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FORMULATION AND EVALUATION OF A CURCUMIN–THYMOQUINONE–RESVERATROL-LOADED NANOEMULSION AND NANOEMULGEL FOR TOPICAL MANAGEMENT OF PSORIASIS AND ACNE VULGARIS

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ABSTRACT:
Background: Curcumin, thymoquinone and resveratrol suffer from the issues of limited aqueous solubility which makes it challenging to be delivered as a topical agent. This work was designed to develop a nanoemulsion co-loaded with these three bioactives and to develop the nanoemulgel and evaluate its skin permeation and biological activity in vivo.
Methods: Nine nanoemulsion formulations were screened after performing a pseudo-ternary phase-diagram analysis and excipient-solubility assessment along with a simultaneously developed UPLC assay. The chosen formulation was then incorporated into a gel formulation of Carbopol 940 and its physicochemical, ex vivo skin delivery, antimicrobial activity, cell based responses, in vivo activity in a mouse model of psoriasis like inflammation and storage stability was assessed.
Results: The selected nanoemulsion (10% Capryol 90, 35% Tween 80–Transcutol P [2:1] and 55% water) had a mean droplet size of 92.4 ± 2.7 nm, polydispersity index of 0.214 ± 0.018, zeta potential of −18.6 ± 1.9 mV and transmittance of 98.6 ± 0.4%. The amount of curcumin, thymoquinone and resveratrol in nanoemulgel was 99.1 ± 0.8%, 99.5 ± 0.7% and 99.0 ± 0.9% respectively. At 24 h, the corresponding skin deposition values were 51.8 ± 3.9, 58.7 ± 4.2 and 45.6 ± 3.5 µg/g, representing 2.64-, 2.37- and 2.55-fold increases over the conventional gel. The inhibition zone values of Cutibacterium acnes and Staphylococcus aureus were 23.4 ± 1.2 and 20.8 ± 1.0 mm, respectively. The nanoemulgel group PASI score was 2.8 ± 0.6 on day 7 and for the disease-control group, it was 9.2 ± 0.7 (reported $p < 0.001$). After 6 months storage at 40°C and 75%RH, the bioactive contents of these samples were still found to be not less than 94%.
Conclusion: The tri-bioactive nanoemulgel improved the deposition of drug on skin when compared to conventional gel and exhibited promising activity on the preclinical models studied. Further research is needed to establish the clinical efficacy, stability and safety of long term use of the product.

KEYWORDS: Curcumin, Thymoquinone, Resveratrol, Nanoemulsion, Nanoemulgel, Topical delivery, Psoriasis, Acne vulgaris.

1. INTRODUCTION

Psoriasis and acne vulgaris are inflammatory diseases of the skin, with different pathophysiological characteristics. In psoriasis, excess proliferation of the keratinocytes and abnormal keratinocyte differentiation are observed and in acne vulgaris, there is increased sebum production and inflammatory response by Cutibacterium acnes [1–6]. The stratum corneum barrier to access to relevant cutaneous compartments can be problem when using 'Topical therapy' (direct application of agents to affected skin). Topical dosage forms may also pose some formulation related issues and may cause local irritation. Anti-inflammatory and antioxidant activity of curcumin, thymoquinone and resveratrol have been studied. Antipsoriatic activity of curcumin was studied in ex vivo preparations and psoriasis model systems [8,9,14,10]. Nigella sativa contains a bioactive compound called thymoquinone which was found to have pharmacological activities in preclinical studies [11,12]. Resveratrol is one of the polyphenols found in grapes that has been studied for the presence of several biological activities including in vitro activity towards acne associated bacteria [5,13]. They are poorly soluble in water, and may be chemically unstable, thus complicating attempts to develop suitable topical dosage forms. Nanoemulsion delivery systems can improve the apparent solubility of less water soluble substances and partition it in the skin [7-10]. It is possible to incorporate a nanoemulsion into a semisolid gel to enable the topical administration and retention at the administration point. Even if the nanoemulsions and nanocarrier gels have been studied for drug delivery in the skin [7–9,16–18,20–22], it was worth to investigate the formulation of a combined system of curcumin, thymoquinone and resveratrol for preliminary evaluation in in vivo. The main objectives of the present study was the formulation and characterization of a tri-bioactive nanoemulsion and nanoemulgel and to study the ex vivo skin

permeation/deposition of the formulation and skin irritation and response in imiquimod-induced mouse model of psoriasis-like inflammation. In parallel, exploratory antibacterial tests were conducted against C. acnes and Staphylococcus aureus and stability tests, excipients screening and HET-CAM vascular screening and tests in HaCaT keratinocytes were performed.

2. MATERIALS AND METHODS

2.1 Materials

Curcumin, thymoquinone and trans-resveratrol were used as the bioactive compounds. The oil phase was capryol 90, the surfactant was Tween 80 and the cosurfactant transcutol P. The gelling polymer was dissolved Carbopol 940 and the neutralizer was dissolved Triethanolamine (TEA). Analytical-grade reagents and HPLC/UPLC-grade acetonitrile and methanol were used for the experimental procedures.

2.2 Preformulation characterization and drug-excipient compatibility

Their appearance, melting range, ultraviolet absorption maximum, Fourier-transform infrared (FTIR) spectrum and differential scanning calorimetry (DSC) profile were used to characterize each bioactive. The FTIR spectra and DSC thermograms of the physical mixtures of each of the bioactives and the selected excipients were also compared with that of the individual components. Changes in characteristic absorption bands and thermal transitions were evaluated to determine change under conditions studied.

2.3 Simultaneous UPLC method

A C18 column (100 × 2.1, 1.7 um), water as mobile phase containing 0.1% formic acid and flow rate 0.35 mL/min were used for the simultaneous ultra-performance liquid chromatography (UPLC) method with injection volume 5µL and photodiode-array (PDA) detection system. The calibration range for curcumin, thymoquinone and resveratrol were 0.5-50, 0.25-40 and 0.5-50 µg/mL, respectively. Linearity, recovery, precision, specificity, limit of detection (LOD) and limit of quantification (LOQ) were determined. The UV absorption maximums given in Table 1 are preformulation and do not necessarily correspond to the detection wavelength for chromatography.

2.4 Solubility screening and pseudo-ternary phase diagrams

Solubility of the selected bioactive material in different oils, surfactant and cosurfactants was determined by putting excess quantity of the drug in each of the oils, surfactant and cosurfactants and allowed to equilibrate for 48 h at 25 ± 1°C. Equilibrated concentration was quantified by using UPLC. The excipients that showed the positive solubilizing property were selected for the phase-diagram screening. The pseudo-ternary phase diagrams showed that the monophasic regions of n-octane, P123, and water would be suitable for the preparation of nanoemulsion by using the ratio of 1:1 Smix, 2:1 Smix, and 3:1 Smix.

2.5 Preparation and optimization of nanoemulsion

Nine formulations of nanoemulsion (F1–F9) were prepared, by varying the concentration of the oil, Smix and water around the screened composition range (Table 3). The concentration of each bioactive in each liquid nanoemulsion was 2 mg/g of curcumin, thymoquinone and resveratrol. The bioactives were dissolved in the oil/Smix phase and then the aqueous phase was slowly introduced at the oil/Smix phase in about 30 min with magnetic stirring. The formulations were subjected to heating/cooling, freeze/thaw and physical stability screenings (4°C and 40°C, −20°C and 25°C respectively). Based on the screening results, formulation F6 was chosen; they did not perform a formal design-of-experiments optimization, but rather a comparative formulation screening.

2.6 Physicochemical characterization

Droplet size and polydispersity index (PDI) were measured after dilution by dynamic light scattering while zeta potential was measured by electrophoretic light scattering. Refractive index, pH, viscosity and transmittance at 650 nm were measured and transmittance measurements were made in three replicates. Transmission electron microscopy (TEM) was used to analyze the droplet morphology. The formulation was based on physical stability (drop size and PDI, transmittance, Ph and viscosity).

2.7 Nanoemulgel preparation and evaluation

Chosen nanoemulsion was incorporated into 1.0% w/w Carbopol 940 gel and neutralized with the help of triethanolamine (TEA), to get a homogeneous semisolid nanoemulgel. Appearance, homogeneity, pH, viscosity, spreadability, extrudability and content of curcumin, thymoquinone and resveratrol was evaluated. Phenol based conventional hydroalcoholic gel containing all three bioactives was evaluated as comparator.

2.8 Ex vivo skin permeation and deposition

Deposition and permeation in human skin ex vivo were determined by using Franz cells. The skin was placed with outside skin surface towards the donor compartment. An equal amount of conventional gel, liquid nanoemulsion and nanoemulgel was applied under the nonocclusive condition. Receptor-fluid samples were collected over the 24 h experiment and, if needed, replaced; the concentrations of the analytes (three of them) were measured through UPLC. The skin samples were washed, homogenized and extracted after 24 h to determine the bioactive deposition. Cumulative permeation was normalized based on the diffusion area and steady-state permeation was determined from the linear part of the permeation–time curve.

2.9 Antimicrobial activity

Agro-Well Diffusion and Broth Micro-Dilution assays were used to determine the antibacterial activity against the bacteria, C. acnes and S. aureus. Blank gel was used as vehicle control and clindamycin and mupirocin were comparator controls against C. acnes and S. aureus, respectively. Inhibition zones (in mm) were measured. Formulations were represented as a combination of the three bioactives for Minimum inhibitory concentration (MIC) value.

2.10 HET-CAM antiangiogenesis and cell-based evaluation

Vascular morphology changes were investigated by hen's egg chorioallantoic membrane (HET-CAM) assay and changes in vessel branching were compared to the saline controls and bevacizumab, as a reference comparator. A human keratinocytes cell line HaCaT was employed for cell-based experiments. The MTT assay was used to determine cell viability. To investigate inflammatory responses, cells were stimulated with the cytokines, namely, TNF-α/IL-17A and the production of the cytokine, namely, IL-6, was evaluated in the culture supernatant. HET-CAM endpoint was used less as a direct measure of a particular antiangiogenic mechanism, but more as a morphological screening tool.

2.11 Skin irritation and in vivo antipsoriatic activity

The Draize-type procedure was performed and data summarized using primary irritation index due to the presence of skin irritation. Psoriasis-like inflammation was induced with imiquimod in BALB/c mice. Six experimental groups were tested (normal control and imiquimod disease control; blank nanoemulgel, conventional tri-bioactive gel, tri-bioactive nanoemulgel and clobetasol reference). Erythema, scaling and skin thickening were daily assessed on a 0-4 scale and composite scores were computed (PASI-like). Thickness of the epidermis and histopathological inflammation were assessed on day 7.

2.12 Stability study and statistics

The storage stability was evaluated at 25°C/60 %RH and 40°C/75 %RH. The 25°C/60% RH series includes months 0, 1 and 3, while the 40°C/75% RH series includes months 1, 3 and 6 (Table 8). The droplet size value and the PDI value at month 0 for the stored nanoemulgel was not confidently assigned as these values were close to the droplet size value and the PDI value of the liquid nanoemulsion at month 0, respectively, and hence these values are indicated by an unverified mark. Data is shown as mean ± standard deviation (SD). Specifications of the tests and statistical outputs were not always available for reporting, and statistical significance notation for comparisons to the mouse models as defined in Table 7.

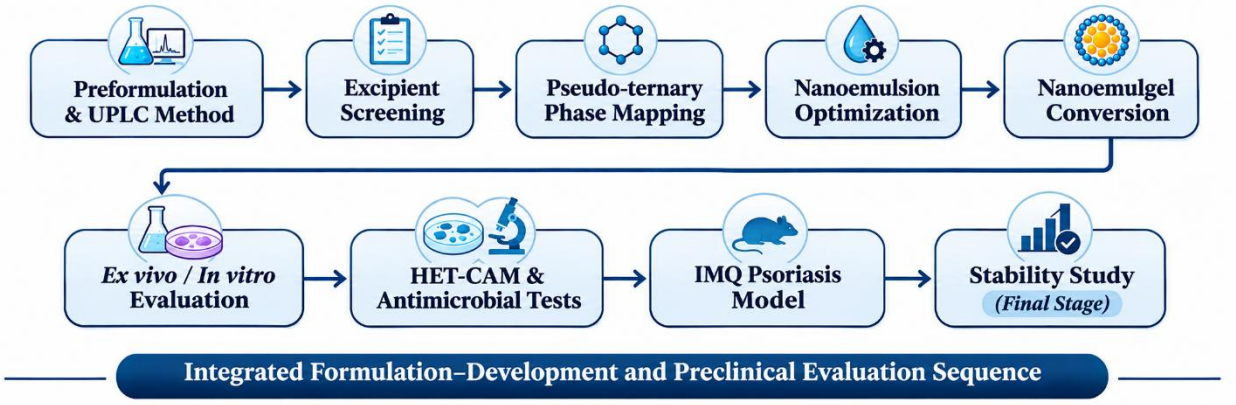


Figure 1. Integrated experimental workflow used for formulation development and preclinical evaluation.

3. RESULTS

3.1 Preformulation and UPLC performance

The Preformulation and UPLC performance showed good bio-similarity to the Reference product. The preformulation properties and performance of simultaneous UPLC assay are listed in table 1. The retention times varied with the three analytes and the coefficient of determination (r^2) of the calibrations were ≥0.9994. The recoveries were 99.3-100.2% and the intraday precision was ≤ 1.08% RSD. The detection and quantification limits obtained for each compound have been displayed.

Table 1. Preformulation characteristics and simultaneous UPLC assay parameters.

Parameter	Curcumin	Thymoquinone	Resveratrol
Appearance	Yellow-orange crystalline powder	Pale-yellow oily/crystalline material	Off-white crystalline powder
Melting range (°C)	181.4-183.0	44.8-46.1	254.2-256.0
UV λmax (nm)	425	254	306
UPLC retention time (min)	4.36 ± 0.04	2.18 ± 0.03	3.12 ± 0.03
Linearity (r^2)	0.9997	0.9996	0.9994
Recovery (%)	99.6 ± 0.8	100.2 ± 0.7	99.3 ± 0.9
Intraday precision (%RSD)	0.84	0.91	1.08
LOD (µg/mL)	0.09	0.07	0.11

Parameter	Curcumin	Thymoquinone	Resveratrol
LOQ (μg/mL)	0.27	0.21	0.33

3.2 Excipient screening and nanoemulsion optimization

During the excipient-solubility screen (Table 2), Capryol 90 achieved the greatest measured solubility of all three bioactives in all of the oils tested. Surfactant and cosurfactant were chosen to be Tween 80 and Transcutol P, respectively. Based on the phase-diagram assessment and solubilization screening a 2:1 Smix ratio was chosen for formulation screening.

Table 2. Equilibrium solubility of the three bioactives in selected formulation excipients (mg/mL, mean ± SD; n=3).

Excipient	Class	Curcumin	Thymoquinone	Resveratrol
Capryol 90	Oil	18.7 ± 0.9	46.2 ± 1.8	13.9 ± 0.7
Labrafac PG	Oil	13.5 ± 0.7	39.4 ± 1.5	10.6 ± 0.5
Oleic acid	Oil	11.8 ± 0.6	42.1 ± 1.7	8.9 ± 0.4
Tween 80	Surfactant	34.6 ± 1.3	52.8 ± 2.1	27.4 ± 1.1
Cremophor RH40	Surfactant	30.1 ± 1.1	48.5 ± 1.8	24.6 ± 0.9
Transcutol P	Co-surfactant	71.3 ± 2.7	83.6 ± 2.9	64.8 ± 2.4
PEG 400	Co-surfactant	49.5 ± 1.8	61.2 ± 2.4	45.7 ± 1.7

A total of nine nanoemulsion trial products were tested (Table 3). For the compositions studied, droplet size and PDI in general, decreased with increasing Smix fraction and increased with increasing oil fraction. Formulation F6 (10% oil, 35% Smix and 55% water) was chosen based on its mean droplet size of 92.4 ± 2.7nm, PDI of 0.214 ± 0.018, high transmittance and satisfactory performance on the applied stress tests. There was no experimental design, only screening tests, used to select the method.

Table 3. Composition and screening characteristics of trial nanoemulsions.

Batch	Oil (%)	Smix (%)	Water (%)	Size (nm)	PDI	Transmittance (%)	Stress-test outcome
F1	8	25	67	128.6 ± 5.2	0.298 ± 0.021	96.2 ± 0.7	Pass
F2	10	25	65	141.3 ± 6.1	0.321 ± 0.026	95.3 ± 0.8	Pass
F3	12	25	63	166.8 ± 7.5	0.352 ± 0.031	93.4 ± 1.0	Fail: freeze-thaw
F4	8	30	62	103.8 ± 3.8	0.246 ± 0.019	97.8 ± 0.5	Pass
F5	10	30	60	99.7 ± 3.4	0.231 ± 0.017	98.1 ± 0.5	Pass
F6	10	35	55	92.4 ± 2.7	0.214 ± 0.018	98.6 ± 0.4	Pass
F7	12	35	53	111.9 ± 4.2	0.238 ± 0.020	97.5 ± 0.6	Pass
F8	14	35	51	136.5 ± 5.9	0.284 ± 0.024	95.8 ± 0.8	Pass
F9	16	35	49	175.6 ± 8.2	0.337 ± 0.029	92.9 ± 1.1	Fail: centrifugation

3.3 Characterization of optimized nanoemulsion and nanoemulgel

The chosen liquid nanoemulsion was optically transparent with mean diameter of the droplets in the nanorange as well as a relatively small droplet size distribution (Table 4). Transmission electron microscopy was used to observe the droplets which appeared to be approximately spherical in shape. Liquid nanoemulsions were incorporated in Carbopol 940 which resulted in increased viscosity and small changes in droplet size after dilution and PDI as compared to the liquid nanoemulsion. The actual level of bioactive components provided in both formulations were fairly close to their stated assay.

Table 4. Physicochemical properties of optimized nanoemulsion and nanoemulgel.

Attribute	Optimized nanoemulsion	Optimized nanoemulgel
Composition	10% Capryol 90; 35% Tween 80:Transcutol P (2:1); 55% water; 2 mg/g of each bioactive	Nanoemulsion incorporated in 1.0% Carbopol 940 gel
Droplet size after dilution (nm)	92.4 ± 2.7	95.8 ± 3.1
PDI	0.214 ± 0.018	0.221 ± 0.019
Zeta potential (mV)	-18.6 ± 1.9	-17.9 ± 1.7
pH	5.68 ± 0.06	5.61 ± 0.05
Refractive index	1.407 ± 0.002	Not applicable
Viscosity	42.8 ± 2.6 mPa·s	12,650 ± 540 mPa·s
Transmittance at 650 nm	98.6 ± 0.4%	Not applicable
Spreadability	Not applicable	6.9 ± 0.4 g·cm/s
Extrudability	Not applicable	91.8 ± 2.3%
Curcumin content	99.3 ± 0.7%	99.1 ± 0.8%
Thymoquinone content	99.7 ± 0.6%	99.5 ± 0.7%
Resveratrol content	99.2 ± 0.8%	99.0 ± 0.9%

3.4 Ex vivo permeation and cutaneous deposition

The steady-state flux and cumulative permeation (Q24) of all three bioactives in the liquid nanoemulsion were also found to be higher after 24 hrs when compared to conventional gel as shown in Table 5. When incorporated into the gel all the permeation and flux values were decreased in comparison to the liquid nanoemulsion, and for each bioactive the nanoemulgel exhibited the higher skin deposition. The nanoemulgel resulted in 2.64-time, 2.37-time and 2.55-time higher deposition of curcumin, thymoquinone and resveratrol, respectively, in the skin as compared to conventional gel. These results are from studies of ex vivo delivery conditions and do not directly demonstrate clinical efficacy/exposure.

Table 5. Ex vivo 24-h permeation and skin deposition (mean ± SD, n=6 diffusion cells).

Bioactive	Formulation	Q24 (µg/cm²)	Flux (µg/cm²/h)	Skin deposition (µg/g)	Deposition enhancement ratio
Curcumin	Conventional gel	28.4 ± 2.7	1.12 ± 0.11	19.6 ± 2.1	1.00
	Nanoemulsion	68.9 ± 4.1	2.85 ± 0.17	39.7 ± 3.0	2.03
	Nanoemulgel	54.7 ± 3.6	2.18 ± 0.15	51.8 ± 3.9	2.64
Thymoquinone	Conventional gel	36.2 ± 3.0	1.46 ± 0.12	24.8 ± 2.4	1.00
	Nanoemulsion	82.6 ± 4.8	3.41 ± 0.20	46.5 ± 3.6	1.88
	Nanoemulgel	66.5 ± 4.0	2.70 ± 0.17	58.7 ± 4.2	2.37

Bioactive	Formulation	Q24 (µg/cm²)	Flux (µg/cm²/h)	Skin deposition (µg/g)	Deposition enhancement ratio
Resveratrol	Conventional gel	24.7 ± 2.3	0.98 ± 0.10	17.9 ± 1.8	1.00
	Nanoemulsion	59.8 ± 3.9	2.46 ± 0.16	34.2 ± 2.8	1.91
	Nanoemulgel	47.3 ± 3.2	1.91 ± 0.13	45.6 ± 3.5	2.55

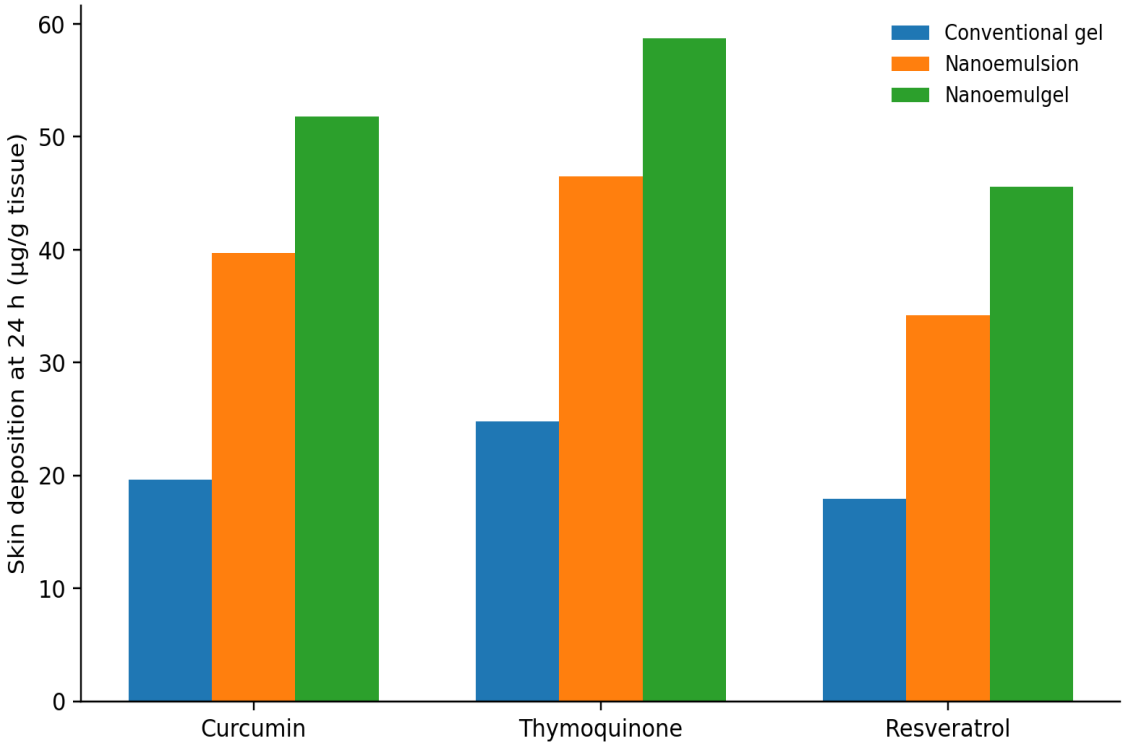


Figure 2. Comparative 24-h skin deposition of the three bioactives from conventional gel, nanoemulsion and nanoemulgel.

3.5 Antimicrobial, HET-CAM and cell-based findings

The zones of inhibition of the conventional tri-bioactive gel compared with the tri-bioactive nanoemulgel showed that larger zones were obtained when tri-bioactive nanoemulgel was tested against the test organisms than the conventional tri-bioactive gel while the similar antibiotic reference control gave larger ZOI values (Table 6). The MICs of nanoemulgel were lower compared with the conventional gel. It is observed that treatment with nanoemulgel resulted in a 43.6 ± 4.1% reduction in endpoint (vessel-branching) as compared to saline, in HET-CAM assay. This is just a vascular morphology study and by no means an identification of an antiangiogenic pathway. The cell viability of HaCaT cells was 87.9 ± 4.0% under the conditions tested. Following the inclusion of TNF-α/IL-17A, stimulated control did not differ significantly with nanoemulgel group (218 ± 20 pg/mL vs 486 ± 31 pg/mL respectively).

Table 6. Antimicrobial, HET-CAM, and HaCaT screening outcomes for the tri-bioactive nanoemulgel.

Assay	Control/comparator	Conventional tri-bioactive gel	Tri-bioactive nanoemulgel	Reference control
C. acnes inhibition zone (mm)	Blank gel: 6.0 ± 0.0	18.1 ± 1.0	23.4 ± 1.2	Clindamycin: 27.6 ± 1.1
C. acnes MIC (mg/mL total bioactives)	-	1.25	0.625	Clindamycin: 0.008
S. aureus inhibition zone (mm)	Blank gel: 6.0 ± 0.0	16.7 ± 0.9	20.8 ± 1.0	Mupirocin: 26.3 ± 1.2

Assay	Control/comparator	Conventional tri-bioactive gel	Tri-bioactive nanoemulgel	Reference control
S. aureus MIC (mg/mL total bioactives)	-	2.50	1.25	Mupirocin: 0.016
HET-CAM vessel inhibition (%)	Saline: 0	28.1 ± 3.5	43.6 ± 4.1	Bevacizumab model control: 61.8 ± 4.7
HaCaT viability (%)	Untreated: 100	90.7 ± 3.8	87.9 ± 4.0	-
TNF-α/IL-17A-induced IL-6 (pg/mL)	Stimulated: 486 ± 31	284 ± 24	218 ± 20	Unstimulated: 96 ± 12

3.6 Skin irritation and antipsoriatic efficacy

The primary irritation index of nanoemulgel was found to be 0.28 ± 0.12. No erythema or oedema reactions were seen in the irritation protocol tested. The erythema, scaling and thickening of the skin were more evident in the disease-control group in comparison to the nanoemulgel group in the imiquimod-induced mouse model. The composite PASI-like score was 2.8 ± 0.6 on day 7 in nanoemulgel group and 9.2 ± 0.7 in disease control (reported $p < 0.001$). Moreover, lower epidermal thickness and lower histological inflammation score (table 7) were noticed in the nanoemulgel group. Equivalence or noninferiority to clobetasol was not evaluated, but the numerical composite score was lower for the clobetasol group (2.1 ± 0.5).

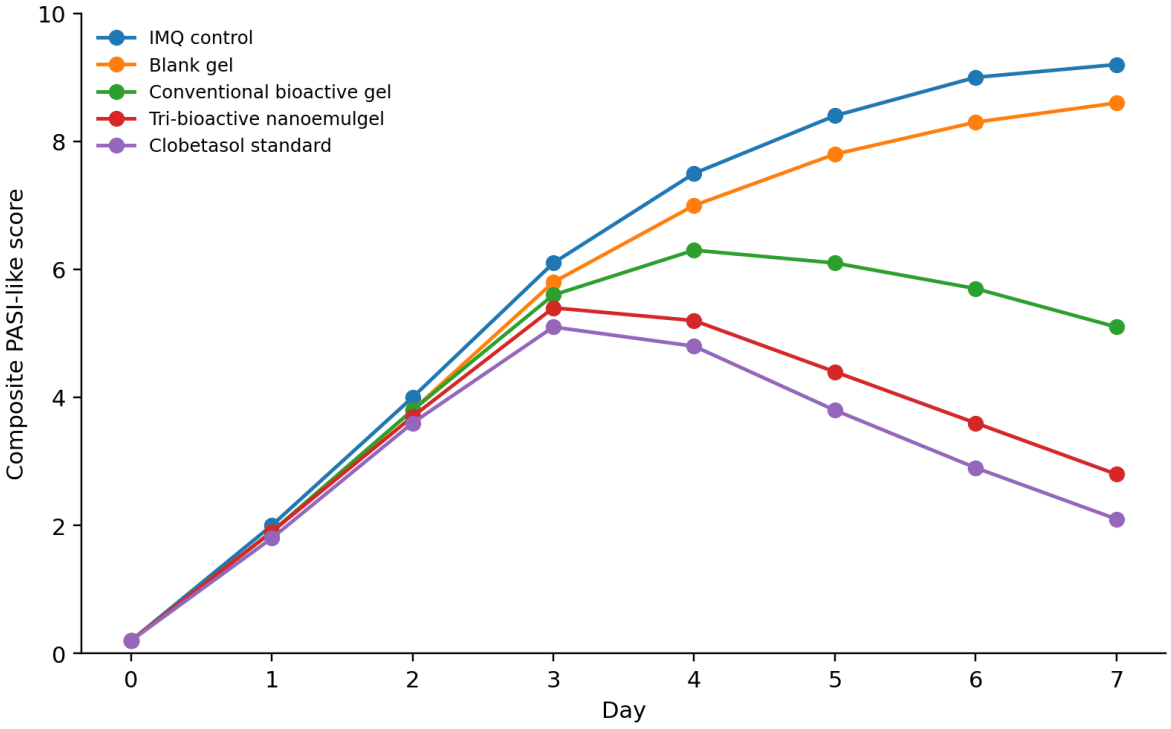


Figure 3. Daily composite PASI-like scores in the imiquimod-induced model (n = 6 per group).

Table 7. Day-7 antipsoriatic and histopathological outcomes.

Group	Day-7 PASI-like score	Epidermal thickness (µm)	Histological inflammation score (0-3)	Interpretation
Normal control	0.2 ± 0.2	34.1 ± 4.8	0.2 ± 0.2	Normal epidermal architecture
IMQ control	9.2 ± 0.7	143.5 ± 12.6	2.8 ± 0.3	Marked acanthosis and inflammation

Group	Day-7 PASI-like score	Epidermal thickness (µm)	Histological inflammation score (0-3)	Interpretation
Blank nanoemulgel	8.6 ± 0.8	136.8 ± 11.4	2.6 ± 0.4	Minimal vehicle effect
Conventional tri-bioactive gel	5.1 ± 0.7*	86.4 ± 8.9*	1.7 ± 0.4*	Partial normalization
Tri-bioactive nanoemulgel	2.8 ± 0.6*†	59.7 ± 6.8*†	0.9 ± 0.3*†	Marked reduction in acanthosis/inflammation
Clobetasol standard	2.1 ± 0.5*	52.8 ± 6.1*	0.7 ± 0.3*	Strong reference response

Table 7: Statistical annotations: *p<0.001 vs imiquimod disease control; †p<0.01 vs conventional tri-bioactive gel. Threshold values are not the actual p-values and the associated test statistics are not reported, but are instead comparisons reported. There was no equivalence or noninferiority analysis compared to clobetasol.

3.7 Stability

During the storage periods examined there was no creaming, cracking or gross phase separation noticed. The various physicochemical properties and bioactive contents values at all the assessment points are given in Table 8. For storage at 25°C/60% RH, it was not possible to confidently attribute the droplet size and PDI at Month 0 to the nanoemulgel as the droplet size and PDI obtained were the same for both the nanoemulgel and the liquid nanoemulsion. The stability series did not have an individual month-0 measurement for 40°C/75% RH. At six months under 40°C/75% RH, measured curcumin, thymoquinone and resveratrol contents were 94.9 ± 1.2%, 95.7 ± 1.1% and 94.3 ± 1.3%, respectively. Only droplet-size measurements are shown in figure 4 for which there also is a report in table 8. These observations are not definitive in verifying a product shelf life.

Table 8. Physicochemical characteristics and drug contents during storage under the evaluated conditions.

Condition	Month	Size (nm)	PDI	pH	Curcumin (%)	Thymoquinone (%)	Resveratrol (%)
25°C/60% RH	0	Not verified‡	Not verified‡	5.61 ± 0.05	99.1 ± 0.8	99.5 ± 0.7	99.0 ± 0.9
	1	93.1 ± 2.8	0.216 ± 0.017	5.60 ± 0.05	98.9 ± 0.9	99.2 ± 0.8	98.7 ± 0.9
	3	95.0 ± 3.0	0.220 ± 0.019	5.58 ± 0.06	98.4 ± 0.9	98.7 ± 0.9	98.1 ± 1.0
40°C/75% RH	0	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported
40°C/75% RH	1	95.8 ± 3.1	0.224 ± 0.020	5.57 ± 0.06	98.2 ± 0.9	98.6 ± 0.9	97.9 ± 1.0
	3	100.9 ± 3.7	0.237 ± 0.021	5.53 ± 0.06	96.8 ± 1.1	97.3 ± 1.0	96.1 ± 1.2
	6	106.3 ± 4.2	0.251 ± 0.024	5.49 ± 0.07	94.9 ± 1.2	95.7 ± 1.1	94.3 ± 1.3

The droplet size and PDI of the above mentioned droplet size (92.4 ± 2.7 nm) and PDI (0.214 ± 0.018), were data for the previously mentioned liquid nanoemulsion and newly characterized nanoemulgel data were 95.8 ± 3.1 nm and 0.221 ± 0.019 respectively. Because of the inability to confirm identity of sample from available information, no values were given to the storage sample. The results for “Not reported” do not represent zero values, but are unavailable measurements.

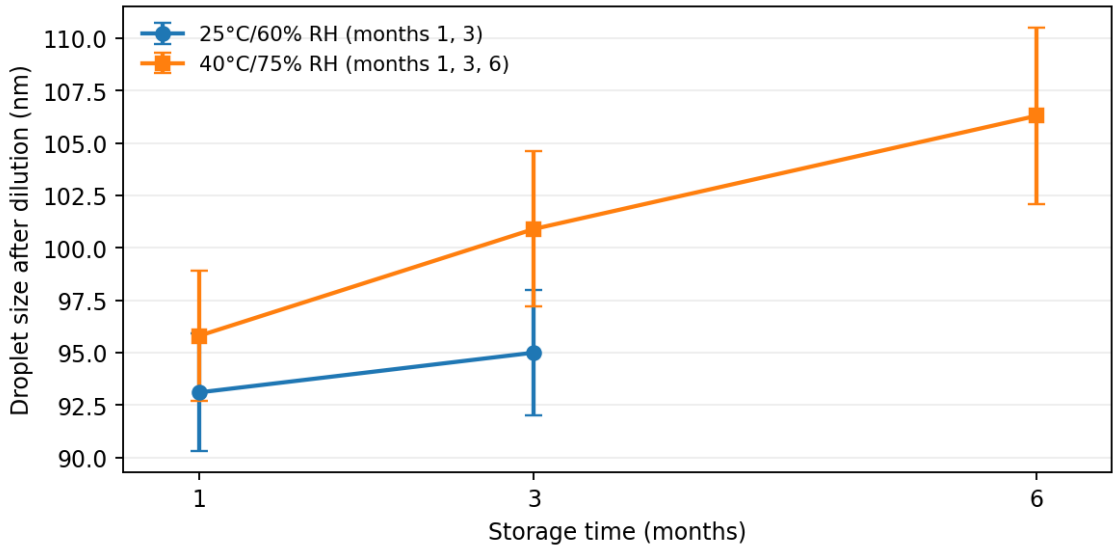


Figure 4. Droplet size during storage at 25°C/60% RH and 40°C/75% RH (mean ± SD); plotted time points correspond to the measurements listed in Table 8.

4. DISCUSSION

A nanoemulsion of curcumin, thymoquinone and resveratrol was prepared and Carbopol based nanoemulgel for cutaneous delivery and preclinical evaluation was prepared. The selected liquid nanoemulsion had a mean droplet size of 92.4 ± 2.7 nm and a PDI of 0.214 ± 0.018 (Table 4). Although it has a moderately negative zeta potential which gives some indication of the properties of the interface of the dispersion, the nature of the surfactant system and steric stabilization is also important. In addition to the long-term stability assessment, the physical stress tests which were performed for the formulation selection were used. Table 5 showed the variation in delivery profile of the liquid nanoemulsion and nanoemulgel ex vivo experiments. The results indicated that the liquid formulation had higher cumulative permeation and flux whereas, nanoemulgel with Carbopol had higher deposition in skin after 24 h. These results are relevant for local use, but don't on their own provide information on the therapeutic efficacy or reduction of systemic exposure. The active-dose equivalence of the formulation being tested is also important when making comparisons between formulations. The antimicrobial screening revealed that nanoemulgel performed better for both test bacteria C. acnes and S. aureus in terms of larger inhibition zones and lower MIC values (conventional tri-bioactive gel).For antimicrobial screening, the nanoemulgel exhibited higher inhibition zones and lower MIC values than conventional tri-bioactive gel against both test bacteria, while the antibiotic reference controls demonstrated higher inhibition zones. The antibacteriological activity of curcumin and resveratrol has been studied [5,15]. The present experiments did not test synergism in pharmacology nor clinical or broad spectrum antimicrobial activity. The nanoemulgel correlated with a decrease in the stimulated HaCaT cells IL-6. The vessel-branching changes in the HET-CAM assay is a morphological endpoint and does not necessarily signify a direct inhibition of vascular endothelial growth factor signaling. In the mouse model with imiquimod-induced disease, a reduction in PASI-like scores, epidermal thickness and histological inflammation scores was observed in the nanoemulgel group compared to the disease control. The numerically lower scores in the clobestasol reference group were not confirmed by the experiment to demonstrate equivalence or noninferiority. Differences in the composition of the topical carrier, the compounds studied, skin models and endpoints limit comparisons with previous studies on other topical carriers [7,16–18,20–22]. The bioactive contents remained at more than 94% after 6 months under accelerated storage conditions (40C/75%RH). However, these droplet-size differences must be interpreted with caution since the sample tested in each month of the year has not yet been identified and a baseline test has not been performed in the series with accelerated storage. A full, traceable stability data set, is required to make a shelf-life claim. In the reporting provided there is some missing information relating to the experimental operating conditions, information regarding comparator doses, as well as some of the inferential statistical output, that may affect the interpretation of the results of the reproducibility and between groups.

5. CONCLUSION

Nanoemulsion and nanoemulgel formulation containing curcumin, thymoquinone and resveratrol were formulated and their physicochemical, ex vivo and preclinical evaluation was performed. After selection the droplet size of the prepared liquid nanoemulsion was 92.4 ± 2.7 nm, while that of the freshly prepared nanoemulgel was 95.8 ± 3.1 nm (Table 4). The nanoemulgel resulted in the higher localization of all three bioactives, as compared to the conventional gel and exhibited antibacterial activity for the organisms tested. A decrease in psoriasis-like inflammatory responses in the imiquimod-induced mouse model was seen. These results are supportive for further formulation and pharmacological investigation and do not determine clinical efficacy or equivalence to clobetasol or validate the product shelf life.

DECLARATIONS

Animal ethics and other approvals: BALB/c mice (in vivo) were used in an imiquimod-induced psoriasis-like model of inflammation. The source written material for this manuscript does not provide detail, approval reference or information about biological-specimen and HET-CAM permissions which are provided by the institutional animal-ethics committee. The approval references in this manuscripts have not been identified.

Data availability: Tables and figures show summary results of all the formulation, physicochemical, ex vivo, microbiological, cell-based, animal-model and stability studies. Supporting raw instrument records, individual-animal observations, manufacturing records and complete statistical outputs are not included in the manuscript. For any supporting records, inquires should be addressed to the corresponding author, contingent on institutional and ethical restrictions.

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