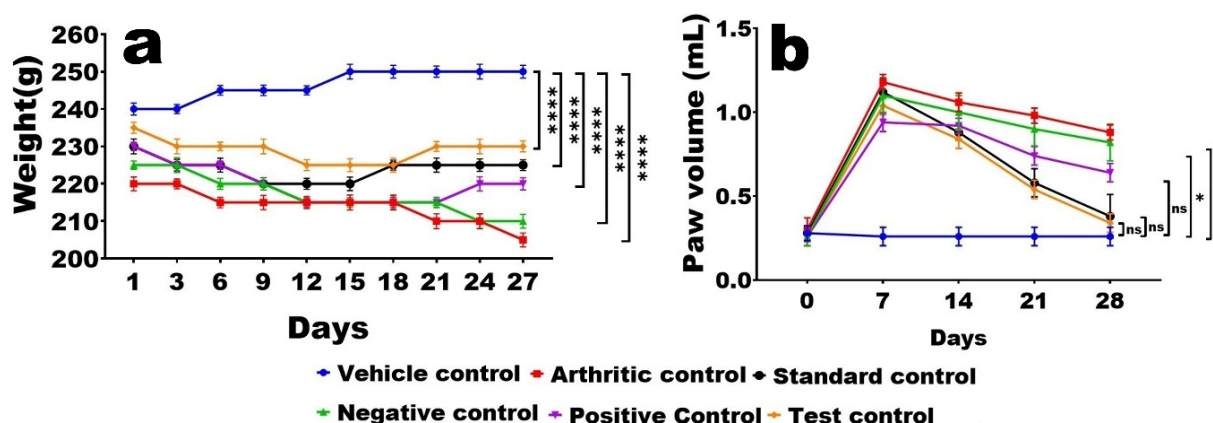


Figure 7. Assessment of arthritis and in vivo anti-inflammatory activity of the CH-TH-BG in animals from physical parameters. (a) variation in body weight; (b) variation in paw volume. Results were presented as mean $\pm$ SE ($n = 5$). Statistical analysis was performed by one-way ANOVA followed by the Dunnett multiple-comparison test. Means were significantly different when compared with the vehicle control group by considering $p < 0.05$ as statistically significant. ns represents non-significant, * $p < 0.05$, **** $p < 0.0001$.

2.8.2. Estimation of TNF- α and IL-6

TNF- α and IL-6 are key pro-inflammatory cytokines leading to joint swelling and inflammation, and they also stimulate osteoclast formation and activate matrix metallo-proteases (MMP), contributing to cartilage and bone degradation in affected joints, which limits mobility and causes stiffness [4]. Therefore, it is required to inhibit the production of these pro-inflammatory cytokines for managing RA. As a result, the level of IL-6 and TNF- α in rat blood serum from different groups was measured after Day 28 of treatment, and the results are displayed in Figure 8. The results revealed that serum levels of IL-6 (Figure 8a) and TNF- α (Figure 8b) were significantly ($p < 0.0001$) increased in the arthritic group compared to the control group. Topical application of positive control, test control, and standard control reduced serum TNF- α and IL-6 levels compared to the arthritic group. Interestingly, the CH-TH-BG and standard control exhibited a significant reduction ($p < 0.0001$) in the expression of both cytokines compared to the arthritic control group. However, the negative control and positive control groups also exhibited slightly reduced serum TNF- α and IL-6 levels compared to the arthritic group. This reduction may be due to the anti-inflammatory activity of β -D-glucosamine (a key component of CH) and TH [47,50]. The reduction in pro-inflammatory cytokines indicates that TH exhibited anti-arthritic activity, and incorporation of TH-loaded BLs into gel magnified its therapeutic efficacy. Additionally, the CH coating also synergizes the anti-inflammatory activity of the developed formulation owing to its anti-inflammatory activity.

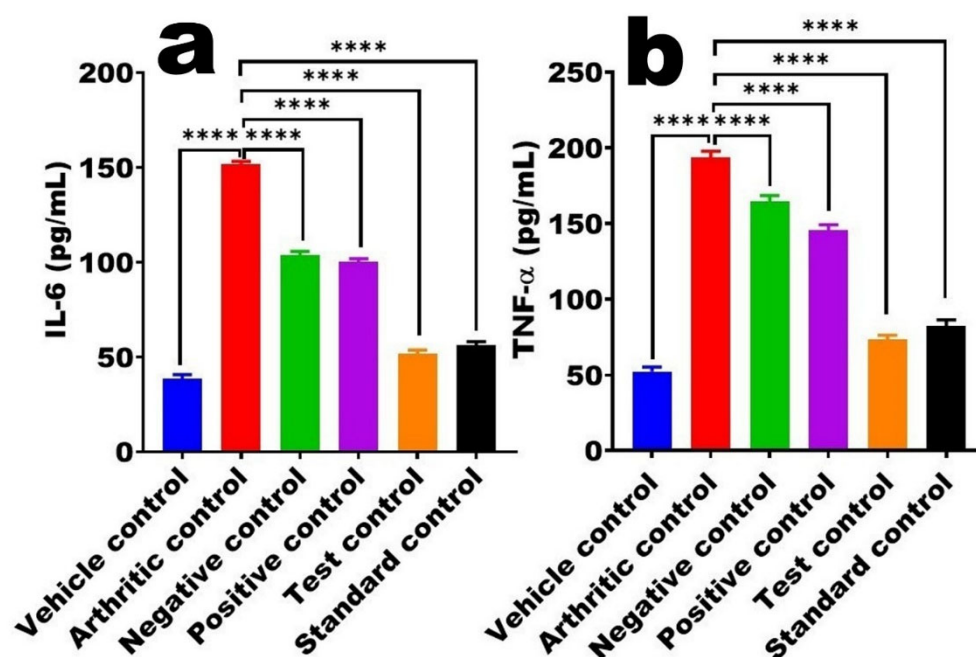


Figure 8. Effect of CH-TH-BG on serum level of IL-6 (a) and TNF- α (b). Results were presented as mean $\pm$ SE ($n = 5$). Statistical analysis was performed by one-way ANOVA followed by the Dunnett multiple-comparison test. Means were significantly different when compared with the arthritic control group by considering $p < 0.05$ as statistically significant. **** $p < 0.0001$.

2.8.3. Histopathological Analysis

Histopathological analysis of ankle joints was carried out to assess the antiarthritic activity of the developed formulation (Figure 9). The photomicrograph of the vehicle control group exhibited a dense articular surface with subchondral bone and normal articular cartilage (i) and a thinner synovial membrane (ii), indicating that the ankle joint [51] was non-inflamed, confirming the biocompatibility of the carbopol gel (Figure 9a). In contrast, the arthritic control exhibited an irregular and thin articular surface (iii), sub-

chondral bone erosion with visible pits (iv), and joint space filled with pannus formation (v) (Figure 9b), confirming arthritis induction. The negative control group also exhibited cartilage degradation with an irregular surface (vi) and subchondral bone damage (vii), indicating inflammation (Figure 9c). The microphotograph of the test control group exhibited a slightly rough and low-to-mildly degenerated articular surface (viii) and a thinner synovial membrane (ix), compared with the arthritic control group (Figure 9d), indicating recovery of the ankle joint. However, the standard control group exhibited cartilage erosion (x) and thinning (xi) as compared to the test control group (Figure 9e). The results confirmed the biocompatibility and antiarthritic potential of the developed CH-TH-BG.

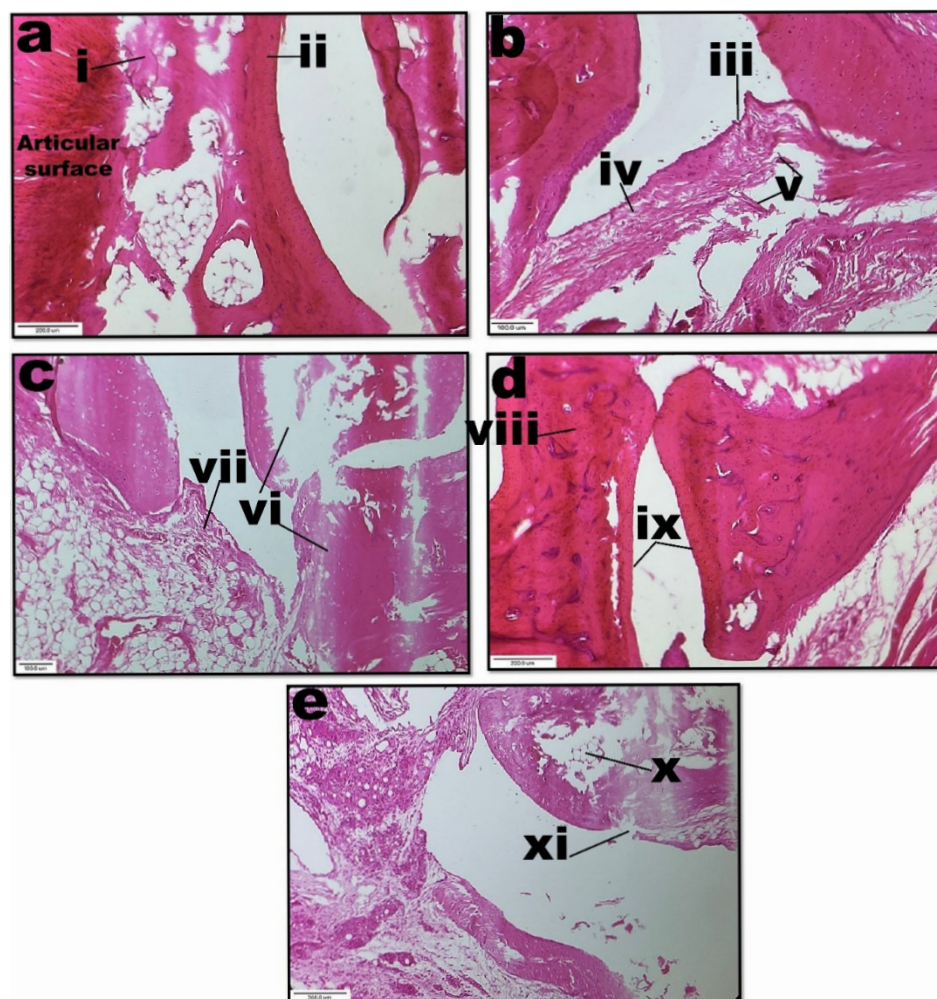


Figure 9. Photomicrograph of ankle joints stained with hematoxylin and eosin ($\times 100$). (a) vehicle control group: (i) dense articular surface, (ii) thinner synovial membrane (scale bar: 200 μm); (b) arthritic control group: (iii) thin articular surface, (iv) subchondral bone erosion, (v) pannus formation (scale bar: 100 μm); (c) negative control group: (vi) cartilage degradation with an irregular surface, (vii) subchondral bone damage (scale bar: 100 μm); (d) test control group: (viii) mild degenerated articular surface, (ix) thin synovial membrane (scale bar: 200 μm); (e) standard control group: (x) cartilage erosion, (xi) cartilage thinning (scale bar: 200 μm).

2.9. Storage Stability

The developed CH-TH-BGs were assessed for long-term stability studies as per International Conference on Harmonization (ICH) guidelines. The developed gel was transparent and homogeneous throughout the study, along with minor variation in viscosity, pH, and drug content (Supplementary Table S2).

3. Conclusions

This study marks the first exploration of the therapeutic potential of transdermal nanobilosomal gel-bearing CH-coated, TH-loaded bilosomes (CH-TH-BG) for the effective management of RA. CH-TH-BLs were prepared by the thin film hydration method and optimized using a BB design, then the optimum lyophilized formulation was incorporated into carbopol gel for prolonged retention of the drug. CH-TH-BLs and CH-TH-BG were physicochemically and therapeutically characterized. The results revealed that the optimized CH-TH-BLs were smooth and spherical in shape without any aggregation, along with enhanced EE, which will be beneficial for augmented therapeutic effect and precise delivery of TH. Moreover, the optimized formulation exhibited a positive zeta potential due to the CH coating, which facilitates improved interaction with the *stratum corneum*, resulting in higher drug permeation. Furthermore, the CH-TH-BG exhibited enhanced drug content, optimal viscosity, excellent spreadability, extrudability, and a pH similar to that of human skin, making the gel effective for TDDS. The developed gel exhibited a biphasic release pattern with a rapid release followed by sustained release and controlled release, in contrast to the CH-TH-BLs, due to the presence of the carbopol matrix, facilitating localization of the drug molecules and extending their therapeutic effect at the target site. Furthermore, ex vivo results revealed that CH-TH-BG exhibited enhanced permeability and skin retention, in contrast to conventional TH gel, which allows the BLs to penetrate the *stratum corneum* and deliver the TH to deeper tissue. Additionally, in vivo results suggested that the developed gel exhibited a significant reduction in paw swelling and restoration of body weight on day 21 compared to the arthritic group. The CH-TH-BG exhibited superiority for managing arthritis and inflammation with a significant reduction in the serum level of TNF- α and IL-6. Histology results also confirmed the antiarthritic activity of CH-TH-BG through the recovery of ankle joints. In conclusion, the developed bilosomal gel offers a reasonable therapeutic strategy for transdermal delivery of TH for the effective management of RA.

4. Materials and Methods

4.1. Materials

Thymol, soy lecithin (SL), cholesterol, Span 60, Tween 80, Carbopol 940, sodium deoxycholate (SDC), chloroform (HPLC), methanol (HPLC), chitosan (CH, MW: 150,000), Freund's complete adjuvant (FCA), and dialysis bags (MW cut off: 12,000 Da) were purchased from Sigma-Aldrich, Bangalore, India. The other excipients and chemicals are of analytical quality and procured from SD-Fine Chemicals, Mumbai, India.

4.2. Preparation of Chitosan-Coated, Thymol-Loaded Bilosomes (CH-TH-BLs)

CH-TH-BLs were prepared by the thin film hydration method with minor modifications using a two-step process [20]. Initially, TH-BLs were prepared and further coated with different concentrations of CH. For TH-BLs, thymol (100 mg), cholesterol (20 mg), and surfactants (span 60: tween 80, and other quantities of SL) were dissolved in 2:1 (40 mL) of chloroform: methanol. The resultant organic mixture was transferred to a round-bottom flask (RBF) and evaporated for 30 min at 50 ± 5 °C using a rotary evaporator (Physilab Rotatory Vacuum Film Evaporator, Haryana, India) at 90 rpm under reduced pressure to form a dry film. Further, the RBF was placed in a desiccator for 24 h to ensure complete removal of organic solvents, followed by the rehydration of the film with 10 mL of SDC solution in phosphate-buffered saline (PBS, pH 7.4) for 1 h under atmospheric pressure at 100 rpm agitation. The resulting TH-BLs dispersion was subjected to a probe sonicator (Sartorius, Labsonic@ P, Singapore) in an ice bath for 4 min at 30% amplitude with a 0.2 s on/off pulse. For CH-TH-BLs, different concentrations of CH were dissolved in 0.5% v/v

glacial acetic acid. Further, 2 mL of CH solution was added dropwise (0.2 mL/min) to the dispersion of TH-BLs under continuous stirring (100 rpm) at room temperature for 4 h [20,32]. Further, the formed CH-TH-BL dispersion was lyophilized and stored at 4 °C for further investigation [17,33].

4.3. Optimization of Processing Variables for CH-TH-BL Preparation

The quality-by-design (QbD) approach was used to develop a robust formulation. Initially, a quality target product profile (QTPP) was established to define the desired performance of the CH-TH-BLs. Based on the QTPP, the critical quality attributes (CQAs) were identified, including particle size, zeta potential, and EE, due to their direct impact on in vitro drug release and ex vivo permeability of the formulation. Preliminary experiments were performed to detect significant formulation variables. Factors such as SL concentration (%w/v), SDC concentration (mg), and CH concentration (%w/v) were selected as independent variables for the optimization study based on their significant effect on the CQAs.

The BB design was used to optimize the formulation variables of CH-TH-BLs with three independent variables at three levels, utilizing Design Expert (version 13.0, Stat-Ease Inc., Minneapolis, MN, USA) (Table 5). This experimental design requires fewer runs than other designs, which makes it more cost-effective and avoids extreme combinations of factors that could fail in the experimental phase. Moreover, BB design evaluated the main effects, interaction effects, and quadratic effects of the formulation variables on CQAs. The optimized formulation was selected based on the minimum particle size, maximum zeta potential, and maximum EE, and with a high desirability index. The experimental data were analyzed using one-way ANOVA to evaluate the significance of processing variables on the responses and their interactions. Moreover, the best-fit model was assessed based on a high R^2 with $p < 0.0001$ [20,33].

Table 5. Independent and dependent variables of the Box–Behnken design for the formulation of CH-TH-BLs.

Independent Variables	Levels		
	Low (−1)	Medium (0)	High (+1)
A: SL concentration (%w/v)	1.0	2	3
B: sodium deoxycholate concentration (mg)	10	20	30
C: Chitosan Concentration (%w/v)	0	0.2	0.4
Dependent variables	Goal		
Particle size (nm),	Minimize		
Zeta potential (mV)	Maximize		
EE (%)	Maximize		

4.4. Physicochemical Characterization of CH-TH-BLs

4.4.1. Particle Size and Zeta Potential

The particle size and zeta potential were analyzed at 25 °C by differential light scattering (DLS) using Malvern Nano-ZetaSizer (Malvern Instrument Ltd., United Kingdom). Aliquots from all bilosomes were diluted with distilled water to attain the proper light scattering. The zeta potential was determined by estimating the electrophoretic dispersion of charged particles in an electrical field [18,33]. All the samples were measured in triplicate, and the average was reported.

4.4.2. Entrapment Efficiency (EE)

The % EE was estimated by measuring the concentration of the un-entrapped drug (free TH) in the dispersion and calculated using Equation (4). To separate free TH from the bilosomes, the formulations were centrifuged at 4 °C and 10,000 rpm for 2 h with a cooling centrifuge (Sartorius-SIGMA 3-18 K, Osterode am Harz, Germany) [33]. The supernatant was withdrawn, diluted to 10 mL, and analyzed spectrophotometrically at λ_{max} 275 nm using a validated UV–visible spectrophotometer (Shimadzu 2202, Japan) (see Supplementary File) [10,11]. All the samples were measured in triplicate.

$$\%EE = \frac{(\text{Total amount of thymol added} - \text{Unentrapped thymol})}{\text{Total amount of thymol added}} \times 100 \quad (4)$$

4.5. Preparation and Characterization of CH-TH-BL Loaded Gel (CH-TH-BG)

The optimized CH-TH-BLs were incorporated into a 1% *w/v* carbopol 940 gel base to prolong their retention time over the epidermis. Initially, the carbopol gel was prepared by dispersing carbopol (100 mg) into 10 mL of distilled water and left to stand for 24 h, followed by continuous stirring for 3–4 h to form a homogeneous gel. Tri-ethanolamine, propylene glycol (5% *w/w*), and methyl–paraben (0.10%) were added to adjust the pH (6.0 ± 0.2), plasticity, and subsequent storage, respectively [20]. Further, 160 mg of the optimized lyophilized CH-TH-BLs was incorporated into 840 mg of gel base to prepare CH-TH-BG (1% *w/w* TH). For comparative analysis, a conventional TH gel was also prepared using the same procedure, by adding 10 mg pure TH into 1 gm (1% *w/v*) of carbopol 940 gel base.

The nanobilosomal gel was characterized for appearance, consistency, and homogeneity by visual inspection. The viscosity of the prepared gel was measured at 25.0 ± 0.5 °C by a Brookfield Viscometer (Brookfield RVT, Bangalore, India) at four different speeds of 10, 20, 50, and 100 rpm, using spindle RV 7 [52]. Further, the adhesive strength of the developed gel was measured using a Texture Analyzer (Texture-TA-XT2 Analyser, Stable Micro Systems, Surrey, England). Briefly, adhesion testing was performed by placing the gel and a two-sided probe within a Mucorig assembly on the instrument stage. The probe was inserted vertically into the Mucorig, avoiding side-wall interference. The software (Texture Expert Exceed, Version 1.00, Surrey, England) directly recorded the detachment force, representing the highest force necessary to break the contact between the probe surface and the gel [35]. For pH, 1 g of gel was diluted with 100 mL of double-distilled water and evaluated by a calibrated pH meter at 25 °C [53]. Spreadability was determined by placing 0.5 g of gel on a Petri plate, and the initial diameter was recorded. Further, another Petri plate was placed over the gel, and a 100 g weight was placed on the top of the upper plate for 5 min, and an increase in diameter was recorded [54]. For extrudability, 6 aluminum tubes were filled with gel (10 g each) and held between two fingers to apply finger pressure. The extrudability for each formulation was calculated by measuring the weight of the extruded gel. To assess the TH content in gel, 1 g of CH-TH-BG was dissolved in methanol and then centrifuged at 6000 rpm for 20 min, followed by filtration. The solution was diluted with methanol and analyzed spectrophotometrically using a UV–visible spectrophotometer at 275 nm [55]. All the samples were measured in triplicate.

4.6. Solid-State Characterizations

The compatibility between TH, bilosomal ingredients (CH, SDC, SL, cholesterol, physical mixture), optimized CH-TH-BLs, and CH-TH-BG was examined using an FTIR spectrophotometer (Bruker, alpha-II, Berlin, Germany) between 400 and 4000 cm^{-1} and differential scanning calorimetry (DSC, Shimadzu, DSC-60Plus, Kyoto, Japan) [17].

4.7. Morphological Characterization of Optimized CH-TH-BLs and CH-TH-BG

A transmission electron microscope (TEM, Thermo Scientific Talos L120C G2 (S), Waltham, MA, USA) was used to analyze the surface morphology of optimized CH-TH-BLs and CH-TH-BG. An aliquot of the diluted sample was placed on a carbon-coated grid and allowed to dry for 15 min at 25 °C. Further, the grid was sprayed with a 2% aqueous solution of phosphotungstic acid and allowed to stand for 3 min, followed by the elimination of excessive solution. The grid was examined under the microscope at 20–120 kV [17].

4.8. In Vitro Drug-Release Study of Optimized CH-TH-BLs and CH-TH-BG

The dialysis method was used to study the in vitro release of TH from CH-TH-BLs and CH-TH-BG, conventional TH gel, and pure TH solution in ethanol: PBS (3:7). Briefly, a dialysis membrane (Hi, media, molecular cutoff 12 kDa–14 kDa, Mumbai, India), pieces of 5 cm length and 2.5 cm width, were soaked overnight in PBS (pH 7.4) to achieve full swelling, enabling consistent pore size during the study. The formulation equivalent to 1% *w/w* of TH was placed in a dialysis bag, and the bag was sealed on both sides to avoid leakage, followed by immersion in 50 mL of ethanol and PBS (pH 7.4) in a ratio of 3:7 at 100 rpm and 37 °C $\pm$ 1 °C using a thermostated shaking water bath (Memmert, SV 1422, Schwabach, Germany). In total, 2 mL of each sample was withdrawn at 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, and 24 h of the study and replenished with an equal volume of fresh release medium to maintain sink conditions. The collected samples were analyzed using a UV–visible spectrophotometer at $\lambda_{\max}$ 275 nm, and the cumulative amount of TH was quantified [11]. Furthermore, the in vitro release data were fitted to various release kinetics models, and the best fit model was determined by the highest regression coefficient (R^2) [31].

4.9. Ex Vivo Study of Optimized CH-TH-BLs and CH-TH-BG

4.9.1. Preparation of Rat Skin

The animal study was performed following the approval granted by the Institutional Animal Ethical Committee (IAEC) of the Faculty of Pharmacy, Babu Banarasi Das Northern India Institute of Technology, Lucknow (U.P.), (BBDNIIT/IAEC/Aug/2025/06) under the regulation of the Committee for Control and Supervision of Experiments on Animals (CCSEA). Wistar rats (200–250 gm) were housed in the animal house at a controlled temperature of 25 $\pm$ 2 °C and 55 $\pm$ 5% humidity with ad libitum access to food and water as per the standard guidelines of the CCSEA, India. Animals were acclimatized for one week to stabilize in the environment. The rats were euthanized after the removal of hair using an electrical clipper, followed by the separation of the dorsal side. Subcutaneous fats and tissues were scraped carefully and then examined for integrity. The skin was sliced into appropriate circular segments, washed with normal saline, and dried at 25 °C to remove excessive moisture [33].

4.9.2. Skin Deposition and Permeation Study

A Franz diffusion cell (permeation area 3.14 cm²) was used to insert the skin membrane between the donor and receiver compartments. The optimized CH-TH-BLs, CH-TH-BG, conventional TH gel, and pure TH solution (control) were placed in the donor compartment and immediately occluded with Parafilm to prevent TH volatilization, and 35 mL of ethanol and PBS (pH 7.4) in a ratio of 3:7 was placed in the receiver compartment at 32 $\pm$ 0.5 °C at 50 rpm to maintain the sink conditions. The drug concentration in each sample was 1% *w/w*. In total, 1 mL of the samples was withdrawn from the receiver compartment and replaced with fresh ethanolic PBS at assigned intervals of 0, 1, 2, 3, 4, 6, 8, and 12 h. The samples were passed through a 0.25 μ m membrane filter and were analyzed using a UV–visible spectrophotometer at $\lambda_{\max}$ 275 nm to quantify the TH concentration [11]. Permeation

parameters, including permeation flux, permeation coefficient (PC), and enhancement ratio (ER), were calculated using Equations (5)–(7), respectively [31,33].

$$\text{Permeation flux (Jss)} = \frac{\text{Concentration of permeated drug}}{\text{Time} \times \text{Area}} \quad (5)$$

$$\text{Permeation coefficient (PC)} = \frac{\text{Jss}}{\text{Initial drug concentration of the donor compartment}} \quad (6)$$

$$\text{Enhancement ratio (ER)} = \frac{\text{PC sample}}{\text{PC control}} \quad (7)$$

After 12 h, the samples were removed, and the skin sample was washed and rinsed with water. It was then sectioned into small pieces and sonicated with 2 mL of ethanol for 5 min. The resultant sample was centrifuged for 2 min, and the supernatant was passed through a 0.25 µm membrane filter and analyzed using a UV–visible spectrophotometer at λ_{max} 275 nm to quantify the skin deposition of TH [18]. All the measurements were performed in triplicate.

4.10. In Vivo Study of CH-TH-BG

4.10.1. FCA-Induced Arthritis Model

A comparative analysis was performed to evaluate the in vivo efficacy of CH-TH-BG (1% w/w) for treating FCA-induced arthritis versus conventional TH gel (1% w/w), and marketed Voltaren emulgel (diclofenac: 1% w/w). Thirty Wistar rats (200–250 gm) were acquired from the animal house and allocated to six groups (n = 5) (Table 6), then shaved before FCA induction [56]. Briefly, rats were sensitized with 0.1 mL of FCA in the sub-plantar space of the right hind paw of all groups (2–6), except the vehicle control group (1). All the measurements were performed in triplicate [56]. The sample size for each experimental group (n = 5) was selected based on published protocols for FCA-induced arthritis and to ensure adequate statistical power [57]. A post hoc justification using the Resource Equation ($E = \text{total no. in the study (30)} - \text{total no. of groups (6)}$) resulted in an E value of 24. Since an E value between 10 and 20 is usually considered enough for exploratory animal research, this sample size provided sufficient degrees of freedom for ANOVA to detect meaningful differences between the 6 experimental groups while remaining consistent with the 3Rs (Replacement, Reduction, and Refinement) principle of animal reduction.

Table 6. Allocation of experimental animals in different groups and treatment modalities.

Group No.	Group Name	Interventions
1.	Vehicle control	1% w/v carbopol 940.
2.	Arthritic control	0.1 mL of FCA
3.	Negative control	0.1 mL of FCA+ 1% w/v carbopol gel comprising CH-BLs without TH
4.	Positive control	0.1 mL of FCA+ conventional TH gel (1% w/w TH)
5.	Test control	0.1 mL of FCA+ CH-TH-BG (1% w/w TH)
6.	Standard control	0.1 mL of FCA+ Voltaren emulgel (1% diclofenac)

4.10.2. Assessment of Arthritis and In Vivo Efficacy of CH-TH-BG

Arthritis was assessed by visual inspection of the affected areas for signs of inflammation (redness, erythema, and swelling) every 3rd day between the 0–27 days in FCA-induced animals [58]. Furthermore, the body weight of all animals was recorded every 3rd day using an electronic balance. Additionally, a mercury-based plethysmometer was used to measure

the right hind paw volume every 7th day. The method relies on the measurement of the level of mercury displaced after the immersion of a rat's paw up to the marked level in an immersion tube containing mercury connected to a measuring column with a graduated scale. The variation in body weight and paw volume in the groups was compared to the arthritic control group, and day 0 was marked as the day of FCA induction [51,56].

To assess the efficacy of the developed gel, the formulations were topically applied to the inflamed area, except for Groups 1 and 2. Briefly, on the 8th day, Group 3 was treated with a placebo gel (CH-BLs without TH), Group 4 with a conventional TH gel (1% *w/w* TH), Group 5 with CH-TH-BG (1% *w/w* TH), and Group 6 with Voltaren emulgel (1% diclofenac) (Table 6). The TH-loaded gel (1% *w/w*) was applied topically at a standardized dose of 500 mg/animal, equivalent to 5 mg of TH. This dose was calculated to achieve a target concentration of 20 mg/kg based on an average body weight of 250 gm. The formulation was spread as a thin film over the inflamed joint once daily for a period of 27 days [11]. The formulations were gently applied using the index finger and massaged in a circular motion, followed by covering with a plastic sheet to allow enough time for permeation. All the animals were closely monitored for 1 h after the treatment to decrease the probability of plastic sheet removal during their movement, followed by visual inspection every 3rd day between the 0–27 days for inflammation [18].

4.10.3. Estimation of TNF- α and IL-6 and Histopathological Analysis

The anti-inflammatory activity of CH-TH-BG was evaluated by measuring serum levels of inflammatory biomarkers TNF- α and IL-6. Briefly, blood samples were withdrawn from the retroorbital plexus using heparinized capillary tubes under anesthesia after completion of treatment, i.e., on the 28th day. The blood sample was centrifuged at 3000 rpm for 10 min, which was followed by the collection of the supernatant, and it was stored at $-20\text{ }^{\circ}\text{C}$ until further analysis. The serum was analyzed for TNF- α and IL-6 using ELISA kits (Raybiotech, GA, USA) following manufacturer instructions [18].

4.10.4. Histopathological Analysis

For histological analysis, the positive control group (conventional TH gel) was excluded to adhere to the 3Rs principle and because of non-significant therapeutic efficacy in previous observations (paw volume, body weight, and pro-inflammatory cytokines). Briefly, the rats were anesthetized and sacrificed for excision of the ankle joints after the completion of the study. Further histopathological studies were done using the FFPE (formalin-fixed paraffin-embedded) method. The articular joints were fixed in formaldehyde solution (10%), decalcified in formic acid, and embedded in paraffin blocks. Further, 5 μm slices were cut and stained with hematoxylin and eosin (HE), followed by an examination under an optical microscope at $100\times$ [17].

4.11. Storage Stability

The storage stability of CH-TH-BG was assessed for long-term accelerated stability studies as per ICH guidelines. Briefly, 10 gm of gel was sealed in a glass vial and stored in a stability chamber (Newtronics Limited, New Delhi, India) at $25 \pm 2\text{ }^{\circ}\text{C}/60 \pm 5\%$ RH for 6 months. Samples were collected at 2, 4, and 6 months and evaluated for physical appearance, viscosity, pH, and drug content. All the measurements were performed in triplicate.

4.12. Statistical Analysis

Statistical analysis was carried out by one-way ANOVA (parametric) with post hoc Dunnett multiple-comparison test using GraphPad Prism (version 8.0.1), and $p < 0.05$ was

considered statistically significant. All values were expressed either as mean $\pm$ standard error of mean (SEM) or mean $\pm$ standard deviation (SD), as mentioned in the figure legends.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/gels12020156/s1>, Figure S1: Particle size (a) and zeta potential of optimized CH-TH-BL; Figure S2: Rheogram of TH-gel and CH-TH-BG; Figure S3: Texture analysis of CH-TH-BG (a) and TH-gel; Table S1: Mathematical model to determine the release kinetics; Table S2: Long-term stability study of CH-TH-BG for 6 months as per ICH guidelines.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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