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Article

Propolis-Loaded Niosomes for Dermopharmaceutic and Cosmetic Applications: Development, Stability, Safety, and *In Vitro* Bioactivity Studies

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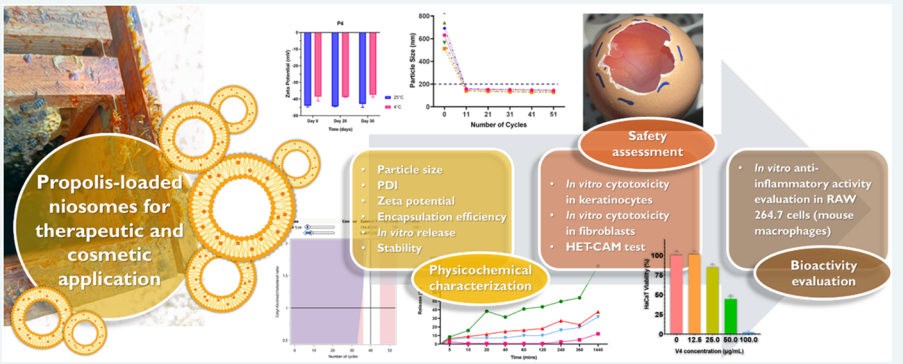

ABSTRACT: Propolis, produced by *Apis mellifera* bees, is composed of several biologically relevant phenolic compounds with known analgesic, anti-inflammatory, antitumor, antioxidant, immunomodulatory, wound healing, and antibacterial effects, having gained significant interest for therapeutic and cosmetic purposes. Niosomes, self-assembled vesicular nanosystems, are highly researched for topical delivery due to providing controlled and sustained release, protecting encapsulated compounds from degradation, improving stability, and having good biocompatibility and biodegradability. This work aimed to develop novel propolis-loaded niosomes with small and homogeneous particle size, high encapsulation efficiency, controlled release, adequate stability, relevant bioactivity, and high safety for topical application, for therapeutic and/or cosmetic purposes. Aided by quality by design (QbD) analysis, niosomes containing Tween 20, Kolliphor RH 40, cetyl alcohol, and/or cholesterol were produced by thin-film hydration followed by extrusion, with small particle size (between 100 and 200 nm), homogeneous distribution (PDI below 0.2), relevant ζ-potential (around −38 mV), good stability both under refrigeration and at room temperature, high encapsulation efficiency (ranging from 78.8 to 87.4%), and a controlled release profile, relevant to ensure prolonged bioactivity at the application site. Adequate concentration-dependent *in vitro* safety in keratinocytes and fibroblasts (up to 12.5 or 25 μg/mL) was demonstrated as well, with some niosomal formulations also showing a low irritative potential in a HET-CAM test, and relevant *in vitro* anti-inflammatory potential (IC_{50} from 14.90 to 17.89 ng/mL). These novel nanoplatforms, containing a nature-derived hydrophobic compound with various relevant bioactivities, could serve as versatile and advantageous formulations in both pharmaceutical and cosmetic contexts of application.

KEYWORDS: *anti-inflammatory, controlled release, niosomes, propolis, QbD, topical delivery*

While topical administration has considerable advantages over other drug delivery routes, such as avoiding gastric pH and first-pass metabolism, having reduced systemic side effects, and leading to enhanced drug bioavailability at the therapeutic target site when used for the treatment of skin conditions, the skin's low permeability hinders the molecules' permeation and deposition.[1−3] Additionally, conventional topical therapeutic and cosmetic formulations, usually being semisolid dosage forms, such as creams, ointments, gels, pastes, or lotions, present relevant downsides such as poor absorption, low skin permeability, decreased bioavailability, the need for repeated administration to achieve the intended effects, local

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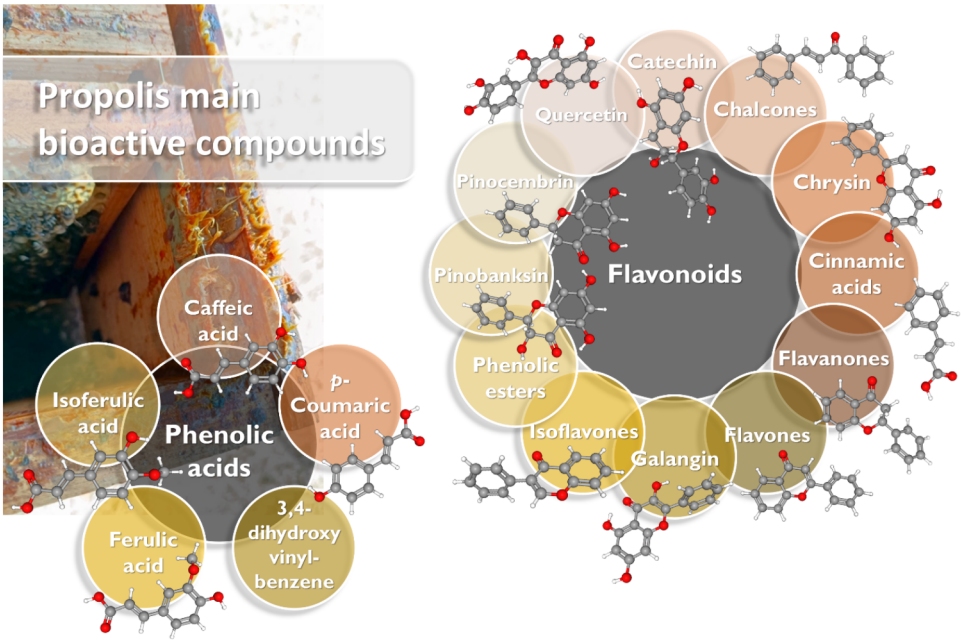

Figure 1. Propolis' main bioactive molecules (flavonoids and phenolic acids), with an added photograph (top left) of propolis in the walls of a beehive taken at the Polytechnic Institute of Bragança's apiary (orange residue) (molecular structures sourced from PubChem).

toxicity, and drug degradation. For this reason, the development of novel technologies to improve skin administration has been a main goal for current research, with many therapeutic and cosmetic formulations already including nanotechnology.[4−6]

Nanoformulations offer many advantages over conventional dosage forms, including the chance to improve the bioactive molecule's delivery efficiency, successfully penetrating through the *stratum corneum* and reaching deeper skin layers thanks to their nanometric size, protecting the encapsulated molecules from degradation, and allowing controlled release, which can in turn lead to prolonged therapeutic or bioactive effects.[7−11] Lipid-based nanocarriers have the additional advantages of possessing increased biocompatibility and biodegradability, and increased skin penetration and/or retention, due to the structural resemblance between their components and the skin's lipids.[12−15] Niosomes are unilamellar or multilamellar vesicular lipid-based nanocarriers, being made of nonionic surfactants, amphiphilic molecules that have the ability to self-assemble into highly organized concentric bilayers when faced with water. These highly versatile nanosystems allow the coencapsulation of molecules with opposite characteristics, with hydrophilic molecules being inserted into the aqueous core, and lipophilic molecules being enclosed into the bilayer membrane.[16−18] Aside from nonionic surfactants, niosomes also often have cholesterol in their composition, which leads to the nanovesicles having enhanced membrane stability, adequate fluidity, minimized passive permeability, and an overall improved entrapment efficiency.[18−20]

Although a variety of synthetic, semisynthetic, and natural molecules can be formulated into niosomes, it is in nature that we are able to find some of the most promising compounds, with multiple bioactivities and high biocompatibility. Bees produce a wide range of substances with beneficial properties, some having already proven to be effective in treating skin conditions, including honey, bee pollen, bee venom, beeswax, royal jelly bee bread, and propolis.[21−26] Propolis has been extensively researched for its diverse benefits in both medicine and cosmetics. Propolis is a hydrophobic, resinous, sticky, and dark substance (Figure 1) produced by the*Apis mellifera*bee species, created when these bees collect resin from plants, mix it with enzymes present in their saliva, and semidigest and combine it with beeswax.[27−29] Depending on the intended chemical profile of the extract, the solvent and the extraction method can differ, and as a result the biological and pharmaceutical properties will also vary.[28,30] Additionally, the complex composition of raw propolis also strongly depends on the geographic origin of the samples, climate conditions, bee species, collection time, seasons, and botanic composition, with more than 180 different chemical compositions having already been recognized around the globe.[31,32] Propolis mostly consists of resins (50%), wax (30%), essential oils (10%), and pollens (5%), and its most relevant chemical components are phenolic and polyphenolic compounds, mainly flavonoids (catechin, chalcones, chrysin, cinnamic acids, flavanones, flavones, galangin, isoflavones, phenolic esters, pinobanksin, pinocembrin, quercetin) and phenolic acids (caffeic acid, *p*-coumaric acid, 3,4-dihydroxy vinylbenzene, ferulic acid, isoferulic acid), which are both key indicators of propolis' quality and the main bioactive molecules present in its composition (Figure 1). Terpenoids, fatty acids, enzymes, minerals, and vitamins have also been identified. Thanks to this complex chemical composition, propolis has been linked to a diverse range of biological properties, including analgesic, anti-inflammatory, antitumor, antioxidant, immunomodulatory, wound healing, and antibacterial effects, having gained significant interest as an active ingredient for skin care and cosmetic formulations.[30,32,33] Among other applications, propolis has been proven effective in treating acne vulgaris (antibacterial and anti-inflammatory properties), for photoprotection (significant UV light absorption capacity and antioxidant action), and for antiaging purposes (neutralization of free radicals' damaging effects).[27,34−36]

Given this contextualization, the aim of the present work was to develop propolis-loaded niosomes, with small and homogeneous particle size, high encapsulation efficiency (EE%),

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controlled bioactive compound release, suitable stability, and adequate bioactivity and safety, to serve as a novel nanotechnological platform for topical application for therapeutic and cosmetic purposes. The work comprised preformulation and optimization studies based on a Quality by Design (QbD) approach. Niosomes' physicochemical characterization, including particle size, polydispersity index (PDI), ζ-potential, EE%, *in vitro* release, and real-time stability, bioactivity and safety evaluation in *in vitro* models, namely in HFF-1 cells (human skin fibroblasts) and HaCaT cells (human immortalized keratinocytes) for safety evaluation, and in RAW 264.7 cells (mouse macrophages) for anti-inflammatory bioactivity evaluation, and additional safety assessment using a Hen's Egg Test-Chorioallantoic Membrane (HET-CAM).

MATERIALS AND METHODS

Materials. Tween 20 (polysorbate 20), Kolliphor RH 40 (PEG-40 hydrogenated castor oil), dexamethasone, trichloroacetic acid (TCA), lipopolysaccharide (LPS), sulforhodamine B (SRB), tris(hydroxymethyl)aminomethane (Tris) buffer, trypan blue, caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, benzoic acid, quercetin, *p*-coumaric acid methyl ester, caffeic acid isoprenyl ester, pinocembrin, and chrysin were bought from Sigma-Aldrich (Saint Louis, Missouri). Transcutol P (diethylene glycol monoethyl ether) was a gift sample from Gattefossè (Saint-Priest, France). Cetyl alcohol was purchased from DS Produtos Qumicos, Lda. (São Domingos de Rana, Portugal). Chloroform was acquired from Sigma-Aldrich (Darmstadt, Germany). Cholesterol 95% was purchased from Thermo Fisher Scientific (Bleiswijk, The Netherlands). Methanol (purity ≥ 98.9%), sodium chloride and sodium hydroxide were bought from Merck (Darmstadt, Germany). Ethanol (purity ≥ 96%) was obtained from LabChem (Zeliniple). Dulbecco's modified Eagle's medium (DMEM), streptomycin, penicillin, fetal bovine serum (FBS), L-glutamine, and trypsin were acquired from Gibco Invitrogen Life Technologies (Carlsbad, California). Apigenin, kaempferol, isorhamnetin, galangin, and acacetin were bought from Extrasynthese (Genay, France). HPLC-grade ethanol, acetonitrile, and analytical grade formic acid, acetic acid, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Leicester, United Kingdom). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Houston, Texas). Propolis was kindly gifted from Mountain Research Center (CIMO) (Polytechnic Institute of Bragança, Bragança, Portugal). Cell lines were purchased from the European Collection of Authenticated Cell Cultures (ECCAC) and the German Collection of Microorganisms and Cell Cultures (DSMZ).

Propolis Production and Harvesting and Phenolic Compounds LC/DAD/ESI-MSn Analysis. Propolis, used as the active compound to be encapsulated within the developed niosomes, was supplied by AALBA−Cooperativa de Produtores de Mel, CRL. The sample was collected through a plastic screen from *A. mellifera* hives, located in Leiria, Portugal, and stored at −20 °C, pending future use. To prepare the propolis extract, we previously crushed raw propolis and homogenized it. The propolis sample was combined with 80% ethanol in water, and extracted for 1 h at 70 °C. The obtained mixture underwent filtration, and the residues were subjected to re-extraction under identical conditions.[32,37]

Liquid chromatography with diode array detection coupled with electrospray ionization tandem mass spectrometry (LC/DAD/ESI-MSn) was applied for the analysis of the phenolic compounds present in the harvested propolis, performed according to a previous study.[38] A Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, San Jose, California) equipped with a diode-array detector and coupled to a mass spectrometer operating in negative ion mode using a Linear Ion Trap LTQXL mass spectrometer (Thermo Scientific, California) equipped with an electrospray ionization source was used. Chromatographic separation was achieved in a Macherey-Nagel Nucleosil C18 column (250 mm 4 mm id, 5 mm particle diameter, end-capped), with temperature being maintained at 30 °C. The employed liquid chromatography parameters were derived from prior work.[38] Spectral data for all peaks were accumulated in the range of 190 to 600 nm. For the mass spectrometry analysis, the defined parameters were the following: ion source voltage, 5 kV; capillary voltage, 20 V; tube lens voltage, 65 V; capillary temperature, 325 °C; and sheath and auxiliary gas flow (N2) were set as 50 and 10 (arbitrary units), respectively. Mass spectra were acquired on full-range acquisition, covering 100 to 1000 m/z.

For the fragmentation study, a data-dependent scan was performed by deploying a collision-induced dissociation set at 35 (arbitrary units). Data acquisition was carried out with the Xcalibur data system (Thermo Scientific, San Jose, California). The determination of the phenolic compounds was achieved by comparison of their chromatographic profile, UV spectra, and mass spectrometry information to those of the standard compounds. When standards were not available, the structural information was validated using UV data in combination with the mass spectrometry fragmentation patterns documented in the scientific literature. For the phenolic compounds' quantification, the following standards were used [including calibration curves' concentration ranges and coefficients of determination (R^2)]: caffeic acid (0.0187 to 0.4 mg/mL; R^2 = 0.996), *p*-coumaric acid (0.0187 to 0.4 mg/mL; R^2 = 0.999), kaempferol (0.075 to 1.6 mg/mL; R^2 = 0.997), pinocembrin (0.0375 to 0.8 mg/mL; R^2 = 0.997), and chrysin (0.0375 to 0.8 mg/mL; R^2 = 0.999). When the standard compound was not available, the quantification was expressed in equivalent terms to the structurally closest compound. The assays were performed in duplicate, and the results were expressed as milligrams per gram of extract.

QbD-Aided Niosome Formulation Development and Optimization. Thin-film hydration was used to prepare the developed niosomes. The nonionic surfactant (either Tween 20 or Kolliphor RH 40) and lipid (either cholesterol, cetyl alcohol, or a mixture of both), at a 3.5:2 molar ratio, were weighed into a round-bottomed flask and then dissolved in 5.5 mL of chloroform under light magnetic stirring for 40 min. This mixture was evaporated in a rotary evaporator (Rotavapor R-210/215, BÜCHI, Switzerland) combined with a vacuum pump (Vacuum Pump V-700/710 with Vacuum Controller V-850/855, BÜCHI, Switzerland) in a 60 °C bath, with a rotation speed of 8 rpm, under reduced pressure (120 mbar), for 60 min. After the organic solvent was evaporated, the formed thin layer was hydrated with deionized water (5.5 mL) heated at the same temperature as the hydration temperature, under mild magnetic stirring, for 60 min. In this phase, different hydration temperatures were tested, including 40, 45, 50, 55, and 60 °C. For the encapsulation of the hydrophobic compound, propolis (1 mg/mL) was included in the round-bottom flask along with the other constituents and dissolved in chloroform as well.

After the initial niosomes were formed, a miniextruder kit (Avanti Mini-Extruder, Avanti Polar Lipids, INC.) was used to

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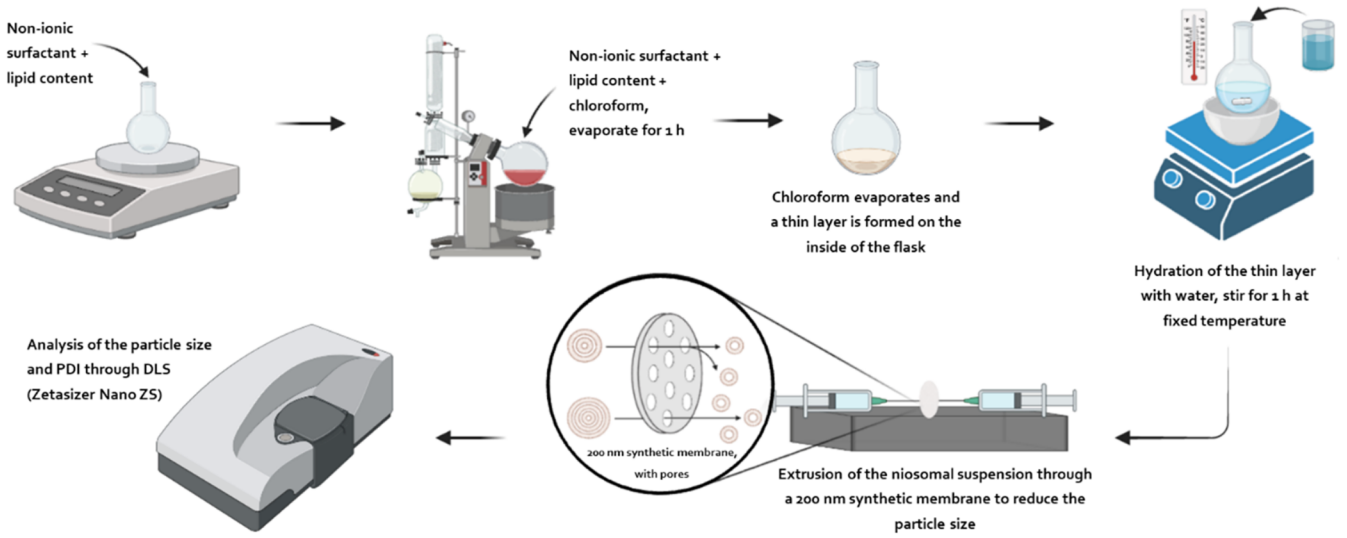

Figure 2. Schematic representation of the production of the developed niosomes using the thin-film hydration method (produced with BioRender).

perform extrusion on the niosomal suspension in order to reduce the particle size of the niosomes, using a polycarbonate membrane with a 200 nm pore size (Avanti Polar Lipids, INC.). Various extrusion cycles were performed (0, 11, 21, 31, 41, or 51), and for every performed cycle series, the particle size and PDI were measured. The niosomal formulations were stored in a refrigerator at 4 °C until further characterization. The described niosome production method can be better understood through the schematization in Figure 2.

For the development and optimization of niosomal formulations, a design of experiments (DoE) was performed using JMP Pro (version 17.0.0). A full factorial design was designed (Table 1), and the data was analyzed using a standard least-squares fit model for effect screening. Only first-order effects were considered. The correlation of the factors with the response was gauged by the *p*-values associated with the regression coefficients. R^2, root-mean-square error, and analysis of variance were analyzed to demonstrate the suitability of the model. Prediction and contour profilers were traced to better predict and define the design space. Different types of nonionic surfactant, types of lipids, hydration temperatures, and number of extrusion cycles were tested. The model was optimized based on the particle size and PDI measurements.

Table 1. Full Factorial Design Parameters

factors			
independent variable	factor name	factor type	variation domain
X_1	nonionic surfactant	categorical factor	[Tween 20, Kolliphor RH 40]
X_2	lipid composition	categorical factor	[cetyl alcohol, or mix (mixture of cetyl alcohol and cholesterol)]
X_3	cetyl alcohol/cholesterol ratio	numeric ordinal factor	[0.5; 1.0; 2.0]
X_4	hydration temperature (°C)	numeric ordinal factor	[40; 45; 50; 55; 60]
X_5	number of extrusion cycles	numeric ordinal factor	[0; 11; 21; 31; 41; 51]
responses			
dependent variable	response name	goal	
Y_1	particle size (nm)	minimize	
Y_2	PDI	minimize	

Particle Size, Polydispersity Index, and ζ-Potential. Particle mean hydrodynamic size, PDI, and ζ-potential of the empty niosomes, and propolis-loaded niosomes were measured by Dynamic Light Scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, Malvern, United Kingdom). Particle size and PDI were determined by an analysis of dynamic light scattering data, and the ζ-potential was obtained by electrophoretic light scattering. Samples were diluted 1/40 in deionized water and then measured in disposable poly(methyl methacrylate) 12 mm^2 diameter cuvettes, at 25 °C, for particle size and PDI. To measure the ζ-potential, a disposable folded capillary cell (DTS1070) was used. All samples were measured in triplicate, and the mean value was reported.

Encapsulation Efficiency. The EE% of the propolis-loaded niosomes was determined using the indirect method by ultrafiltration−centrifugation. First, 3.5 mL of the extruded niosomal suspension was added to an ultrafiltration-centrifuge tube (Amicon Ultra-4 centrifugal filter Ultracel 50 K, Merck Millipore, Germany), and then, the samples were centrifuged at 5445*g*, for 40 min, at 4 °C (Sigma Laborzentrifugen 3 K15, Sigma, Germany). Afterward, the filtrate, already separated from the pellets on the tube, was analyzed for compound quantification, using UV−vis spectrophotometry at a wavelength of 401 nm, by a microplate reader (BioTek Synergy HTX Multimode Microplate Reader, Vermont). This led to the quantification of the amount of free compound (nonencapsulated), which was estimated using a previously obtained concentration curve. The EE% was then calculated using eq 1[39,40]

$$\text{EE\%} = \frac{\text{total amount of propolis} - \text{free amount of propolis quantified in filtrate}}{\text{total amount of propolis}} \times 100 \quad (1)$$

***In Vitro* Release.** The *in vitro* propolis release profile was evaluated using vertical Franz diffusion cells (PermeGear, Pennsylvania). After extrusion and centrifugation, the obtained pellets were resuspended in 3.5 mL of deionized water and this suspension was used for drug release assessment. The experiment was carried out for 24 h at 35 °C (mean skin

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temperature), using a dialysis tubing cellulose membrane (Sigma-Aldrich, Darmstadt, Germany), previously dampened for 1 h in the assay medium, and put between the receptor chamber and the flat ground joint of the donor chamber. The receptor compartment was filled with 5 mL of phosphate buffered saline (PBS), the assay medium, ensuring *sink* conditions. The donor chamber was filled with 200 μL of the selected formulations and covered with parafilm to avoid evaporation, while both chambers were clamped together. At specific time points (5, 10, 20, 40, 60, 120, 240, 360, and 1440 min), samples (300 μL) were collected from each cell and stored in a tube for later analysis. After the samples were collected, the volume in each cell was restored with PBS, up to the meniscus marked on the sampling port.

By the end of the experiment, all of the collected samples were analyzed using UV−vis spectrophotometry (BioTek Synergy HTX Multimode Microplate Reader, Vermont, United States of America), at a wavelength of 285 nm. Every experiment was conducted in sextuplicate, ensuring a total of six replicates.

Niosome Physical Stability Assays. The real-time stability of optimized propolis-loaded niosomes was evaluated during a time frame of 30 days while stored at either 4 °C (under refrigeration) or 25 °C (room temperature). The particle size, PDI, and ζ-potential of each sample were measured after 20 and 30 days, as described in the Particle Size, Polydispersity Index, and ζ-Potential section, and compared with the values measured right after niosome production (day 0). Each measurement was done in triplicate, and the mean values were reported.

***In Vitro* Cell Assays.** The bioactivity and safety of propolis encapsulated into the developed optimized niosomes were tested in *in vitro* cell assays and compared with the free compound (propolis solution). Biocompatibility was assessed through cytotoxicity assays in two human skin cell lines, HFF-1 cells (human foreskin fibroblasts) and HaCaT cells (human immortalized keratinocytes). The vesicles' bioactive properties were assessed in RAW 264.7 cells (mouse macrophages) to test for their anti-inflammatory potential. All used cell lines are commercially available and were bought from authorized cell line sources, namely either the German Collection of Microorganisms and Cell Cultures or the European Collection of Authenticated Cell Cultures. All assays were performed according to the Guidance on Good Cell Culture Practice (GCCP), to ensure high technical requirements.[41]

***In Vitro* Healthy Skin Cell Viability Assays.** To evaluate the cytotoxicity of the propolis-loaded niosomes in healthy skin cells, a colorimetric SRB assay was performed in two human cell lines, HFF-1 (human skin fibroblasts) and HaCaT (human immortalized keratinocytes), based on previously described methods.[42−44] The cells were cultured in DMEM, supplemented with 10% FBS, glutamine, and antibiotics (penicillin and streptomycin 1%), in an incubator at 37 °C, with 5% CO_2, and with saturated controlled humidity. Trypsin was used to detach the cells from the bottom of the flask. The resulting cell suspension was centrifuged at 3000 rpm for 5 min, resuspended in fresh culture medium, and 10,000 cells per well were plated in a 96-well plate and left to incubate for 24 h, so that the cells could adhere to the bottom of the plate. After 24 h, water-dispersed propolis-loaded niosomes' and empty niosomes' samples, with concentrations of 0.1, 0.05, 0.025, and 0.0125 mg/mL, were added to the plate. The same concentrations were also prepared for the free propolis (diluted in DMSO/H_2O, 1:1 ratio). The plates were then incubated for 48 h, again at 37 °C and with 5% CO_2. After 48 h, the cells were fixed with trichloroacetic acid for 1 h at 4 °C. Afterward, the liquid inside the plate was discarded, and the plate was washed 3 times with tap water and left to air-dry. Once dry, 100 μL of SRB was added to each well and kept for 30 min at room temperature. Acetic acid 1% was used to remove the unbounded dye from the cells, and the bounded dye was dissolved with Tris buffer 10 mM. The results were expressed in μg/mL of the concentration of each formulation that caused a 50% inhibition of cell growth (IC_{50}). A microplate reader (ELX800 Biotek microplate reader, BioTek Instruments, Inc., Vermont, United States of America) was used to read the samples' absorption at 540 nm. Triton X (1%), a detergent, served as a positive control due to its capacity to disrupt and destroy all cells. For the negative control group, only cells and the medium were added. Each concentration was tested in triplicate.

***In Vitro* Anti-Inflammatory Potential.** To evaluate the anti-inflammatory potential of the propolis-loaded niosomes, a cell assay was conducted, using the mouse macrophage cell line RAW 264.7, and carried out according to previously defined conditions.[43,45] The propolis-loaded niosomes were dispersed in water in order to obtain a final concentration of 1 μg/mL, from which successive dilutions were carried out, obtaining the concentrations to be tested (0.05−0.0008 mg/mL). The RAW 264.7 mouse macrophage cell line was grown in DMEM medium, supplemented with heat-inactivated FBS (10%), glutamine, and antibiotics (penicillin and streptomycin 1%), and kept in an incubator at 37 °C, with 5% CO_2, and under a humid and controlled atmosphere. Cells were detached by a cell scraper. An aliquot of the macrophages' cell suspension (300 μL), with a cell density of 5×10^5 cells/mL, and with a proportion of dead cells below 5% according to the trypan blue exclusion test, was plated in each well. The microplate was then incubated for 24 h under the previously indicated conditions to allow adequate adherence and multiplication of the cells. After that period, the cells were treated with different concentrations of the niosomal formulation (15 μL) and incubated for 1 h. Inflammatory stimulation was performed with the addition of 30 μL of a LPS solution (1 mL/mL), and incubation for an additional 24 h period. Dexamethasone (50 mM) was used as a positive control, and samples in the absence of LPS were used as a negative control. Quantification of nitric oxide (NO) was performed using a Griess reagent system kit (nitrophenamide, ethylenediamine, and nitrite solutions) and using a nitrite calibration curve ($y = 0.0069x + 0.1062$) also prepared in the 96-well plate. The produced NO was determined by reading absorbances at 540 nm (ELX800 Biotek microplate reader, BioTek Instruments, Inc., Vermont) and by comparison with the standard calibration curve. The results were calculated through the graphical representation of the percentage of inhibition of NO production versus sample concentration and expressed concerning the concentration of each of the formulations that caused a 50% inhibition of NO production (IC_{50}).

Safety Evaluation through the HET-CAM Test. The HET-CAM test was done in accordance with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended test method.[46,47] Forty Leghorn fertilized chicken eggs were incubated for 10 days in an automatic rotating incubator at 37 °C and 65% humidity. To confirm the presence of an embryo, a flashlight was used to detect each egg. If no embryo was detected, then that egg would not be used. After 10 days, the top part of the eggshells was cut open to expose the chorioallantoic membrane, so that the

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formulation could be applied. For each tested formulation, a total of three eggs were used. All of the membranes were hydrated with 0.9% sodium chloride (NaCl) for 30 min prior to the application of the formulations. Three formulation drops (0.3 mL, approximately 0.1 mL per drop) were applied to the chorioallantoic membrane of each egg. The negative control was the eggs, to which only 0.9% NaCl was applied. The positive control group was the eggs to which 10% sodium hydroxide (NaOH) was applied, causing an expected severe inflammatory reaction. The irritancy potential of each formulation was monitored through the observation of the occurrence of the following events: hemorrhage (bleeding of the vessels), lysis (disintegration of the vessels), and coagulation (intra- and extravascular protein denaturation). The occurrence of these events was observed at specific time points, namely 0.5, 2, and 5 min after formulation application, and depending on which one of these events occurred, and when, the sum of the scores attributed to each event added up to a maximum score of 21, corresponding to a level of irritability (Table 2).[46,48] After 5 min, an irritation score (IS) was calculated for each formulation, and a number ranging from 0 to 21 was assigned, representing a level of irritability: 0 to 4.9 for slightly/nonirritative, 5 to 8.9 for moderately irritative, and 9 to 21 for strongly irritative potential.

Table 2. HET-CAM Test Scoring Scheme

	score		
event	0.5 min	2 min	5 min
lysis	5	3	1
hemorrhage	7	5	3
coagulation	9	7	5

Statistical Analysis. For a more robust result interpretation, and to help choose the optimal formulation composition, a two-way ANOVA analysis was conducted on the results from the niosomes' physicochemical characterization parameters, followed by a Tukey's multiple comparisons test. For these statistical tests and for the production of all graphical data representations, the software GraphPad Prism was used (GraphPad, San Diego, California), version 8.0. Additionally, a more in-depth analysis was conducted using the JMP software (JMP Pro, Version 17.0.0.4, SAS Institute Inc., Cary, North Carolina). A 95% confidence level was considered in all analyses.

RESULTS AND DISCUSSION

Propolis Extract Phenolic Compounds' Identification and Quantification. The chemical composition of propolis is largely determined by the types of plants and flowers that exist near the beehive, resulting in a diverse mix of chemical compounds, particularly phenolic compounds, which are the key contributors to its bioactive properties. To thoroughly identify and quantify the phenolic compounds present in the harvested propolis extract, LC/DAD/ESI-MS^n was used. Results are presented in Table S1 and Figure 3. It was possible to identify 42 phenolic compounds, including 14 phenolic acids, 12 dihydroflavonols, 9 flavonols, 4 flavones, and 3 flavanones. The major compounds were the flavanone pinocembrin (m/z 255; 146.42 ± 0.01 mg/g extract), followed by the flavonols galangin (m/z 269; 95.26 ± 0.23 mg/g extract) and quercetin

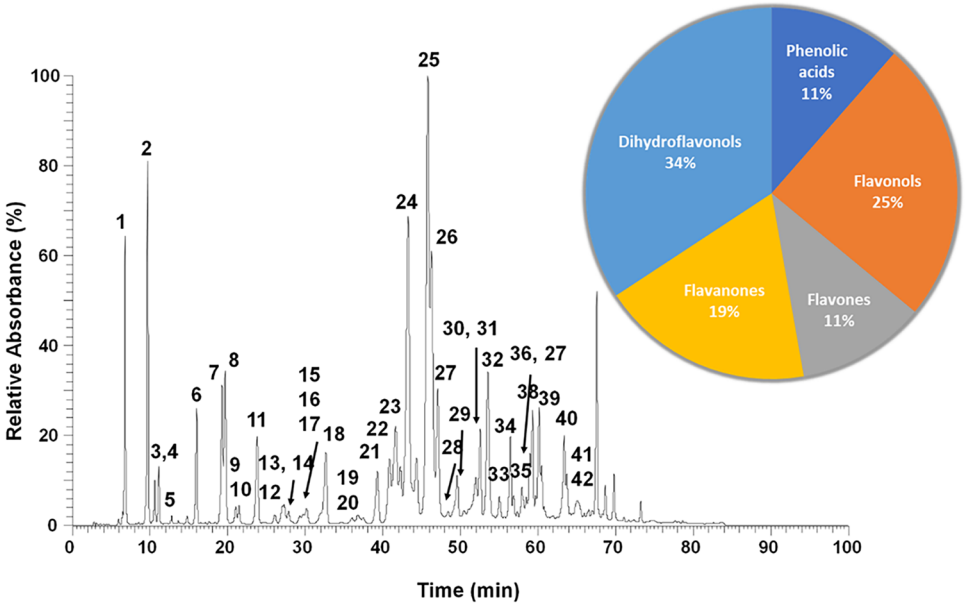

Figure 3. Chromatographic profile at 280 nm of the analyzed propolis phenolic extract, and main phenolic compound classes identified in it (in terms of percentage of the extract); 1—caffeic acid; 2—*p*-coumaric acid; 3—ferulic acid; 4—isoferulic acid; 5—benzoic acid; 6-3,4-dimethyl-caffeic acid; 7—pinobanksin-5-methyl-ether; 8—quercetin; 9—*p*-coumaric acid methyl ester; 10—quercetin-3-methyl-ether; 11—pinobanksin; 12—pinocembrin-5-methyl-ether; 13—apigenin; 14—kaempferol; 15—isorhamnetin; 16—kaempferol-methyl-ether; 17—quercetin-dimethyl-ether; 18—cinnamyldenacetic acid; 19—rhamnetin; 20—galangin-5-methyl-ether (isomer); 21—caffeic acid isoprenyl ester; 22—caffeic acid isoprenyl ester (isomer); 23—caffeic acid benzyl ester; 24—pinocembrin; 25—chrysin; 26—pinobanksin-3-*O*-acetate; 27—galangin; 28—acacetin; 29—chrysin-6-methyl-ether; 30—*p*-coumaric acid isoprenyl ester; 31—caffeic acid cinnamyl ester; 32—pinobanksin-3-*O*-propionate; 33—pinobanksin-5-methyl-ether-3-*O*-pentanoate; 34—3-hydroxy-5-methoxyflavanone; 35—pinobanksin-methyl-ether-3-*O*-phenylpropionate; 36—pinobanksin-methyl-ether-3-*O*-phenylpropionate (isomer); 37—caffeic acid derivative; 38—pinobanksin-3-*O*-pentenoate; 39—pinobansin-3-*O*-acetate-5-*O*-hydroxyphenylpropionate (isomer); and 40—pinobanksin-3-*O*-pentanoate or 2-methylbutyrate; 41—pinobanksin-3-*O*-phenylpropionate; 42—pinobanksin-3-*O*-hexanoate.

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(m/z 301; 78.87 ± 0.12 mg/g extract), the flavone chrysin (m/z 253; 93.01 ± 0.18 mg/g extract), and the dihydroflavonol pinobanksin-3-*O*-acetate (m/z 313; 92.67 ± 0.18 mg/g extract) (Table S1 and Figure 3).

In general, the obtained phenolic composition matched the typical profile seen in propolis from temperate regions, which has its origin mainly from *Populus* spp. resins. This sample, which originated from the central coast area of Portugal, showed a higher content of quercetin when compared with previously analyzed samples from the same region.[38] The sample also presented a higher content of main phenolic compounds, with the highest amounts corresponding to dihydroflavonols (34% of the extract), followed by flavonols (25% of the extract), flavanones (19% of the extract), and flavones (11% of the extract), which highlights a higher general concentration of flavonoids when compared to phenolic acids (Table S1 and Figure 3). As mentioned, among the biologically active substances commonly present in propolis, flavonoids are considered to be the main bioactive components, having been linked to several beneficial properties such as antimicrobial, anticancer, antioxidant, anti-inflammatory, antifungal, neuroprotective, and chemoprotective effects, among others.[49]

Niosomal Vehicles' Formulation Development and Physicochemical Characterization. Niosomal formulations were produced using the thin-film hydration method, the most commonly used niosome production method, mainly due to being a simple technique. In this method, the niosomes are formed when water (or another aqueous medium) is added to hydrate the previously formed thin layer, which was in turn formed by the evaporation of the organic solvent, a solvent that was used to dissolve all the components necessary for niosomal composition (surfactants and other lipids or hydrophobic components).[16,50,51]

Niosomes for topical therapeutic or cosmetic applications should have a size range between 100 and 200 nm in order to ease the permeation into the epidermis, and to have a good retention once there.[52−54] Hence, this was the chosen target size range for the developed niosomal vesicles. To reduce the particle size, extrusion was the selected technique in which the liquid dispersion was submitted to numerous passes through a 200 nm pore-size polycarbonate membrane filter. The performed number of extrusion cycles and the size of the polycarbonate filter pores tend to have a substantial influence on the mean size of the resulting particles and their size distribution homogeneity.[17,55]

Following preliminary experiments, two hydrophilic nonionic surfactants were selected, Tween 20 and Kolliphor RH 40, to be tested in combination with cholesterol, cetyl alcohol, or a mixture of both lipids. The molar ratio was fixed at 3.5:2 of nonionic surfactant to lipid. Typically, niosomal compositions tend to work better when having a higher quantity of nonionic surfactant compared to the amount of lipids, depending on the surfactant type, particularly its hydrophilic−lipophilic balance (HLB) value. In several studies, the particle size of niosomal formulations testing different nonionic surfactants has been revealed to increase with the increase of the cholesterol ratio in the formulation.[56−60] Hence, in our work, a relatively higher amount of nonionic surfactant was used when compared to the lipid proportion. Additionally, the inclusion of the right lipids can have a strong impact on the niosome's bilayer structure. Cholesterol has a substantial influence on membrane rigidity, and cetyl alcohol tends to enhance the overall stability of the niosomes. Cetyl alcohol is a polar lipid and fatty alcohol that has been reported in the scientific literature as a common component in the constitution of niosomes, alone or in combination with cholesterol.[61,62] On the other hand, Kolliphor RH 40 is a nonionic solubilizer and emulsifying agent, resulting from reacting 1 mol of hydrogenated castor oil with 40 mol of ethylene oxide, which can form clear mixtures with fatty alcohols, like cetyl alcohol, at high temperatures. It has been reported to have low toxicity, although there is limited research on its use in niosomal formulations, thereby bringing novelty into the field.[63−65] Tween 20 *is another nonionic surfactant*, composed of fatty acid esters of polyoxyethylene sorbitan, and quite commonly used in nanosystem production, including some studies regarding niosome development.[66−68]

At first, the formulation vehicles were developed (no encapsulated compound). Four combinations were made for each nonionic surfactant, with different lipid mixtures: cetyl alcohol only, a mixture of cholesterol and cetyl alcohol in a 1:1 ratio, a mixture of cholesterol and cetyl alcohol in a 1:2 ratio, and a mixture of cholesterol and cetyl alcohol in a 2:1 ratio. Additionally, five different hydration temperatures were tested, including 40, 45, 50, 55, or 60 °C. After the primary niosomal suspensions were created, all the samples underwent up to 51 cycles of extrusion to reduce the particle size, including 0, 11, 21, 31, 41, or 51 cycles. After extrusion, the particle size and PDI of the vesicles were measured. A summary of these eight formulations' composition, as well as tested hydration temperatures and performed number of extrusion cycles, is described in Table 3. The experimental results of the DoE analysis are summarized in Tables S2 and S3.

Table 3. Summary of the Developed Niosomal Vehicle Formulations' Composition, As Well As Corresponding Tested Hydration Temperatures and Performed Number of Extrusion Cycles

niosomal formulation components		tested hydration temperatures	performed number of extrusion cycles
nonionic surfactant	lipids		
Kolliphor RH 40	cetyl alcohol	40, 45, 50, 55, or 60 °C	0, 11, 21, 31, 41, or 51 cycles
	cetyl alcohol + cholesterol (1:1)		
	cetyl alcohol + cholesterol (1:2)		
	cetyl alcohol + cholesterol (2:1)		
Tween 20	cetyl alcohol		
	cetyl alcohol + cholesterol (1:1)		
	cetyl alcohol + cholesterol (1:2)		
	cetyl alcohol + cholesterol (2:1)		

The two selected nonionic surfactants both have high HLB values (both above 14). A lower hydrophobicity of the nonionic surfactants tends to cause an increase in the surface free energy, which can increase the particle size of the niosomes, something that has been reported in several studies, including in niosomal formulations having Tween 20 in their composition, which originated larger particle sizes due to its low hydrophobicity in comparison with other nonionic surfactants with lower HLB values (and hence increased hydrophobicity).[69,70] Therefore, bigger particle sizes before extrusion were expected and observed, hence the need for extrusion itself. For all of the

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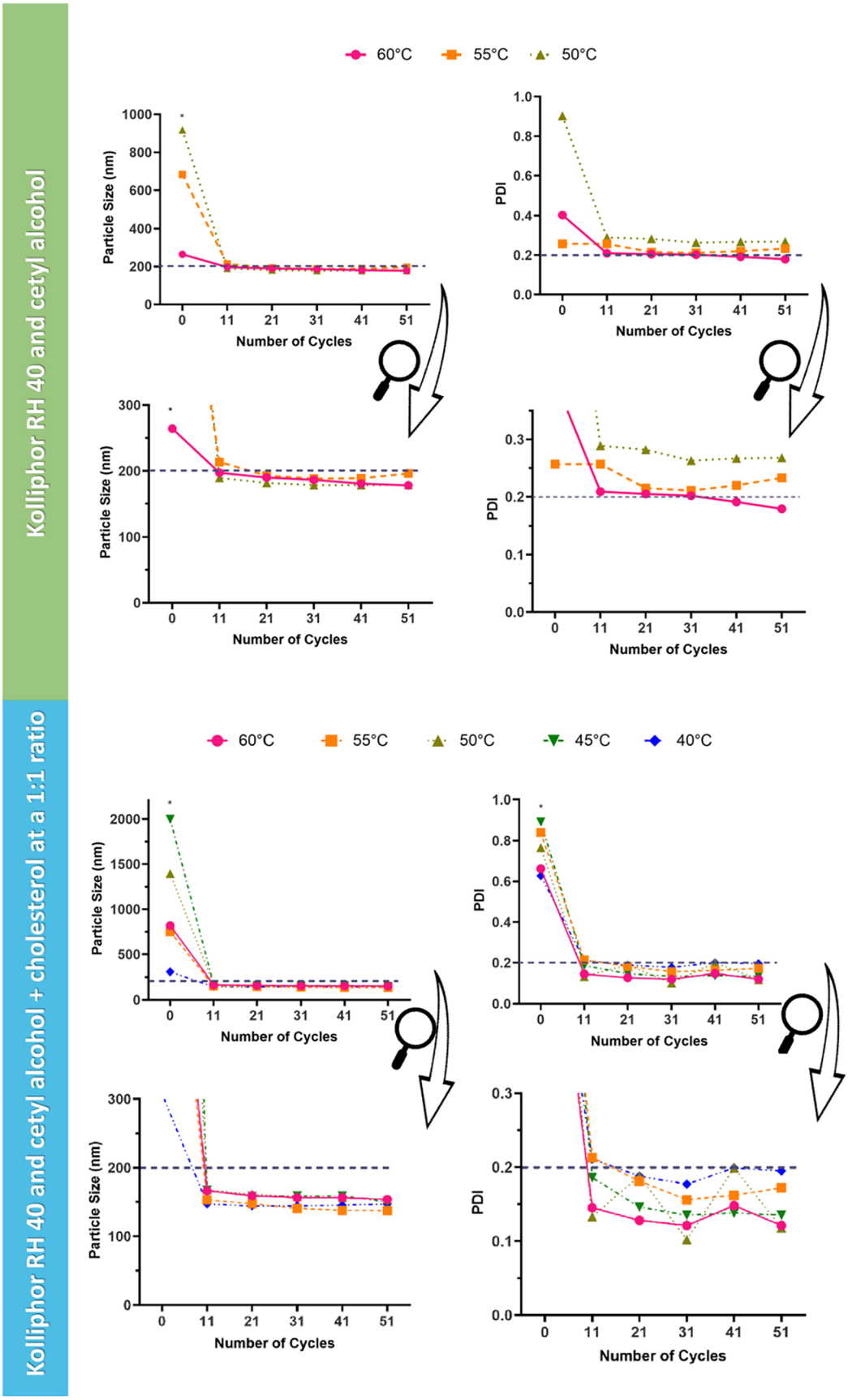

Figure 4. Particle size and PDI values obtained for niosomal vehicles containing Kolliphor RH 40 and cetyl alcohol (results on top, marked in green) or Kolliphor RH 40 and cetyl alcohol + cholesterol 1:1 (results on bottom, marked in turquoise) for all tested temperatures (40 to 60 °C) and number of extrusion cycles (0, 11, 21, 31, 41, or 51); all graphs are provided with an amplification for better result visualization, with scale adaptation.

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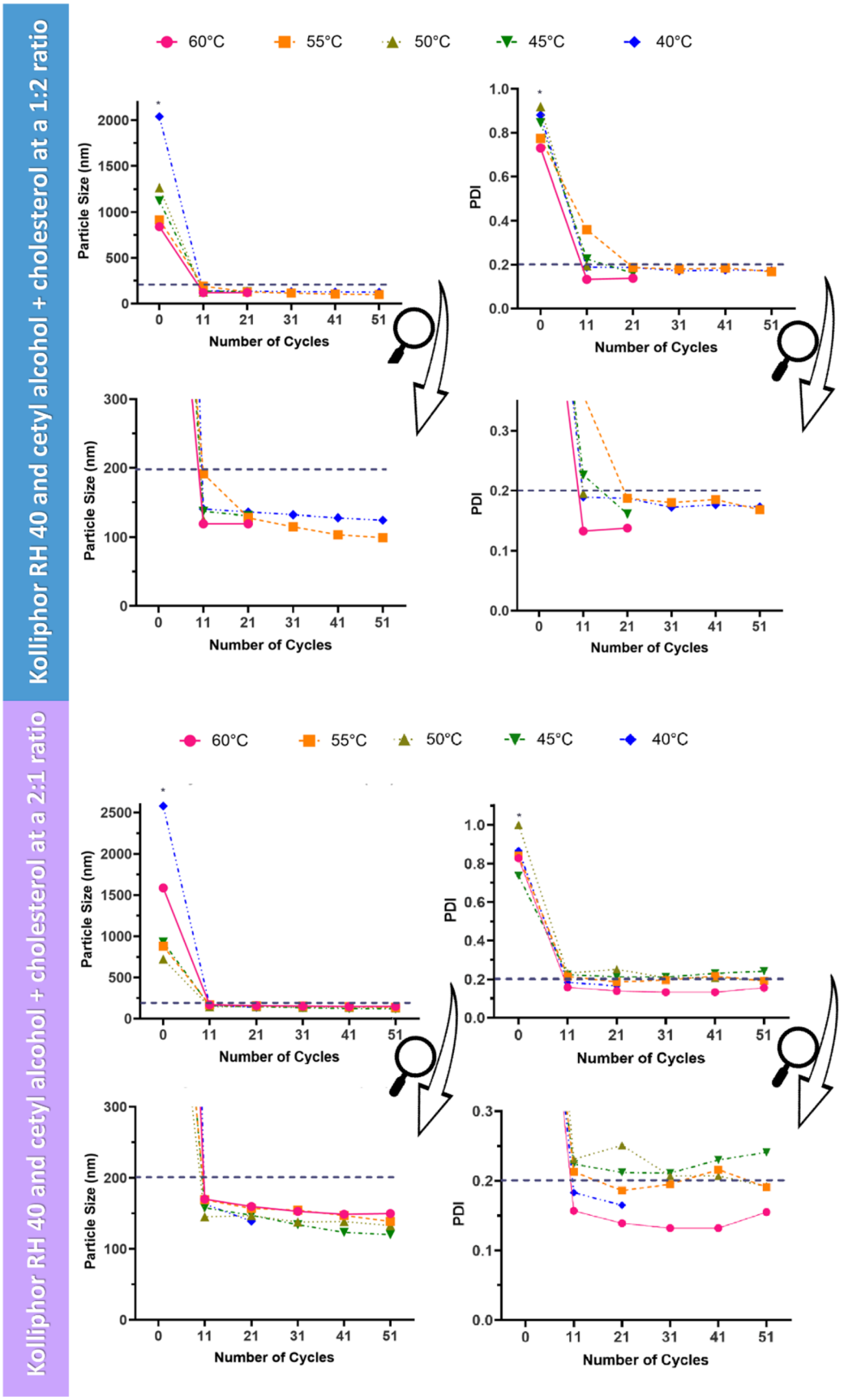

Figure 5. Particle size and PDI values obtained for niosomal vehicles containing Kolliphor RH 40 and cetyl alcohol + cholesterol 1:2 (results on top, marked in blue) or Kolliphor RH 40 and cetyl alcohol + cholesterol 2:1 (results on bottom, marked in purple) for all tested temperatures (40 to 60 °C) and number of extrusion cycles (0, 11, 21, 31, 41, or 51); all graphs are provided with an amplification for better result visualization, with scale adaptation.

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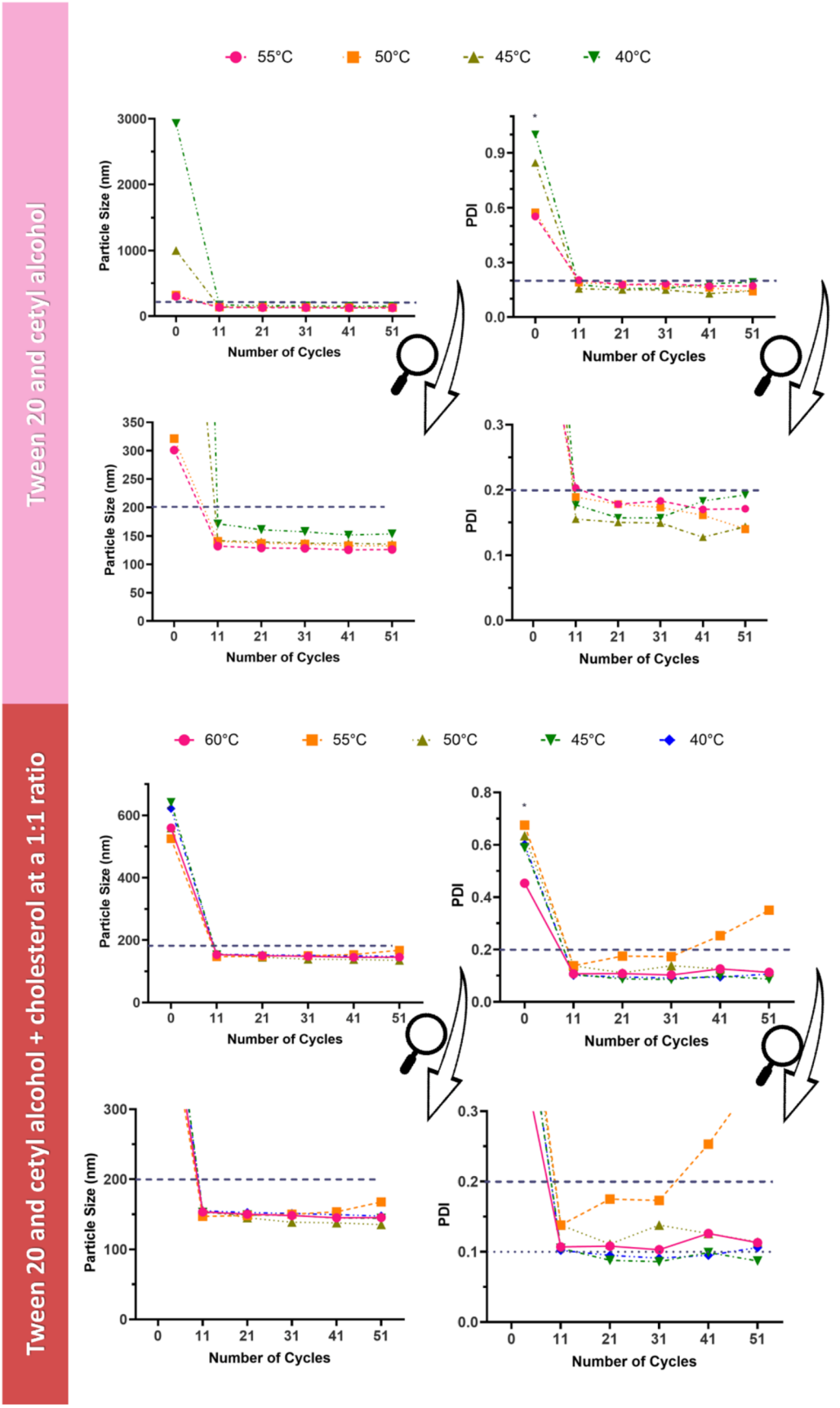

Figure 6. Particle size and PDI values obtained for niosomal vehicles containing Tween 20 and cetyl alcohol (results on top, marked in pink) or Tween 20 and cetyl alcohol + cholesterol 1:1 (results on bottom, marked in red) for all tested temperatures (40 to 60 °C) and number of extrusion cycles (0, 11, 21, 31, 41, or 51); all graphs are provided with an amplification for better result visualization, with scale adaptation.

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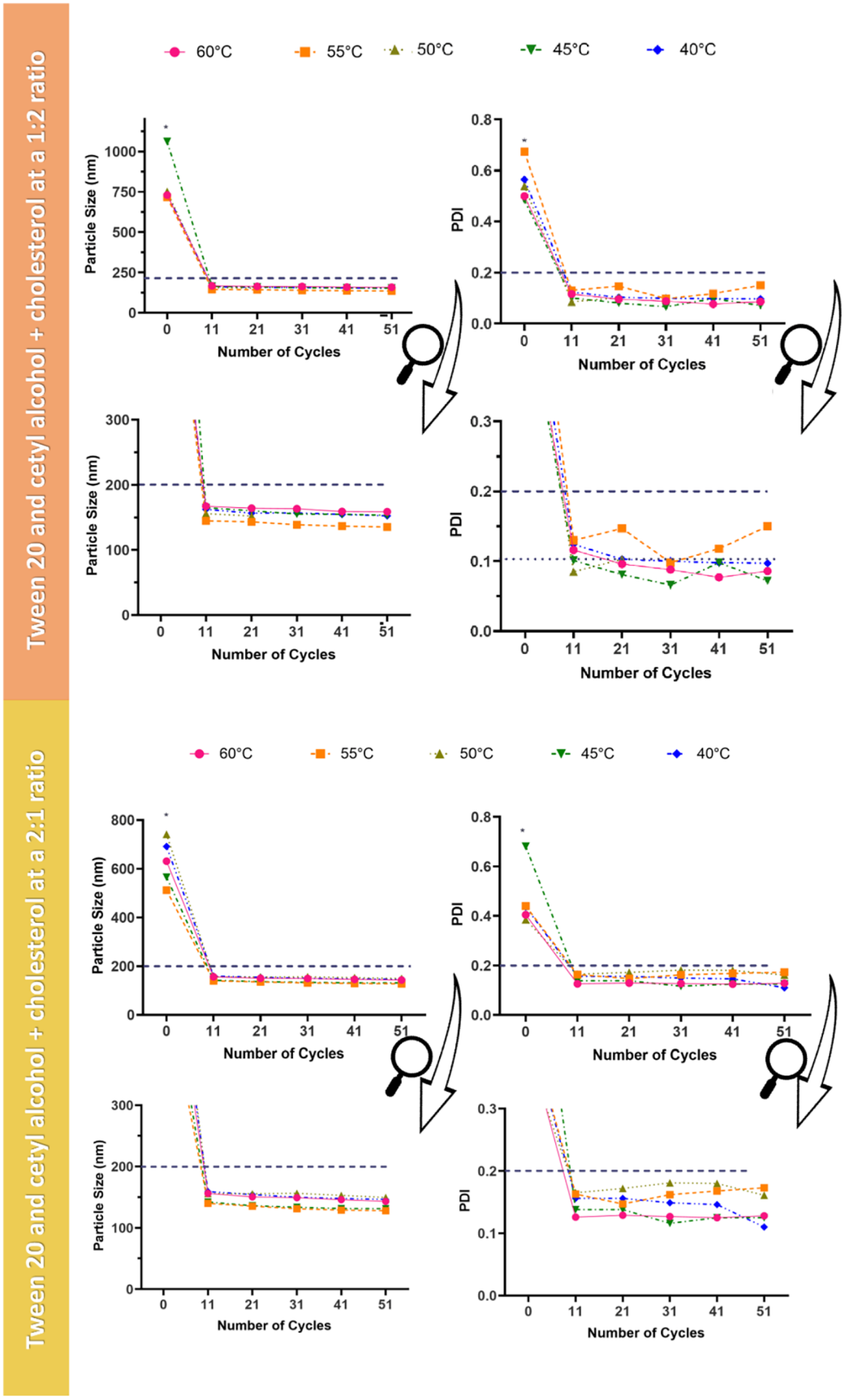

Figure 7. Particle size and PDI values obtained for niosomal vehicles containing Tween 20 and cetyl alcohol + cholesterol 1:2 (results on top, marked in orange) or Tween 20 and cetyl alcohol + cholesterol 2:1 (results on bottom, marked in yellow) for all tested temperatures (40 to 60 °C) and number of extrusion cycles (0, 11, 21, 31, 41, or 51); all graphs are provided with an amplification for better result visualization, with scale adaptation.

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eight different niosomal vehicle formulations, particle size and PDI results, expressing the measured values for each temperature and each different number of extrusion cycles, can be observed in Figure 4 (niosomes made of Kolliphor RH 40 and cetyl alcohol, and niosomes made of Kolliphor RH 40 and cetyl alcohol + cholesterol at a 1:1 ratio), Figure 5 (niosomes made of Kolliphor RH 40 and cetyl alcohol + cholesterol at a 1:2 ratio, and niosomes made of Kolliphor RH 40 and cetyl alcohol + cholesterol at a 2:1 ratio), Figure 6 (niosomes made of Tween 20 and cetyl alcohol, and niosomes made of Tween 20 and cetyl alcohol + cholesterol at a 1:1 ratio), and Figure 7 (niosomes made of Tween 20 and cetyl alcohol + cholesterol at a 1:2 ratio, and niosomes made of Tween 20 and cetyl alcohol + cholesterol at a 2:1 ratio).

In the niosomes having in their composition Kolliphor RH 40 and cetyl alcohol (Figure 4, represented in green), there was a generally significant difference in particle size values resulting from the application of extrusion ($p \leq 0.05$). When making multiple comparisons, there was a statistically significant difference between no extrusion and extrusion; however, no significant difference was observed between the different number of extrusion cycles, which means that 11 extrusion cycles were sufficient for this formulation, leading to adequate small particle sizes for all tested hydration temperatures. As for PDI, extrusion of the niosomal suspension did lead to increased homogeneity with a reduction of PDI values. No statistically significant difference was found when different hydration temperatures. Additionally, while all formulation's particle sizes were below or near 200 nm after extrusion, a PDI value equal to or below 0.2 was only possible to obtain at 60 °C, with 31, 41, or 51 extrusion cycles. Data at hydration temperatures of 45 and 40 °C was not possible to obtain for this formulation since extrusion was not possible to apply due to the high heterogeneity of the nanosystem, giving origin to larger particles, which clogged the syringes of the extruder. As for the niosomal formulation containing Kolliphor RH 40 as a surfactant and a lipid mixture of cetyl alcohol and cholesterol at a 1:1 ratio (Figure 4, represented in turquoise), there was a generally statistically significant difference concerning the influence of extrusion on particle size values ($p \leq 0.0001$), with a significant difference between no extrusion and extrusion, but again no significant difference among the different number of extrusion cycles, meaning that 11 cycles were once more sufficient for this composition, at all tested hydration temperatures. In this formulation, there was also a statistically significant difference of extrusion influence on PDI values ($p \leq 0.0001$), but again the significance was only in applying extrusion or not, and not in the number of extrusion cycles. Again, no statistically significant difference was found when comparing different hydration temperatures. Particle sizes were always below 200 nm for this composition, and PDI was always below 0.2 (after extrusion), except at a 55 °C hydration temperature and 11 cycles of extrusion, conditions for which it was above 0.2.

For the formulation having Kolliphor RH 40 as the surfactant, and cetyl alcohol plus cholesterol at a 1:2 ratio as the lipid fraction (Figure 5, represented in blue), again there was a general statistically significant difference ($p \leq 0.01$) of extrusion on particle size values, but, once more, it resided mainly on applying extrusion or not, with the number of extrusion cycles having no statistical meaning, and therefore it was concluded that 11 cycles was enough, for all tested temperatures. There was also a significant difference ($p \leq 0.001$) of extrusion application on PDI values, but, once more, it was independent of the number of cycles. No statistical significance was found when different hydration temperatures. All formulations' particle sizes were below 200 nm and PDI below 0.2 (after extrusion), except for the 45 °C hydration temperature with 11 cycles of extrusion, conditions for which PDI values were above 0.2. For niosomes containing Kolliphor RH 40, and cetyl alcohol plus cholesterol at a 2:1 ratio (Figure 5, represented in purple), there was also a significant difference ($p \leq 0.0001$) regarding the influence of the application of extrusion on both particle size and PDI, but again 11 cycles were satisfactory. Moreover, no statistically significant difference was observed when different hydration temperatures. All formulations' particle sizes were below 200 nm (after extrusion), but PDI values were below 0.2 only for hydration temperatures of 60 °C, 55 °C, and 45 °C.

Concerning the other tested nonionic surfactant, in niosomes containing Tween 20 and cetyl alcohol (Figure 6, represented in pink), again a statistically significant difference ($p \leq 0.0001$) was found in the influence of the application of extrusion on both niosomal particle size and PDI, but once more 11 cycles were enough to guarantee a small vesicle size and adequate size dispersion homogeneity. There was no statistical significance when comparing different hydration temperatures. For this composition, all formulations' particle sizes were below 200 nm, and almost all PDI values were below 0.2 (after extrusion), except when using 55 °C as the hydration temperature combined with 11 cycles of extrusion, conditions for which PDI values were above 0.2. For niosomes having Tween 20, and cetyl alcohol plus cholesterol at a 1:1 ratio (Figure 6, represented in red), both particle size and PDI were significantly affected by extrusion ($p \leq 0.0001$), but no significance was found between the different number of cycles, meaning that 11 cycles were adequate for this formulation as well. Additionally, while particle size results were generally not significantly affected by the variation of hydration temperature, different hydration temperatures significantly affected PDI values in this formulation ($p \leq 0.001$). Furthermore, all formulation's particle sizes were below 200 nm (after extrusion), but although for PDI one hydration temperature led to values above 0.2 (55 °C), two hydration temperatures, 45 and 40 °C, led to PDI values below 0.1, depicting a very high degree of vesicle size distribution homogeneity.

As for the niosomal vehicles containing Tween 20 plus cetyl alcohol and cholesterol at a 1:2 ratio (Figure 7, represented in orange), there was a significant difference ($p \leq 0.0001$) in the particle size and PDI of the developed vesicles regarding extrusion, but again regardless of the number of cycles, and hence 11 extrusion cycles were satisfactory. No temperature effects were observed, and while all formulations had particle sizes below 200 nm and PDI values below 0.2 (after extrusion), almost all tested conditions also had PDI values below 0.1, representing truly homogeneous formulations with regard to particle size distribution. Finally, the niosomes containing Tween 20 as the surfactant and cetyl alcohol plus cholesterol at a 2:1 ratio in their composition (Figure 7, represented in yellow) had their particle size and PDI values significantly affected by extrusion ($p \leq 0.0001$) in general, again with no effect regarding the number of extrusion cycles, with 11 cycles being sufficient, and with no significant hydration temperature influences. Once more, all formulations' particle sizes were below 200 nm and PDI values below 0.2 (after extrusion).

Furthermore, generally comparing each composition's particle size, niosomes prepared with Kolliphor RH 40 were bigger before extrusion than the ones prepared with Tween 20,

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since while with Kolliphor RH 40 the obtained particle sizes ranged from 264 to 3287 nm with cetyl alcohol only, from 308 to 1998 nm with cetyl alcohol plus cholesterol at a 1:1 ratio, from 724 to 2580 nm with cetyl alcohol plus cholesterol at a 2:1 ratio, and from 566 to 2040 nm with cetyl alcohol plus cholesterol at a 1:2 ratio. On the other hand, with Tween 20 the particle size ranged from 301 to 2931 nm with cetyl alcohol only, from 525 to 641 nm with cetyl alcohol plus cholesterol at a 1:1 ratio, from 513 to 742 nm with cetyl alcohol plus cholesterol at a 2:1 ratio, and from 716 to 1062 nm with cetyl alcohol plus cholesterol at a 1:2 ratio. This can be explained by the fact that Kolliphor RH 40 has a higher HLB value and, therefore, a lower hydrophobicity than Tween 20, thus the particle sizes of the niosomal formulations with Kolliphor RH 40, before extrusion, had a tendency of being bigger. Based on these findings, it is evident that the choice of nonionic surfactant for niosome formulation significantly impacts both its particle size and PDI, which is in accordance with what has been previously described by other studies.[52,71] A previous study by Salem et al. using different nonionic surfactants, including Kolliphor RH 40, had similar results since the niosomes formulated with this surfactant also had an increased particle size when compared to the other tested nonionic surfactants.[71]

A two-way ANOVA analysis, followed by a Tukey's multiple comparisons test, was conducted on the data obtained from the 34 trials (different niosomal compositions with different hydration temperatures, and a different number of extrusion cycles). The objective was to determine the optimal formulation composition, with the smallest particle size (within the intended 100 to 200 nm size range) and minimum PDI value. The observed mean particle size was approximately 130 nm. Based on this analysis, the only other general conclusive finding was the existence of a significant difference between nonextrusion and extrusion of the niosomal vesicles, which affected particle size and PDI values. Nevertheless, in general, no additional improvement was observed with extrusion cycles beyond 11, and no significant trend was found concerning the hydration temperature. Hence, after achieving particle sizes below or near 200 nm, and PDI values below or near 0.3, for all formulations through extrusion, which aligns with the desirable criteria for cosmetic applications and skin permeation facilitation,[52−54] selecting the optimal formulation for further characterization became challenging using the initial analysis. Therefore, a QbD approach based on DoE was conducted by using the JMP software program to further optimize the experimental conditions and define a design space. For that, target values were defined, namely, particle sizes below or near 200 nm and PDI values below or near 0.3. This analysis was conducted using the JMP software program involving two stages.

The first JMP analysis aimed to assess which type of nonionic surfactant led to improved vesicle characteristics, whether the use of one single lipid (cetyl alcohol) or a lipid mixture (cetyl alcohol plus cholesterol, no specific ratios) was better, and which hydration temperature and number of extrusion cycles led to optimal results. Hence, these were the four independent variables. The chosen dependent variables were the niosomes' particle size and PDI, the two responses being affected by the independent variables. A summary of all selected variables is described in Table 4.

Based on Figures 8A, S1, and S2, it is possible to conclude that both responses (particle size and PDI) were only significantly affected by the number of extrusion cycles ($p < 0.05$). Hydration temperature did not have a significant impact on the optimized niosomes' outcomes. Additionally, despite the influence of surfactant type and lipid composition not being statistically significant on the niosomes' particle size and PDI, it was possible to verify a slight tendency of niosomes composed by Tween 20 (instead of Kolliphor RH 40) and a lipid mixture (instead of cetyl alcohol only) for having lower particle size and PDI values.

Table 4. Independent and Dependent Variables Used for the First JMP Analysis, to Assess the Influence of Nonionic Surfactant Type, Lipid Composition, Hydration Temperature, and Number of Extrusion Cycles on Niosome Particle Size and PDI

variable	type of variable	admitted values
type of nonionic surfactant	independent	Kolliphor RH 40 or Tween 20
use of one lipid or a mixture of lipids		cetyl alcohol or lipid mixture (cetyl alcohol plus cholesterol)
hydration temperature		60, 55, 50, 45, or 40 °C
number of extrusion cycles		0, 11, 21, 31, 41, or 51
particle size	dependent	100−200 nm
PDI		0−0.3

Since the initial DoE analysis indicated better outcomes for niosomal formulations composed of Tween 20 and a mixture of lipids, an optimization DoE was conducted to assess the impact of different ratios of the lipidic phase, regarding cetyl alcohol-to-cholesterol proportion [either 0.5 (1:2 ratio), 1 (1:1 ratio) or 2 (2:1 ratio)], nonionic surfactant type, and number of extrusion cycles on niosomes' particle size and PDI (dependent variables). A summary of the variables is described in Table 5.

Based on Figures 8D, S3, and S4, the number of extrusion cycles was the only independent variable that had a significant impact on the particle size values, whereas for PDI, both the number of extrusion cycles and the type of surfactant had a significant impact. Following this analysis, it was evident that niosomes formulated with Tween 20 tended to exhibit higher homogeneity, compared to Kolliphor RH 40, and that the number of extrusion cycles was crucial for the obtention of niosomes with lower particle size and PDI. On the other hand, different lipid ratios had a minimal impact on the results.

In order to define a design space, contour profiles were plotted for both Kolliphor RH 40 (Figure 8B,E) and Tween 20 (Figure 8C,F). It was verified that for Kolliphor RH 40, in niosomes composed of one single lipid only (cetyl alcohol), the desired particle size and PDI values were achieved with approximately 51 cycles of extrusion, whereas for niosomes composed of a mixture of cetyl alcohol and cholesterol, 41 extrusion cycles were ideal at any cetyl alcohol-to-cholesterol ratio. For niosomes composed of Tween 20 and cetyl alcohol, the optimized biophysical properties were achieved by using approximately 41 cycles of extrusion, whereas for niosomes composed of a mixture of cetyl alcohol and cholesterol, 31 extrusion cycles were ideal at any cetyl alcohol-to-cholesterol ratio. After this analysis, it was possible to assign an optimized number of cycles to each formulation composition (Table 6). Since the hydration temperature proved to not significantly influence the particle size or PDI of the niosomes, 40 °C was selected, since it allowed the encapsulation of any kind of molecule, including molecules with a certain degree of thermolability, thus being more versatile. The eight formulations' composition, as well as the selected production conditions, are summarized in Table 6.

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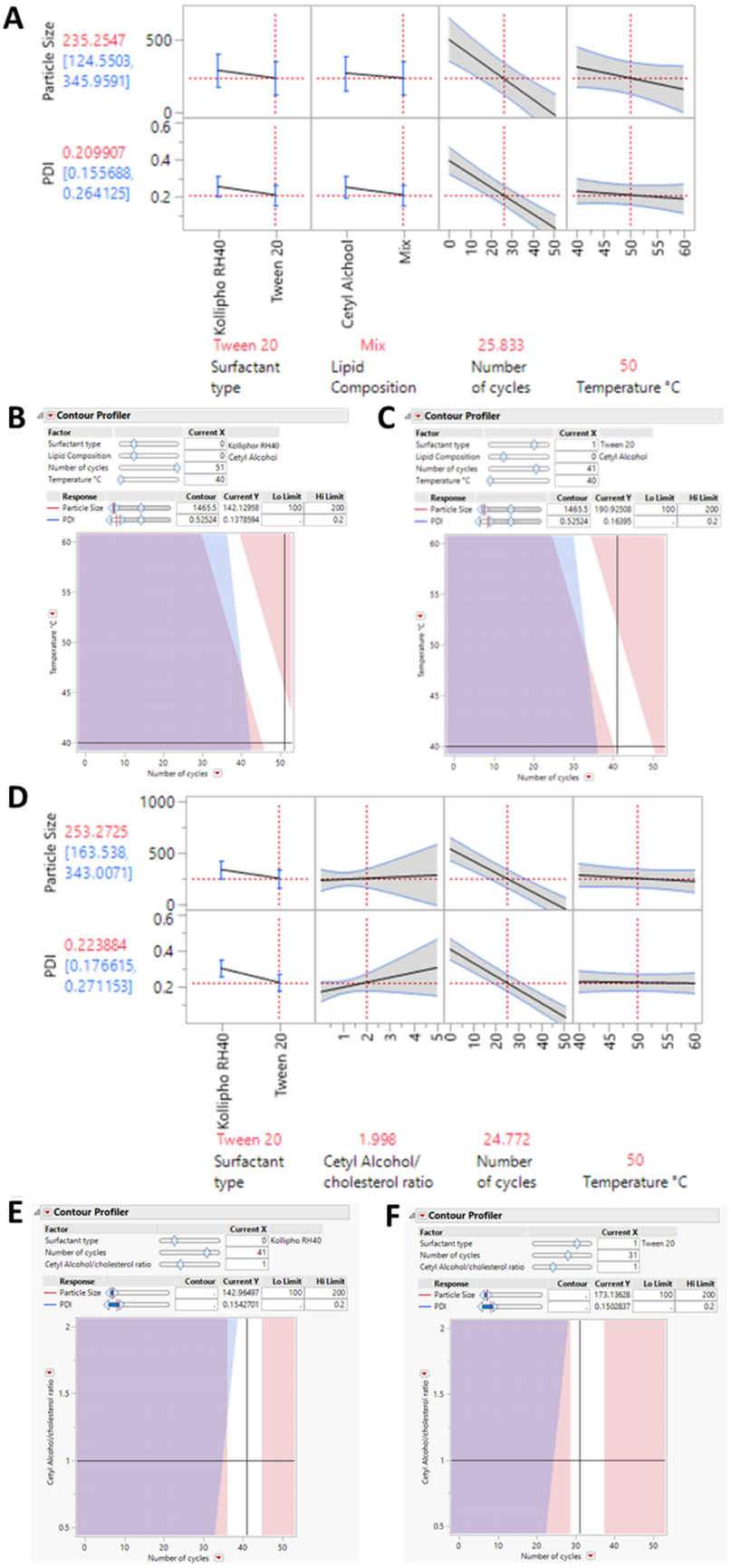

Figure 8. (A) Prediction profile of the impact of different independent variables, namely nonionic surfactant type, lipid composition, hydration temperature, and number of extrusion cycles, on niosomal vehicles' particle size and PDI; (B) contour profile of niosomes composed by one single lipid (cetyl alcohol) and Kolliphor RH 40, in which the red area represents the nonoptimal area for particle size, the blue area represents the nonoptimal area

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Figure 8. continued

for PDI, the purple area is the overlay between the particle size and the PDI, and the white area (design space) is the optimal area to achieve a niosomal particle size between 100 and 200 nm and PDI values below 0.2; (C) contour profile of niosomes composed by one single lipid (cetyl alcohol) and Tween 20, in which the red area represents the nonoptimal area for particle size, the blue area represents the nonoptimal area for PDI, the purple area is the overlay between the particle size and the PDI, and the white area (design space) is the optimal area to achieve a niosomal particle size between 100 and 200 nm and PDI values below 0.2; (D) prediction profile of the impact of different independent variables, namely nonionic surfactant type, lipid ratio, hydration temperature, and number of extrusion cycles, on niosomal vehicles' particle size and PDI; (E) contour profile of niosomes composed by a mixture of lipids (cetyl alcohol and cholesterol) and Kolliphor RH 40, in which the red area represents the nonoptimal area for particle size, the blue area represents the nonoptimal area for PDI, the purple area is the overlay between the particle size and the PDI, and the white area (design space) is the optimal area to achieve niosomal particle size between 100 and 200 nm and PDI below 0.2; (F) contour profile of niosomes composed by a mixture of lipids (cetyl alcohol and cholesterol) and Tween 20 (B), in which the red area represents the nonoptimal area for particle size, the blue area represents the nonoptimal area for PDI, the purple area is the overlay between the particle size and the PDI, and the white area (design space) is the optimal area to achieve niosomal particle size between 100 and 200 nm and PDI below 0.2 (obtained with JMP).

Table 5. Independent and Dependent Variables Used in Optimization of DoE, to Assess the Influence of Nonionic Surfactant Type, Lipid Ratio, and Number of Extrusion Cycles on Niosome Particle Size and PDI

variable	type of variable	admitted values
type of nonionic surfactant	independent	Kolliphor RH 40 or Tween 20
lipid ratio (cetyl alcohol-to-cholesterol)		0.5 (1:2), 1 (1:1) or 2 (2:1)
number of extrusion cycles		0, 11, 21, 31, 41, or 51
particle size	dependent	100−200 nm
PDI		0−0.3

Table 6. Optimized Niosomal Vehicle Formulations' Composition and Selected Formulation Conditions (Number of Extrusion Cycles and Hydration Temperature)

niosomal vehicle formulations' composition	number of extrusion cycles	hydration temperature
Tween 20 and cetyl alcohol	41 cycles	40 °C
Tween 20 and cetyl alcohol + cholesterol (1:1)	31 cycles	
Tween 20 and cetyl alcohol + cholesterol (1:2)	31 cycles	
Tween 20 and cetyl alcohol + cholesterol (2:1)	31 cycles	
Kolliphor RH 40 and cetyl alcohol	51 cycles	
Kolliphor RH 40 and cetyl alcohol + cholesterol (1:1)	41 cycles	
Kolliphor RH 40 and cetyl alcohol + cholesterol (1:2)	41 cycles	
Kolliphor RH 40 and cetyl alcohol + cholesterol (2:1)	41 cycles	

Effects of Propolis Encapsulation, Entrapment Efficiency, and *In Vitro* Release Studies. For the formulation of propolis-loaded niosomes, a concentration of 1 mg/mL of propolis extract was dissolved in chloroform, along with the remaining constituents of the formulation, during the production process. The selected concentration was based on previous studies.[72,73]

Although the formulation comprising Kolliphor RH 40 and cetyl alcohol gave results similar to those of the other selected composition variations in what concerned particle size and PDI values after extrusion, it was excluded before propolis encapsulation due to instability and reproducibility issues. Additionally, upon adding propolis to the formulation containing Kolliphor RH 40 and cetyl alcohol plus cholesterol at a 1:2 ratio, extrusion was hindered due to the presence of larger particles, which caused syringe clogging and hence not allowing extrusion to be performed. Consequently, this formulation was excluded from additional characterization, as well, leaving six formulations for further analysis. Figure 9A showcases the particle size and PDI values of the propolis-loaded niosomal formulations (after extrusion, optimized conditions), and it is clear that, aside from the already mentioned cases, propolis encapsulation did not lead to significant differences in vesicle particle size or PDI, thus still leading to formulations with adequate characteristics.

In order to quantify the amount of encapsulated propolis in the six selected optimized niosomal formulations, a calibration curve was performed in methanol: water (60:40), with concentrations of propolis ranging from 0.05 to 2 mg/mL, reaching a correlation coefficient (R^2) of 0.993. In order to determine the encapsulation efficiency, each formulation was subjected to centrifugation using an ultrafiltration-centrifuge tube. The filtrate obtained after the centrifugation was collected and subsequently diluted in a methanol:water mixture (60:40). Samples were then analyzed by UV−vis spectrophotometry at a wavelength of 401 nm. The entrapment efficiency was expressed in percentages, and the results are summarized in Table 7 (including a summary of the obtained formulation particle size and PDI values as well, along with their respective composition and attributed code).

The EE% of propolis in the developed niosomes ranged between 78.8 and 87.4%, demonstrating to be quite successful. Although there was a range of EE% values, which can be attributed to the variation of niosomal vehicles' composition, the range was small, and all values can be considered as being reasonably high, contrarily to other previously published studies, such as the one by Zhang et al.,[74] in which niosomes with Span 40 and cholesterol reached low EE%, around 30%, despite different ratios between the nonionic surfactant and cholesterol having been tested. In another study, Qumbar et al.[75] developed niosomes using Span 60 and cholesterol, also tested at different ratios, with EE% ranging from 30 to 94%, which is a quite wide range of values. In a study by Li et al.,[76] which focused on the development of niosomes having Tween 40 and Span 60 as nonionic surfactants and cholesterol as the lipid portion, the maximum reached EE% was just 49%. Compared to these studies, our propolis-loaded niosomes reached overall higher and more consistent EE% values, probably not only due to the selected excipients, but also due to the hydrophobicity of propolis, since there is a high affinity of hydrophobic drugs and active molecules to the niosomes' bilayer, and hence EE% is highly dependent on the characteristics of the molecule to be encapsulated inside the vesicles.[64,77]

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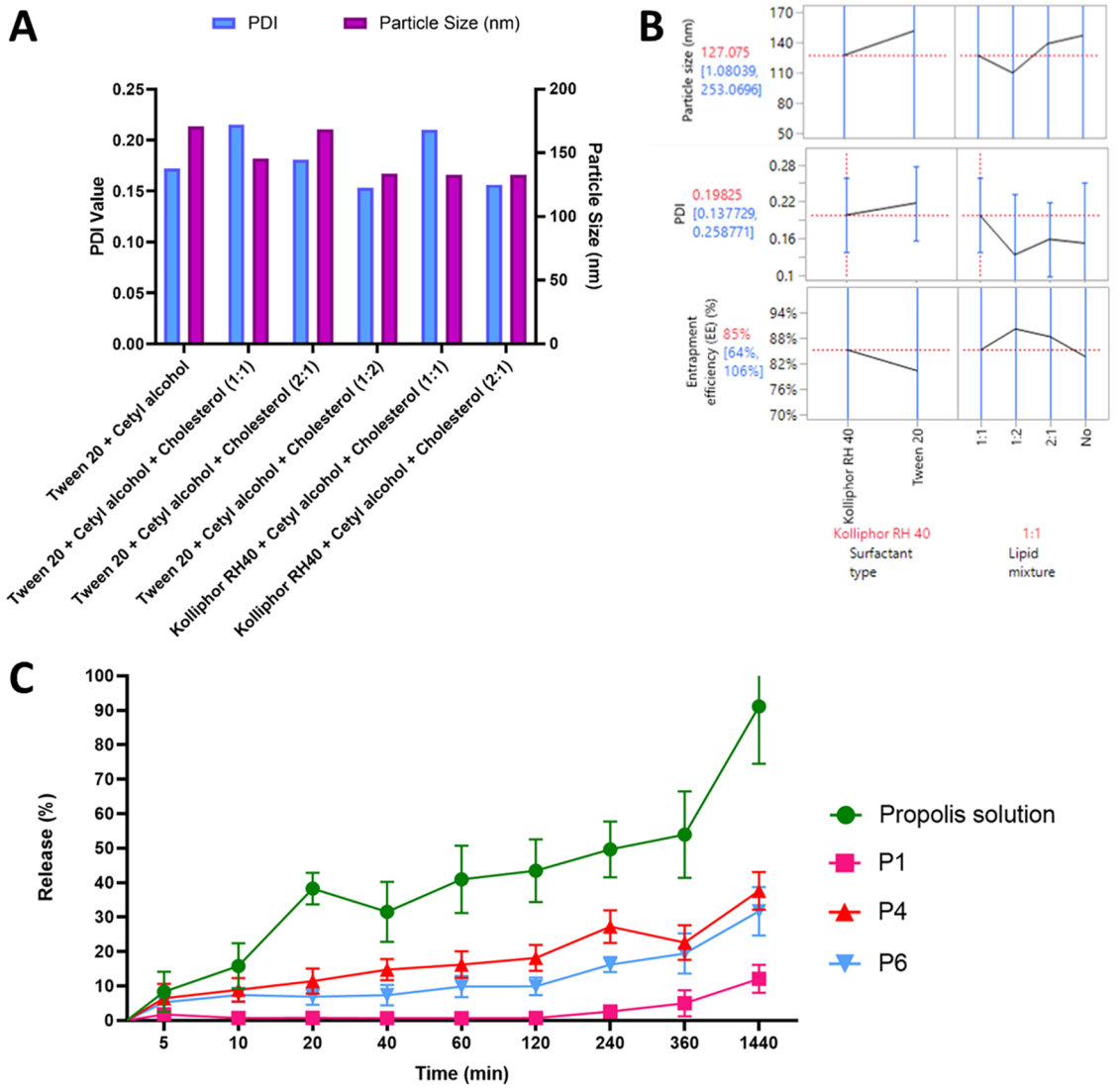

Figure 9. (A) Particle size and PDI of the optimized propolis-loaded niosomal formulations (after extrusion), with different compositions (graphs produced with GraphPad Prism, remaining images derived from BioRender); (B) prediction profile of the impact of different independent variables, namely nonionic surfactant type and lipid ratio (cetyl alcohol + cholesterol at 1:1, 1:2, or 2:1), on niosomal vehicles' particle size, PDI and EE% (obtained with JMP); (C) *in vitro* release profiles of a propolis solution and the developed optimized propolis-loaded niosomes: P1 (Tween 20 and cetyl alcohol), P4 (Tween 20 and cetyl alcohol + cholesterol 1:2), and P6 (Kolliphor RH 40 and cetyl alcohol + cholesterol 2:1).

Table 7. Particle Size, PDI, and Entrapment Efficiency of the Six Optimized Propolis-Loaded Niosomal Formulations (after Extrusion) along with Their Respective Composition and Attributed Code

niosomal formulation code	niosomal formulation composition	particle size (nm)	PDI	EE (%)
P1	Tween 20 + cetyl alcohol + propolis	170.6	0.172	78.8
P2	Tween 20 + (cetyl alcohol + cholesterol 1:1) + propolis	145.4	0.215	79.4
P3	Tween 20 + (cetyl alcohol + cholesterol 2:1) + propolis	168.4	0.181	84.4
P4	Tween 20 + (cetyl alcohol + cholesterol 1:2) + propolis	133.7	0.153	85.3
P5	Kolliphor RH 40 + (cetyl alcohol + cholesterol 1:1) + propolis	132.8	0.201	86.2
P6	Kolliphor RH 40 + (cetyl alcohol + cholesterol 2:1) + propolis	132.9	0.156	87.4

To better comprehend the tendency of the obtained EE% data, another JMP analysis was performed using a standard least-squares fit model for effect screening, where only first-order effects were considered. Based on Figures 9B and S5−S7, formulations containing Kolliphor RH 40 were found to possess higher EE% values than the ones containing Tween 20 as the selected surfactant. This could be related to the length of the alkyl chain of the nonionic surfactants, since it has been reported to influence the permeability of the niosomal membrane. The longer the alkyl chain, the lower the membrane permeability and the higher the entrapment. Kolliphor RH 40 has a longer alkyl chain than Tween 20, hence the increased EE%.[71,78] The addition of cholesterol has also been reported to tend to decrease the permeability of the niosomes, which can enlarge their hydrodynamic diameter, and consequently increase the EE %, which was observed in the formulations with cholesterol

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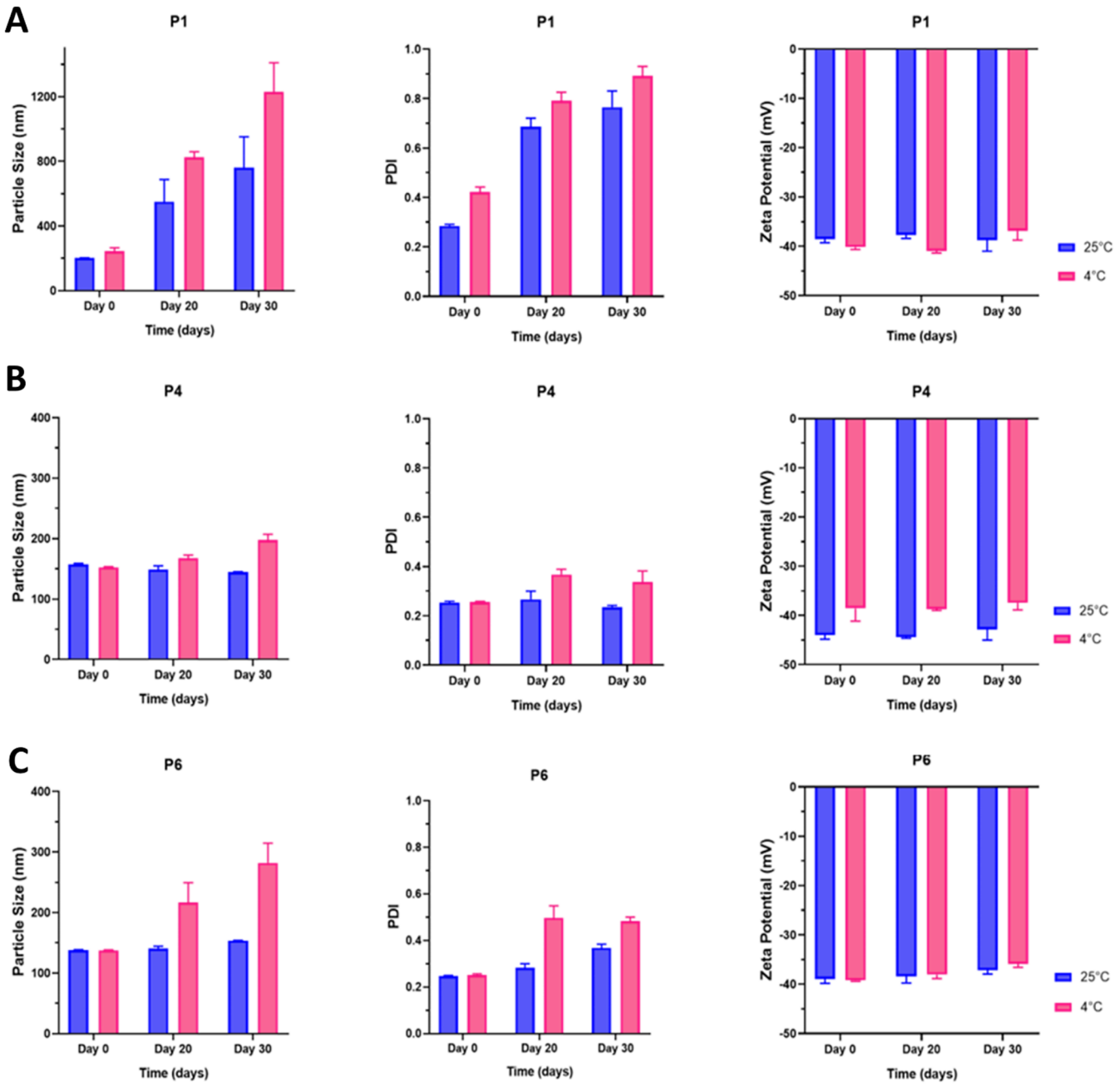

Figure 10. Physical stability of the optimized propolis-loaded niosomes, stored at 4 °C (pink bars) or 25 °C (blue bars), showing the evolution of mean particle size, PDI, and ζ-potential values of formulations P1 (A), P4 (B), and P6 (C), for a time frame of 1 month.

compared with P1, the only formulation that lacks cholesterol.[67,80]

Since all EE% values were so similar between the different niosomal compositions, three formulations were selected to undergo further characterization studies, namely, evaluating their *in vitro* release. Hence, for comparison purposes, three formulations with different compositions were selected: P1, due to being the only formulation containing just one lipid, cetyl alcohol; and P4 (containing Tween 20) and P6 (containing Kolliphor 40), as these two were the formulations that presented higher EE% and smaller particle size and PDI values. Thus, the *in vitro* release of the selected propolis-loaded niosomes was evaluated, using Franz diffusion cells, at 35 °C, a temperature chosen to simulate with the utmost accuracy the mean surface temperature of the human skin.[81] A propolis solution in Transcutol P was used as a positive control, for comparison with the nanometric formulations' performance. Transcutol P was selected due to being a good cosolvent and solubilizer for hydrophobic molecules, also being linked to the improvement of skin penetration in topical formulations.[82] To quantify the amount of propolis released from each formulation, a calibration curve was prepared in PBS, with propolis concentrations ranging from 0.05 to 0.0005 mg/mL, and with an R^2 of 0.9806. The samples were analyzed at a wavelength of 285 nm, and the results are shown in Figure 9C.

The cumulative release percentage for the propolis solution was around 90%, which is expected for a free compound solution. After 24 h, niosome formulations P1, P4, and P6 had cumulative releases of 12.04, 37.61, and 31.67%, respectively. This represents a controlled and sustained release profile, which

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can be beneficial in topical administration, leading to lower toxicity, ensuring an efficient concentration of the active compounds at the application site, and decreasing the need for frequent application, thus ensuring higher compliance.[64,83,84] Additionally, our results are in accordance with previous studies, since in another study by Arafa et al.,[85] involving Span 60 and cholesterol-based niosomes loaded with propolis, the researchers also observed a controlled release behavior, with the release of the encapsulated compound happening either through diffusion or erosion mechanisms.

Moreover, the higher amount of cetyl alcohol in formulations P6 and, especially, P1, is believed to delay the permeation of drugs through the assay membrane, due to being a polar compound.[86] Nevertheless, the incorporation of cetyl alcohol into niosomal formulations instead of cholesterol has proven to be beneficial since it can control the release rate and reduce drug leakage. The chain length of the used fatty alcohols is thought to be related to the release rate of these nanosystems, and since cetyl alcohol has a long chain, it can cause a decrease in the niosomes' release rate.[87] On the other hand, cholesterol tends to lead to an increase in the niosomes' membrane rigidity, allowing for a faster and overall higher release, as observed in P4 and P6, in comparison with P1, without any cholesterol in its composition.[80,88]

Optimized Propolis-Loaded Niosomal Formulations' Physical Stability Assessment. To evaluate the physical stability of the optimized propolis-loaded niosomes, particle size, PDI, and ζ-potential of P1, P4, and P6 were measured for a time period of 1 month when stored at 4 °C (under refrigeration) or 25 °C (room temperature). ζ-potential, a measurement directly related to a particle's surface charge, has a relevant impact on niosomes' behavior and stability. Charged particles in suspension tend to mutually repel each other, being able to avoid or delay the aggregation phenomenon. Therefore, ζ-potential values should preferably be lower than −30 mV or higher than 30 mV, for adequate nanometric vesicle stabilization, achieved through electrostatic repulsion.[50,87,89] Coincidentally, in our study all optimized niosomal formulations had a ζ-potential lower than −30 mV, ranging from −35.87 to −44.43 mV (Figure 10), absolute values that can thus be considered high enough to allow for stabilization to occur due to electrostatic repulsion.

Out of all 3 studied formulations, formulation P1 was the one that was revealed to be the least stable, since after the 30-day time period the niosomes' particle size surpassed 200 nm and the corresponding PDI values exceeded 0.3 considerably, while the mean ζ-potential remained the same, being around −38 mV for both tested temperatures (Figure 10A). Formulation P6 was more stable, since it only had a significant increase in the particle size and PDI values at 4 °C, but it remained stable at room temperature (Figure 10C). On the other hand, after 30 days, formulation P4 had a particle size below 200 nm and a PDI around 0.3 at both temperatures, making it the most stable out of the tested compositions (Figure 10B).

Previously published studies on propolis-loaded niosomes also revealed negative ζ-potential values, ranging from −40.9 to −44.3 mV, which is in accordance with our results. It is believed that these negative ζ-potential values might be due to the absorption of hydroxyl ions on the niosomal surface.[72,90,91] Additionally, while cetyl alcohol is present in all three formulations, cholesterol is present in only formulations P4 and P6. As mentioned, the addition of cholesterol to niosomal composition has an influence on the rigidity of the vesicle's membrane, leading to increased bilayer stability.[61,92] In our study, results confirmed that the presence of cholesterol in P4 and P6 significantly enhanced their stability compared to P1. Furthermore, in what concerns the selected nonionic surfactant, the use of Tween 20 appeared to lead to more stable vesicles when compared to the use of Kolliphor RH 40.

***In Vitro* Safety and Bioactivity Assessment.** Selected propolis-loaded formulations were tested in *in vitro* cell assays to assess their safety and bioactivity. Formulations P1, P4, and P6 were thus chosen, and the respective vehicles (no encapsulated compound) were evaluated as well, for comparison purposes, being coded from now on as V1, V4, and V6. A summary of the tested nanocarrier formulations is presented in Table 8.

Table 8. Composition of the Selected Niosomal Formulations Tested in *In Vitro* Cell Assays and Respective Formulation Code

	formulation code	
niosomal formulation composition	propolis-loaded niosomes	niosomal vehicle (no propolis)
Tween 20 and cetyl alcohol	P1	V1
Tween 20 and cetyl alcohol + cholesterol 1:2	P4	V4
Kolliphor RH 40 and cetyl alcohol + cholesterol 2:1	P6	V6

***In Vitro* Safety Assessment on Healthy Skin Cells.** To evaluate the safety and potential toxicity of the developed optimized niosomal formulations, a colorimetric SRB assay was conducted in two human cell lines, HaCaT (keratinocytes) and HFF-1 (fibroblasts). The cell viability of HaCaT cells after incubation with 25 μg/mL of the developed propolis-loaded niosomes was equal to 87.8, 93.3, and 97.6% for P6, P1, and P4, respectively, as can be seen in Figure 11(A−G). Cell viability decreased with concentrations higher than 25 μg/mL, making this concentration the safest. Additionally, the empty niosomal formulations did not lead to an overall higher cell viability when compared to the propolis-loaded niosomes. Additionally, except for P4, all formulations (loaded with propolis or not) showed a decrease in cell viability lower than 50% at concentrations above 25 μg/mL, making P4 the safest at higher concentrations (above 25 μg/mL). Similarly, the cell viability of HFF-1 cells following exposure to 25 μg/mL of the optimized nanocarriers was highest for formulation P4, exhibiting a cell viability of 94.5%. In contrast, for formulations P1 and P6, the safest concentration was 12.5 μg/mL, with respective viability values of 78.2 and 85.4%, as can be seen in Figure 11(H−N). Thus, again, P4 appeared to be the safest out of the tested niosomal formulations and therefore the most promising one in terms of *in vitro* cytotoxicity, having Tween 20, cetyl alcohol, and cholesterol in its composition. Furthermore, of all the empty niosomal formulations, V4 also appeared to be the safest formulation vehicle.

The IC_{50} values of the propolis-loaded and empty niosomal formulations in HaCaT and in HFF-1 cell lines are presented in Table 9. The propolis solution, used as control, showed an IC_{50} greater than 100 μg/mL in both cell lines, meaning that, at all tested concentrations, pure propolis did not have a significant impact on cell viability, proving to be noncytotoxic. These results are in accordance with other previous studies, which also proved the noncytotoxicity of propolis in concentrations inferior to 125 μg/mL, in HFF-1 and HaCaT cell lines.[93,94] Additionally,

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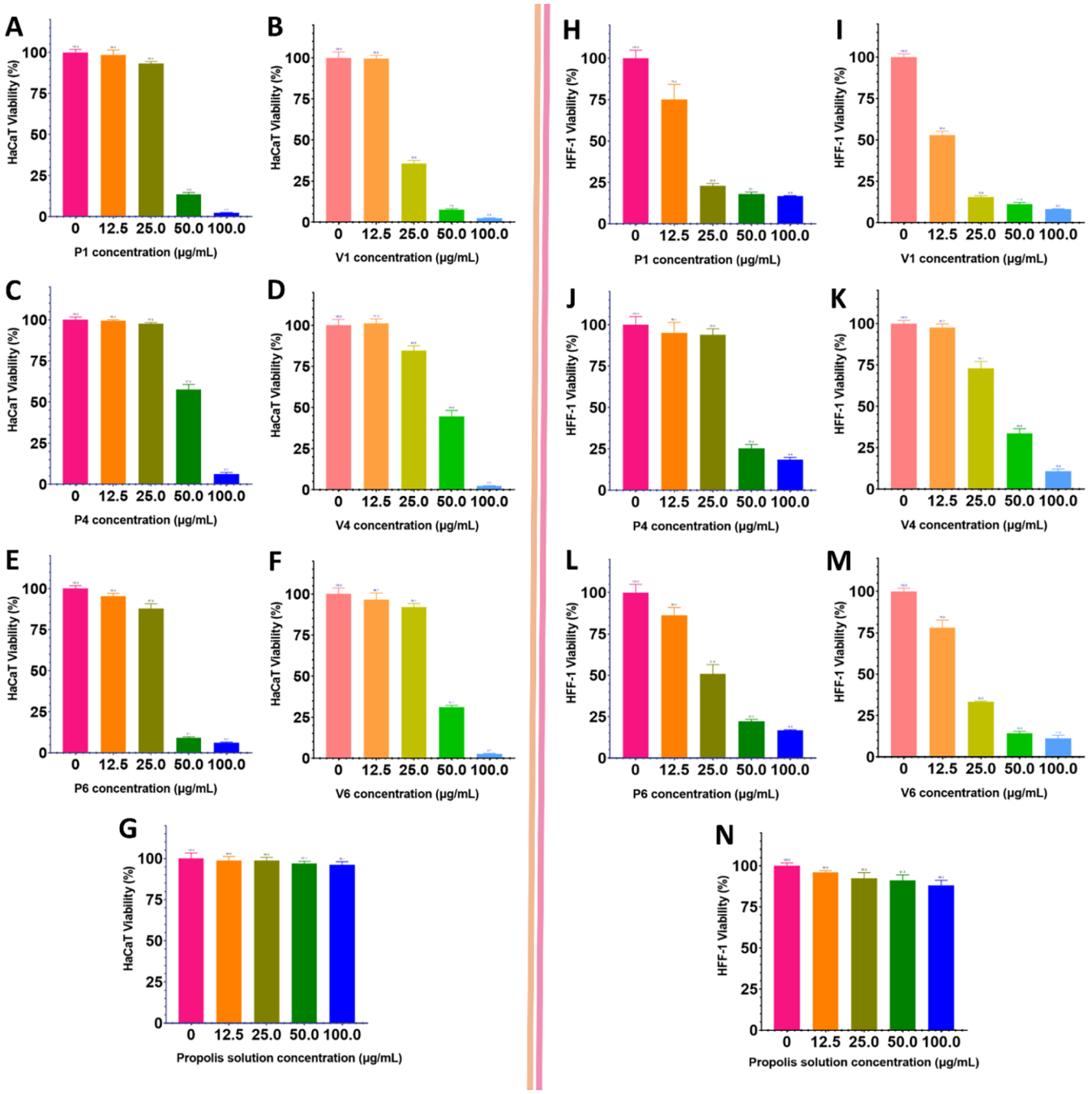

Figure 11. *In vitro* cell viability results on HaCaT cells (keratinocytes) of the optimized propolis-loaded niosomes (A–E), niosomal vehicles (B–F), and propolis solution (G), and on HFF-1 cells (fibroblasts) of the optimized propolis-loaded niosomes (H, J, and L), niosomal vehicles (I, K, and M), and propolis solution (N).

overall IC_{50} values of both propolis-loaded and empty niosomes tended to be higher in the HaCaT cell line than in the HFF-1 cell line. This might be attributed to the difference in the cell's sensitivity to the tested formulations, and thus keratinocytes, cells predominantly present in the epidermis, had a lower sensitivity than fibroblasts, the major skin cells present in the dermis, suggesting that the developed formulations might be safer when retained on more superficial layers of the skin.[95]

Since both propolis-loaded and empty niosomal formulations showed some level of cytotoxicity against the two studied cell lines, at concentrations above 12.5 or 25 μg/mL (depending on formulation composition), and the propolis solution did not show any, the safety of these formulations can be related to their excipients, namely, the used surfactants. While niosomes are generally considered biocompatible, the amount of surfactants used in their composition could be the cause of the observed cytotoxicity, since high surfactant concentrations have been proven to be cytotoxic, including in skin cells, with high concentrations of Tween 20 having demonstrated to be harmful to HaCaT cells and other cell lines, especially after long periods of exposure.[96−99] Nevertheless, since propolis was revealed to be nontoxic in the tested concentrations, and up to 12.5 or 25 μg/mL all formulations can be considered noncytotoxic, at these specific concentrations they are safe for use, with formulation

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Table 9. IC_{50} Values Obtained from *In Vitro* Cell Viability Studies with the Developed Optimized Empty and Propolis-Loaded Niosomes Evaluated in HaCaT and HFF-1 Cells[a]

	cell viability (IC_{50}, μg/mL)	
formulations	HaCaT cells	HFF-1 cells
V1	22.77 ± 0.99	12.75 ± 0.99
V4	45.43 ± 0.99	38.39 ± 0.99
V6	42.12 ± 1.00	20.18 ± 0.98
P1	37.52 ± 1.00	18.20 ± 0.94
P4	53.66 ± 1.00	40.77 ± 0.95
P6	34.47 ± 0.99	27.64 ± 0.97
propolis solution	<100	<100

[a]IC_{50}—formulation concentrations causing a 50% reduction of cell viability or proliferation; data is represented as mean ± standard deviation of tested formulations.

P4, containing Tween 20, cetyl alcohol, and cholesterol, showing the safest profile, being able to be administered at the highest concentration. Additionally, while *in vitro* cytotoxicity assays are relevant and necessary to reduce animal use during the formulation development process (and with animal use being generally prohibited in the case of cosmetics), they come with certain limitations, such as the difficulty in replicating the real conditions of the body and its complex mechanisms.[95,100,101] Hence, in order to further assess the safety of the developed formulations, a HET-CAM test was performed, and the results are shown in In Vitro Bioactivity Assessment on Inflammatory Cells section.

***In Vitro* Bioactivity Assessment on Inflammatory Cells.** The anti-inflammatory properties of both free propolis (solution) and encapsulated propolis (propolis-loaded niosomes) were evaluated in mouse macrophage cell line RAW 264.7. The formulation vehicles were evaluated as well for comparison purposes. Obtained IC_{50} values are shown in Table 10. In these assays, IC_{50} is the concentration of the tested compound, in this case, free propolis or niosomes, required to inhibit 50% of NO production, demonstrating its anti-inflammatory activity. NO is categorized among the labile radical entities known as reactive oxygen species (ROS), and it is synthesized by an enzyme, named nitric oxide synthase (NOS). The lower the IC_{50} value is, the more potent the anti-inflammatory activity of the tested formulation will be, as a lower concentration is required to achieve the desired anti-inflammatory effect.[102−104]

Table 10. IC_{50} Values Obtained from *In Vitro* Anti-Inflammatory Studies, with the Developed Optimized Empty and Propolis-Loaded Niosomes Being Evaluated in RAW 264.7 Cells[a]

formulations	anti-inflammatory activity (IC_{50}, ng/mL)
V1	37.78 ± 1.27
V4	>50
V6	>50
P1	14.90 ± 1.03
P4	15.19 ± 0.71
P6	17.89 ± 0.68
propolis solution	5.95 ± 0.23

[a]IC_{50}—formulation concentrations providing a 50% inhibition of NO production; data is represented as mean ± standard deviation of tested formulations.

The propolis solution presented an IC_{50} of 5.95 ng/mL, which indicates that it has a relevant anti-inflammatory potential, being in accordance with previous studies, which have demonstrated propolis' ability to reduce the expression of cytokines and chemokines induced by LPS in macrophages during inflammation. Compounds such as caffeic acid, a phenolic acid found in the composition of propolis, have proven to be directly linked to the inhibition of NO production during inflammatory processes, providing propolis with its substantial anti-inflammatory potential.[105−107] Moreover, the quantified high amount of flavonols, such as quercetin and galangin, have been linked to a strong anti-inflammatory effect.[49] Quercetin has proved to be a beneficial bioactive substance for the treatment of atopic dermatitis-related symptoms, through anti-inflammatory and antioxidant effects, with additional wound healing properties via ERK1/2 MAPK and NF-kB pathways.[108] Moreover, as expected, the empty niosomes, V1, V4, and V6, did not show any significant anti-inflammatory effects, with IC_{50} values above 37.78 ng/mL. These results were not surprising since the formulation excipients were not expected to have intrinsic bioactivity, with the empty niosomal formulations being mostly used as controls to compare the anti-inflammatory potential of the propolis-loaded niosomes. As for the propolis-loaded niosomes, P1, P4, and P6 showed substantial anti-inflammatory potential, with IC_{50} values of 14.90 15.19, and 17.89 ng/mL, respectively.

Since niosomes without propolis showed no anti-inflammatory activity, these results mean that the anti-inflammatory potential of the propolis-loaded niosomes was due to the presence of propolis and did not originate from the nanocarrier itself. Additionally, the less potent anti-inflammatory activity of the propolis-loaded niosomes when compared to the propolis solution was likely due to the controlled and sustained release of propolis from within the nanovesicles, as supported by the formulations' *in vitro* release profiles (Effects of Propolis Encapsulation, Entrapment Efficiency, and In Vitro Release Studies section). Nevertheless, although having higher IC_{50} values, the niosomal formulations could prolong the bioactivity of propolis at the administration site, with the slower and steady release of propolis from the vesicles leading to longer and more consistent effects. Additionally, the encapsulation of propolis within the niosomal formulations allows its protection from enzymatic or chemical degradation, thus being more readily available to exert its anti-inflammatory activity. Therefore, the encapsulation of propolis into the niosomes did not actually impair its anti-inflammatory potential, but rather prolonged it, while protecting the compound from degradation, a positive outcome that has also been demonstrated by previous studies regarding different nanosystem types loaded with diverse drugs or bioactive molecules.[109,110]

As for the comparison between the different niosomal compositions, the niosomes with the highest anti-inflammatory potential were from formulation P1, with an IC_{50} value of 14.90 ng/mL. However, the value obtained for P4 was similar, 15.19 ± 0.71 ng/mL. Propolis-loaded niosomal formulation P6 had a higher IC_{50} value of 17.89 ± 0.68 ng/mL.

Safety Assessment Using the HET-CAM Test. In addition to the formulations' safety assessment in *in vitro* cytotoxicity assays on healthy skin cells, the irritative potential of both propolis-loaded and empty niosomes was evaluated in a HET-CAM test. This test offers insights into the potential of the tested formulations to cause eye irritation and their impact on the ocular membrane, relying on the similarity between the

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vascularized mucosal tissues of rabbit or human eyes and the CAM of fertilized hen's eggs. Furthermore, it is assumed that if a given formulation is nonirritant to the eye, it will also most likely be safe to administer on the skin.[111,112] After the application of 0.3 mL of each formulation onto the CAM of the fertilized hen's eggs (no prior dilution), the occurrence of adverse effects was observed, especially in what concerned the membranes' blood vessels, during the first 5 min. The results are shown in Table 11 and Figure 12.

Table 11. Results of the HET-CAM Test, Performed with the Developed Optimized Propolis-Loaded Niosomes (P1, P4, and P6) and Respective Formulation Vehicles (V1, V4, and V6)[a]

formulation	average IS	SD	observed vascular reactions	irritation degree
P1	8.7	0.47	lysis	moderately irritative
P4	9.3	0.47	lysis	moderately irritative
P6	4.7	1.20	no lysis	slightly irritative
V1	8.0	0.82	no lysis	slightly irritative
V4	13.7	0.47	hemorrhage	strongly irritative
V6	7.7	0.94	no lysis	slightly irritative
0.9% NaCl	0.0		none	nonirritant
0.1 N NaOH	19.0		hemorrhage	strongly irritative

[a]IS—irritation score; SD—standard deviation.

No irritability was observed when a 0.9% NaCl solution (negative control) was applied, and strong irritability was observed when a 0.1 N NaOH solution (positive control) was applied. Formulations P1 and P4, with IS values of 8.7 and 9.3, respectively, caused some irritation to the vascular membrane, including modest lysis (Figure 12A,B, white arrows). On the other hand, formulation P6, with an average IS of 4.7, only led to a slight irritative response, lower than the response observed with P1 and P4, with no lysis being detected (Figure 12C). Vehicle niosomal formulations V1 and V6 also did not show any vascular reactions, leading to only a slight irritative response as well (Figure 12D,F). However, vehicle niosomal formulation V4 had the highest IS value of 13.7, causing the most severe vascular reaction, including hemorrhage, which is noticeable since blood vessels cannot be observed in the egg's membrane, and instead a dark red stain can be seen (Figure 12E, pink arrow). Nevertheless, except for V4, no hemorrhage was detected in the other CAM, in which clear blood vessels could be observed. Still, in both P1 and P4, fewer blood vessels can be observed, indicating the occurrence of lysis, which can be confirmed by the disappearance or whitening of the vessels and no blood circulation.[113] Yet, it is relevant to point out that P4 and P6 irritation scores decreased compared to those of the corresponding vehicles, V4 and V6. This might suggest that the anti-inflammatory potential of propolis could have a beneficial effect in lowering the formulations' irritation potential. In this study, formulation P6 and the corresponding vehicle, V6, proved to be the safest.

However, while the HET-CAM test is a safety assay necessary to assess the irritative potential of the ingredients used in cosmetic formulations, again, results should be treated carefully, since these assays come with limitations in what concerns translation to the human application context, also being dependent on factors such as time of contact of the tissue with the formulation, applied volume, applied pressure, skin conditions, etc.[47,114,115] Future tests in human volunteers could be the key to truly assessing the safety of the developed formulations. Additionally, the developed niosomes could also be incorporated into a semisolid base, such as a gel matrix or a cream, which could not only ensure suitable topical spreadability for therapeutic or cosmetic application but also reduce the potential irritability of some of the niosomal components.

Final Remarks. Niosomes are a relatively new nanotechnology approach developed to overcome some of the issues related to their predecessors, liposomes, such as instability and higher cost of production. They are lipidic vesicular nanosystems that have had an increased interest due to their relevant potential for skin delivery of drugs or other bioactive molecules, by providing them with a sustained and controlled release and allowing to encapsulate both hydrophilic and lipophilic compounds, making them versatile and advantageous platforms for both pharmaceutical and cosmetic development.

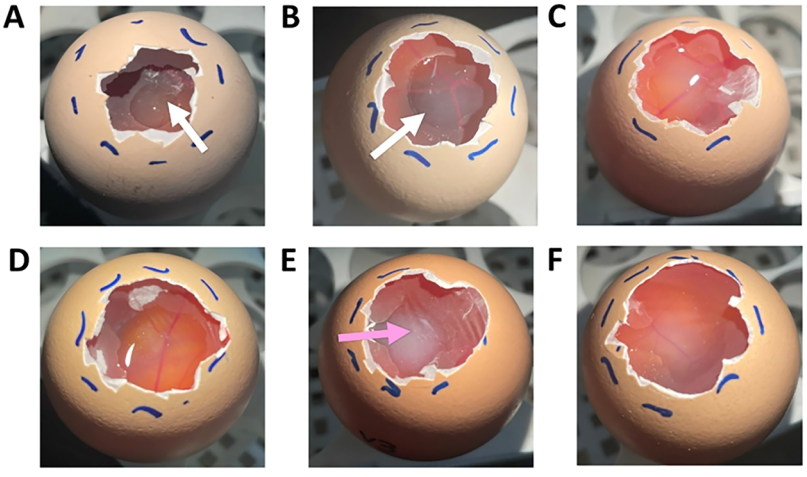

Figure 12. Photographs of the HET-CAM test results, performed with the developed optimized propolis-loaded niosomes (P1, P4 and P6, corresponding to A, B, and C, respectively) and respective formulation vehicles (V1, V4 and V6, corresponding to D, E, and F, respectively); white arrows represent lysis, and pink arrow represents hemorrhage.

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In this work, a hydrophobic compound with various relevant bioactivities, propolis, was successfully encapsulated inside the lipidic bilayer of niosomal vesicles produced using the thin-film hydration method. Besides focusing on formulating niosomes using different combinations of nonionic surfactants, cetyl alcohol, and cholesterol, in order to optimize the particle size and PDI of the obtained nanosystems to meet the desired criteria for topical application, extrusion was used to reduce the niosomes' particle size and improve particle size distribution homogeneity. The number of extrusion cycles was a crucial factor in achieving the desired particle size (below 200 nm) and PDI (below 0.2). Due to their high HLB values, nonionic surfactants Tween 20 and Kolliphor RH 40 were used, and the particle size and homogeneity of the resulting niosomal formulations were considerably affected by both surfactant and lipid ratio choice.

Six optimized niosomal vehicle formulations were selected, namely, having in their composition Tween 20 and cetyl alcohol, Tween 20 and cetyl alcohol plus cholesterol at a 1:1 ratio, Tween 20 and cetyl alcohol plus cholesterol at a 2:1 ratio, Tween 20 and cetyl alcohol plus cholesterol at a 1:2 ratio, Kolliphor RH 40 and cetyl alcohol plus cholesterol at a 1:1 ratio, or Kolliphor RH 40 and cetyl alcohol plus cholesterol at a 2:1 ratio. For the encapsulation of the bioactive compound, 1 mg/mL of propolis extract was added to the niosomes, and high EE% values were achieved, ranging from 78.8 to 87.4%, suggesting that the selected formulations were quite successful at encapsulating the intended compound. Additionally, the EE% appeared to be influenced by the length of the alkyl chain of the used nonionic surfactant, since niosomal formulations containing Kolliphor RH 40, which has a longer alkyl chain than Tween 20, showed greater EE%. Cholesterol also appeared to lead to the improvement of the amount of encapsulated propolis, likely due to a decrease in the niosomal membrane's permeability, which in turn could have led to the enlargement of the hydrodynamic diameter of the vesicles.

Propolis-loaded niosomes' *in vitro* release profiles showed a controlled and sustained release, with the formulation containing Tween 20 and cetyl alcohol plus cholesterol at a 1:2 ratio leading to the best cumulative release value after the 24-h test time period. The stability of the developed niosomal vesicles was also assessed after a 1-month storage period at 4 or 25 °C. Results showed that the inclusion of cholesterol in the niosomes' composition had a significant impact on the stability of the nanovesicles. The formulation composed of Tween 20 and cetyl alcohol plus cholesterol at a 1:2 ratio exhibited the best stability over one month, leading to irrelevant changes in particle size, PDI, and ζ-potential values when stored at both temperatures. Additionally, the mean ζ-potential values were around − 38 mV, a substantially high absolute value, meaning that the developed vesicles' stability could at least partially result from electrostatic repulsion.

The evaluation of the developed formulations in *in vitro* cell assays revealed that while propolis in solution did not have relevant cytotoxicity, thus making the compound itself safe at any of the tested concentrations, when encapsulated into the niosomes, these concentrations were adjusted, with niosomal formulations up to 12.5 or 25 μg/mL being safe in HaCaT and HFF-1 cell lines (keratinocytes and fibroblasts, respectively). Again, the formulation containing Tween 20 and cetyl alcohol plus cholesterol at a 1:2 ratio had the best performance. Furthermore, propolis' anti-inflammatory potential was maintained once encapsulated, although having a lower IC_{50} value when compared to the nonencapsulated compound. This occurred due to the controlled and sustained release of propolis that the encapsulation into the nanosystem provides, leading to additional advantages such as prolonged bioactivity at the application site as well as protection from enzymatic or chemical degradation, hence making propolis more readily available to exert its anti-inflammatory effects. In this assay, once more, the formulation containing Tween 20 and cetyl alcohol plus cholesterol at a 1:2 ratio led to one of the best results. An additional safety assessment using the HET-CAM test showed that the nanovesicles had different degrees of irritative potential, with some formulations depicting only a slightly irritative degree.

Hence, overall, while several of the developed formulations had adequate physicochemical characteristics, safety, and bioactivity, the formulation containing Tween 20 and cetyl alcohol plus cholesterol in a 1:2 ratio led to the best results, having optimal stability, drug release, *in vitro* safety, and anti-inflammatory potential. Furthermore, although not assessed in this specific study, the developed optimized vesicles could serve for dual loading by simultaneously encapsulating propolis in the niosomes' bilayer and a hydrophilic compound into the vesicle's core, thereby allowing the synchronized delivery of two drugs or other bioactive compounds. The developed nanoplatform could also be incorporated into a cream or gel matrix to ensure suitable topical spreadability, for therapeutic or cosmetic purposes. Additionally, for dermopharmaceutical applications, *in vivo* assays could be performed, in order to assess the potential of the developed nanometric formulations in a more complex biological system. Alternatively, if intended for cosmetic use only, *in vivo* assays would not be possible to perform, due to the regulatory framework in which this type of products is inserted, but instead tests using human volunteers could be executed, such as the patch test for assessing the potential of the formulations for leading to skin sensitivity reactions or a sensorial analysis in order to assess the potential acceptability of the formulations as a topical product. The possible translation to a larger scale, namely in an industrial production context, would also be interesting to evaluate, in order to determine whether the niosomal formulation's characteristics would remain ideal after the scale-up process.

CONCLUSIONS

Propolis-loaded niosomes containing Tween 20, Kolliphor RH 40, cetyl alcohol, and/or cholesterol were successfully developed using the thin-film hydration method, followed by extrusion. The vesicles exhibited a small particle size (between 100 and 200 nm), adequate for topical application, with homogeneous distribution (PDI below 0.2), and relevant ζ-potential values (around −38 mV), thus being at least partially stabilized by electrostatic repulsion, which led to good stability both under refrigeration and at room temperature. The vesicles also allowed a high EE% (ranging from 78.8 to 87.4%), which indicates a successful encapsulation of the compound, and led to a controlled release profile relevant to ensure prolonged bioactivity at the application site. The developed nanoplatforms also depicted adequate concentration-dependent *in vitro* safety in keratinocytes and fibroblasts, with some formulations also showing a low irritative potential in the HET-CAM test, and relevant *in vitro* anti-inflammatory potential through the successful inhibition of nitric oxide production. These novel nanometric structures containing a nature-derived hydrophobic compound with various relevant bioactivities could serve as

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versatile and advantageous formulations in both pharmaceutical and cosmetic contexts of application.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.5c00253.

LC/DAD/ESI-MSn parameters and obtained concentrations (mg/g extract) of the phenolic compounds found in the harvested propolis sample (Table S1); an initial DoE analysis, assessing the impact of each nonionic surfactant, either in the presence of one single lipid (cetyl alcohol) or a lipid mixture (cetyl alcohol plus cholesterol, no specific ratios), as well as the impact of the hydration temperature and the number of extrusion cycles on the final biophysical properties of the developed niosomes (particle size and PDI) (Table S2); an initial DoE analysis, assessing the impact of each nonionic surfactant, either in the presence of one single lipid (cetyl alcohol) or a lipid mixture (cetyl alcohol plus cholesterol, no specific ratios), as well as the impact of the hydration temperature and the number of extrusion cycles on the particle size of the developed niosomes (Figure S1); an initial DoE analysis, assessing the impact of each nonionic surfactant, either in the presence of one single lipid (cetyl alcohol) or a lipid mixture (cetyl alcohol plus cholesterol, no specific ratios), as well as the impact of the hydration temperature and the number of extrusion cycles on the PDI of the developed niosomes (Figure S2); the influence of niosome composition and process parameters on particle size and PDI values (Table S3); the optimization DoE analysis, assessing the impact of each nonionic surfactant, lipid composition and lipid ratio, hydration temperature and number of extrusion cycles on the developed niosomes' particle size (Figure S3); the optimization DoE analysis, assessing the impact of each nonionic surfactant, lipid composition and lipid ratio, hydration temperature and number of extrusion cycles on the developed niosomes' PDI (Figure S4); propolis-loaded niosomes DoE analysis, assessing the impact of each nonionic surfactant, lipid composition and lipid ratio, and drug loading on the developed niosomes' particle size (Figure S5); propolis-loaded niosomes DoE analysis, assessing the impact of each nonionic surfactant, lipid composition and lipid ratio, and drug loading on the developed niosomes' PDI (Figure S6); propolis-loaded niosomes DoE analysis, assessing the impact of each nonionic surfactant, lipid composition and lipid ratio, and drug loading on the developed niosomes' entrapment efficiency (Figure S7) (PDF)

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Notes

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ABBREVIATIONS

CIMO- Mountain Research Center of the Polytechnic Institute of Bragança
DLS- dynamic light scattering
DMEM- Dulbecco's Modified Eagle Medium
DMSO- dimethyl sulfoxide
DoE- design of experiments
DSMZ- German Collection of Microorganisms and Cell Cultures
ECCAC- European Collection of Authenticated Cell Cultures
EE%- encapsulation efficiency
FBS- fetal bovine serum
GCCP- Guidance on Good Cell Culture Practice
HET-CAM- Hen's Egg Test-Chorioallantoic Membrane
HLB- hydrophilic−lipophilic balance
IC_{50}- concentration of each tested formulation that caused a 50% inhibition of cell growth (*in vitro* cytotoxicity assays), or a 50% inhibition of nitric oxide production (*in vitro* anti-inflammatory assays)
ICCVAM- Interagency Coordinating Committee on the Validation of Alternative Methods
IS- irritation score
LC/DAD/ESI-MSn- liquid chromatography with diode array detection coupled with electrospray ionization tandem mass spectrometry
LPS- liposaccharide
NaCl- sodium chloride
NaOH- sodium hydroxide
NLC- nanostructured lipid carriers
NO- nitric oxide
NOS- nitric oxide synthase
PBS- phosphate buffered saline
PDI- polydispersity index
QbD- quality by design
R^2- coefficient of determination
ROS- reactive oxygen species
SC- *stratum corneum*
SD- standard deviation
SLN- solid lipid nanoparticles
SRB- sulforhodamine B
TCA- trichloroacetic acid
Tris- tris (hydroxymethyl)aminomethane
UV- ultraviolet

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