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Lycopene from Tomatoes: Vesicular Nanocarrier Formulations for Dermal Delivery

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Supporting Information

ABSTRACT: This experimental work aimed to develop a simple, fast, economic, and environmentally friendly process for the extraction of lycopene from tomato and incorporate this lycopene-rich extract into ultradeformable vesicular nanocarriers suitable for topical application. Lycopene extraction was conducted without a cosolvent for 30 min. The extracts were analyzed and incorporated in transfersomes and ethosomes. These formulations were characterized, and the cellular uptake was observed by confocal microscopy. Dermal delivery of lycopene formulations was tested under *in vitro* and *in vivo* conditions. Lycopene extraction proved to be quite safe and selective. The vesicular formulation was taken up by the cells, being more concentrated around the nucleus. Epicutaneous application of lycopene formulations decreased the level of anthralin-induced ear swelling by 97 and 87%, in a manner nonstatistically different from the positive control. These results support the idea that the lycopene-rich extract may be a good alternative to the expensive commercial lycopene for incorporation into advanced topical delivery systems.

KEYWORDS: *lycopene, extraction method, transfersomes, ethosomes, cytotoxicity, cellular uptake, anthralin-induced dermatitis*

INTRODUCTION

Lycopene is an open-chain unsaturated carotenoid with powerful antioxidant properties superior to those of β-carotene.[1] It is responsible for the red color in several commonly consumed fruits and vegetables, representing more than 80% of total tomato carotenoids.[2] During food processing, all-*trans*-lycopene may be converted into its *cis* configuration, which may modify its biological activity. Lycopene has also been studied for other physiological activities, such as suppression of proliferation of human cancer cells.[3] Because of the beneficial health properties of lycopene, several attempts have been made to isolate lycopene from tomatoes or tomato derivatives and formulate it in suitable dosage forms for human use.[4,5] However, lycopene is not commercially available at high purity grade, and when it exists, the high cost is not compatible with medium- or large-scale pharmaceutical manufacturing processes. On the other hand, lycopene is much more sensitive to light, air, and heat in its pure form. The extraction of lycopene from tomatoes could thus be a good alternative.[6] In fact, because of the potential therapeutic roles of lycopene, extraction of lycopene from tomato and tomato-derived products has received significant attention in recent years.[7] Conventional lycopene extraction methods consume large volumes of organic solvents that are expensive, toxic, and hazardous. AOAC[8] recommends a methanol/tetrahydrofuran mixture [50/50 (v/v)] for extracting carotenoids, while other authors use ethyl acetate (100%) or different mixtures of ethanol and hexane; acetone, ethanol, and hexane; ethyl acetate and hexane; or acetone and hexane.[9,10] Traces of the extractants in the final product make it unsuitable for food, pharmaceutical, and cosmetic uses. For the extraction of carotenoids from the samples, other systems can be used, like liquid−liquid extraction, solid phase extraction, or supercritical fluid extraction (SFE).[11−14] SFE with CO_2 has been proposed because of the weak contamination, nontoxic, and nonflammable characteristics of the technique. SFE of lycopene from a wide variety of raw materials has been reported[15,16] as well as poorly reproducible results due to the degradation and/or isomerization of lycopene during extraction.[7] Besides, SFE demands large investments in the high purity required and the equipment and makes mandatory the presence of a stabilizer and a cosolvent because of its nonpolar character. Every extraction step may result in isomerization and/or degradation. Moreover, not all the analytical methods available for carotenoid analysis in food products are suitable for lycopene-rich foods because of the low solubility of lycopene in some of the solvents employed, as in the case of methanol, and due to the fact that the use of other solvents may interfere with the mobile phases applied for carotenoid separation. There are many high-performance liquid chromatography (HPLC) methods that can be applied for the determination of carotenoids. However, these compounds need very careful and tedious manipulation because of their chemical lability.

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Therefore, the development of new and rapid methodologies of extraction and separation is relevant, and the need for a reliable and rapid analysis method of lycopene in vegetable products has been recognized, with a goal of minimizing the changes in the carotenoid profile of the original sample.[17,18]

According to Ribaya et al.,[19] when applied as a topical medicine in skin care, lycopene is more efficient in preventing oxidative damage than β-carotene. Thus, the delivery of lycopene into the skin may provide beneficial results in terms of photodamage protection. Although topical carotenoids have been previously proposed, their efficient delivery into the skin has not been studied. Lycopene is strongly lipophilic (log P ∼ 15), which makes the penetration of lycopene across the *stratum corneum* (SC, epidermis' outermost layer) into viable skin layers a difficult task because of the affinity of lycopene for SC components and its tendency to be retained in this layer. To improve lycopene deep-skin delivery, few authors reported lycopene formulation strategies.[20−23] Vesicular nanocarriers, such as liposomes, and more deformable ones (UDV), such as transfersomes and ethosomes, could facilitate the deposition of active molecules on the skin and also stabilize it.[24] The versatile deformable lipid carriers have proven to deliver into and across the skin clinically relevant concentrations of low-molecular weight drugs or active molecules, either hydrophilic or hydrophobic, or macromolecular drugs.[25−28]

In this work, we aimed (a) to develop an extraction protocol of lycopene using a simple, rapid, and low-cost procedure, (b) to prepare and fully characterize two extracted lycopene-loaded deformable vesicle (UDV) formulations, (c) to evaluate the cellular uptake of ultradeformable vesicles, using an *in vitro* keratinocyte culture, and (e) to evaluate the *in vivo* antioxidant and anti-inflammatory activity of topically applied lycopene formulations. The combined data will provide valuable information for validating these formulations as good alternatives for antioxidant treatment based on region-specific delivery.

MATERIALS AND METHODS

Materials. Commercial standard lycopene (≥98% pure) was purchased from Extrasynthese (Genay, France). Tomato fruits from *Lycopersicon esculentum* L. (Tomate Pera, Sunstream variety, from organic farming) were purchased from Vitacress (Odemira, Portugal). Soybean phosphatidylcholine (S100) was purchased from Lipoid KG (Ludwigshafen, Germany). Tween 80, anthralin, tetrahydrofuran (THF), butylated hydroxytoluene (BHT), thiazolyl blue tetrazolium bromide (MTT), and sodium succinate were purchased from Sigma-Aldrich (St. Louis, MO). L-α-Phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (rhodamine-PE) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). HPLC-grade solvents were from Fluka (St. Gallen, Switzerland), Sigma-Aldrich, and Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Methods. *Lycopene Extraction.* Tomato fruits (250 g per extraction) were sliced and dried using a freeze-dryer (Edwards, Crawley, U.K.) overnight. Dried tomato was pulverized and extracted at room temperature with ethyl acetate, for 30 min, while being gently shaken and protected from light and air. The solvent/dry tomato ratio was 7/1 (milliliters to grams). The organic solvent containing lycopene was then filtered under vacuum through a 0.2 μm membrane filter and concentrated in vacuum using a rotary evaporator (Buchi, Flawil, Switzerland) at <40 °C. The resulting extract was stored at −20 °C until it was used.

Identification and Quantification of Lycopene in Tomato Extract. Extracted lycopene was identified and compared to a commercial standard using HPLC and mass spectrometry.

Analysis of Lycopene Extract by Reversed Phase (RP-HPLC). The identification of all-*trans*-lycopene in the extract was conducted by comparing the retention time and absorption spectra with those of the reference standard. Five concentrations (93, 186, 466, 931, and 1863 nM) were used to prepare the standard curve of all-*trans*-lycopene. Lycopene was solubilized in hexane. Duplicate analyses were performed, and the mean value was determined. The regression equation and correlation coefficient (r^2) was obtained using Microsoft Excel 2010. The limit of detection (LOD) and limit of quantification (LOQ) were determined according to the method described by the International Conference on Harmonization.[29] Extracted lycopene was quantified according the standard calibration curve and then diluted with hexane to prepare extracted lycopene curves.

The method used was adapted from ref 30. An HPLC system consisting of 32 Karat Software (Beckman Instruments, Fullerton, CA), a Midas Spark 1.1 autoinjector (Spark, Emmen, The Netherlands), and a diode-array 168 detector (Beckman Instruments) was used for all preparations. The injector was fit with a 50 μL injection loop. Chromatographic separations were performed using a Licrospher RP18-5 reversed phase chromatography column (5 μm, 25 cm × 4.0 mm) (Supelco, Bellefonte, PA). The detection wavelength was 472 nm. The mobile phase consisted of methanol and acetonitrile [50/50 (v/v)] at a flow rate of 1.5 mL/min at 25 °C.

Analysis of Lycopene Extract by Mass Spectrometry. Liquid chromatography−electrospray ionization tandem mass spectroscopy (LC−ESI-MS/MS) was performed in an HPLC Alliance 2695 Separation Module, coupled to a diode array detector (PDA 996, Waters, Dublin, Ireland) and a tandem triple quadrupole mass spectrometer (Micromass Quattromicro API, Waters). The separation of lycopene was achieved by reverse phase chromatography on a LiChrospher 100 RP-18 column (5 μm, 100 mm × 4 mm) from Merck operating at 35 °C. The composition of the mobile phase was at the beginning of the analysis 15% eluent A (water and 0.5% formic acid) and 85% eluent B (acetonitrile). After 10 min, the composition changed to 98% eluent B. This composition of the mobile phase was maintained for 30 min. The column was re-equilibrated for 10 min with 85% eluent B. The flow rate of the mobile phase was 0.30 mL/min. The injection volume was 5 μL. The diode array detector operated in the range of 210−600 nm. The ionization of the compounds was performed using an electrospray source operating in positive mode. The ionization conditions were optimized; the source temperature was maintained at 120 °C and the desolvation temperature at 350 °C. A capillary potential of 4.0 kV and a potential source of 30 V were applied. Mass spectra were obtained in "full scan" mode from m/z 250 to 700. MS/MS experiments gave the characteristic fragmentation of the molecular ion, using different collision energies (10−40 eV). Argon was used as the collision gas. For acquisition and data processing, MassLynx version 4.1 was used.

Evaluation of Lycopene Extract Cytotoxicity by the MTT Assay. The cytotoxicity potential of extracted lycopene was evaluated in HaCaT cells, a nontumorigenic immortalized human keratinocyte cell line obtained from Cell Lines Services (Eppelheim, Germany). These cells were grown in Dulbecco's modified Eagle's medium (DMEM), rich in glucose, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin/streptomycin (10000 units/mL), and 1% fungizone (250 units/mL) (Gibco, Grand Island, NY) at 37 °C in a humidified incubator with a 5% CO_2 atmosphere.[31,32] Standard commercial lycopene and extracted lycopene were dissolved in sterilized THF containing BHT. The THF/BHT/lycopene mixture was diluted with FBS at a 1/9 ratio and added to the culture medium over 8 h at final concentrations of 10 μM lycopene, 0.1% THF, 0.001% BHT, and 2% FBS. The cell viability 24 h after lycopene exposure was assessed by the MTT assay[33] in six-well plates. Cells were incubated with 500 μg of MTT and 0.27 mg of sodium succinate and allowed to react for 2 h at 37 °C. The medium was removed, and 1 mL of the crystal solubilization reagent [0.57% acetic acid and 10% SDS in DMSO (Sigma-Aldrich)] was added. The plate was gently shaken for ∼15 min and protected from light. The optical density of reduced MTT was measured at 570 nm by spectroscopy on an automatic microtiter plate reader (Synergy HT Multi-Mode, BioTeK, Winooski,

VT) equipped with Gen5 (BioTek), and the cell metabolic activity (MA ≈ viability/proliferation characteristics) was calculated according to the following equation:

$$\mathrm{MA}\,(\%) = \frac{\text{Abs 570 nm}_{\text{sample}} - \text{Abs 570 nm}_{\text{DMSO}}}{\text{Abs 570nm}_{\text{negative control}} - \text{Abs 570 nm}_{\text{DMSO}}} \times 100$$

At least three independent assays were performed with three replicates. Besides the negative control (nonexposed cells), the assay results were also compared to those of the vehicle used (THF/BHT/FBS) with the same dilution factor.

Preparation of Lycopene Transfersomes. For this work, three independent batches of the same formulation were prepared according to the method of Simões et al.[28] In brief, we mixed the correct amounts of SPC [assumed molar weight (MW) of 800, as a former basic bilayer] and Tween 80 (assumed MW of 1310, as a bilayer softener) to produce the selected SPC/Tween molar ratio (2/1). Both amphipaths were taken up in enough 50 mM sodium phosphate buffer (pH 7.4) to yield a total amphipath concentration of 20 wt %, as well as enough lycopene to yield a concentration of 0.05% (w/w), and the mixture was left to be stirred constantly over 7 days protected from light and air. After that, crude suspensions were filtered under pressure to prepare 100 ± 50 nm vesicles (considered unilamellar), through track-etched polycarbonate (PCTE) membranes under a nitrogen stream. To produce larger vesicles, suspensions were frozen (at −70 °C) and rethawed (at 70 °C) five times, and the final size of vesicles was narrowed to 150 ± 50 nm by sequential filtration.

Preparation of Lycopene Ethosomes. Ethosomal systems consisted of 5% (w/v) SPC, 45% (v/v) ethanol, and up to 100% (v/v) water and were prepared according to the method of Touitou et al.[34] Briefly, SPC and extracted lycopene (~0.05%) were dissolved in ethanol, and water was added slowly in a fine stream with constant stirring, in a well-sealed container. Mixing was continued for an additional 5 min. The system was kept at 30 °C throughout the preparation and was then left to cool for 30 min at room temperature. Finally, the suspension was dimensioned by pressure filtration through PCTE membranes with a 100 nm pore size under a nitrogen stream.

Characterization of Vesicles. Vesicle Size and ζ Potential, Incorporation Efficiency, and Chemical Stability. The vesicle size and vesicle size distribution, in terms of the polydispersity index (PdI), were determined by a dynamic light scattering method (DLS) using a computerized inspection system (Zetasizer Nano-S, Malvern, Worcestershire, U.K.). For this purpose, 10 μL of a vesicular suspension was mixed with 1000 μL of MilliQ water, and the measurements were conducted in triplicate. The ζ potential was determined by a laser Doppler anemometry method, using a computerized inspection system (Zetasizer Nano-Z, Malvern). In this case, ethosomal suspensions were diluted in water and transfersomal suspensions were diluted in 50 mM sodium phosphate buffer (pH 7.4).

Phosphatidylcholine was quantified by the Trinder method, using a CHO-POD enzymatic colorimetric assay kit (Spinreact, Girona, Spain). To determine the incorporation efficiency (IE), the lipid concentrations in both initial and final vesicle suspensions were determined. Final suspensions were obtained by ultracentrifugation on a Beckman L8-60M ultracentrifuge (180000*g* for 2 h at 15 °C). IE (%) was calculated as follows:

$$\mathrm{IE}\,(\%) = [(\text{lycopene})\text{f}/(\text{SPC})\text{f}]/[(\text{lycopene})\text{i}/(\text{Lip})\text{i}] \times 100$$

where (lycopene)f is the final lycopene concentration, (lycopene)i the initial lycopene concentration, (SPC)f the final SPC concentration, and (SPC)i the initial SPC concentration.

Lycopene was quantified by HPLC as described above, solubilizing the samples in the mobile phase before [(lycopene)i] and after [(lycopene)f] centrifugation.

The chemical stability of the vesicle formulations was evaluated under stress light conditions. The formulations were exposed to incandescent light (60 W lamp, 230 V) at a distance of 15 cm, over 24 h. After this time, the amount of lycopene remaining in each sample was assessed by HPLC and compared to the initial amount.

Pressure-Driven Transport. Vesicle suspensions were diluted to a final SPC concentration of 2% (w/v), with water or 50 mM phosphate buffer (pH 7.4) in the case of ethosomes or transfersomes, respectively. The flow of vesicle suspensions through a microporous filter (PCTE) with a 30 nm pore diameter was driven by a 1.0 MPa external pressure, created by a nitrogen stream, and measured as a function of time. These measurements were taken with a 1 mL filtration unit that miniaturizes the commercial available pressure filtration device (home-built). The suspension was collected into a container on a Sartorius LA620P scale (Sartorius, Göttingen, Germany) to determine automatically the weight of the filtered suspension. The data were collected with the Wedge software for Windows (TAL Technologies Inc., Philadelphia, PA).

In Vitro Permeation and Skin Retention Study. An *in vitro* permeation study was conducted by using vertical Franz diffusion cells with a diffusion area of 0.68 cm^2. The vesicular formulations (200 μL, considered a constant and infinite dose) were spread over a fresh pig ear skin under non-occlusive conditions. According to OECD guideline 428, the infinite dose skin absorption experiment corresponds to a topical application (>10 $\mu L/cm^2$) of a liquid formulation on the skin.[35] The receptor phase contained a 3 mL mixture of saline phosphate buffer (pH 7.4) and 10% ethanol to solubilize lycopene[21] in the receptor solution maintained at 32 °C while being stirred (300 rpm) and protected from light. Twenty-four hours later, the receptor solution was analyzed and the skin samples were rinsed to remove the excess formulation and dried with filter paper. For the extraction process, 1 mL of the HPLC mobile phase [50/50 (v/v) acetonitrile/methanol] was added to small skin pieces, stirred for 2 min in a vertical mixer, and sonicated for 20 min to reach the cell lysis. The final solution was centrifuged (2500 rpm for 10 min), and the supernatant was filtered (0.45 μm) and injected via HPLC to quantify the amount of lycopene retained in skin samples (*stratum corneum*, epidermis, and dermis). The same methodology was used previously to evaluate the (extracted) lycopene recovery from skin membrane after solvent evaporation.

Data were expressed as the amount (percent per square centimeter) of lycopene that permeated through the skin, considering the total amount of lycopene applied in each formulation (transfersomes and ethosomes).

UDV Cellular Uptake. To observe the UDV cellular uptake, a formulation containing 0.4 mol % rhodamine PE-loaded transfersomes was prepared (amphipath concentration of 1%). HaCaT cells were seeded on 18 mm Ø coverslips in six-well plates (150000 cells/well) and incubated for 24 h. After this period, the cells were washed with PBS and exposed to 5 mM $CaCl_2$ for 30 min and then to Rhod-UDV at a final concentration of 0.525 μg/mL over 24 h. After being washed twice with PBS, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) (1.5−2.0 mL, 20 min) and the blue fluorescent Hoechst dye (Invitrogen, Barcelona, Spain) was added to cells (1/2000, 3 min at room temperature). Finally, coverslips with the adherent cells were removed from the six-well plates and mounted in slides. Confocal images were acquired on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging Inc., Oberkochen, Germany) using a Plan-Apochromat 100×/1.4 oil objective. Digital images were optimized for contrast and brightness using LSM-510 software (Carl Zeiss MicroImaging Inc.). The fluorescent filters used to observe the Hoechst and rhodamine fluorescence correspond to excitation wavelengths of 330−380 and 510−560 nm, respectively. The results were compared to those of nonexposed cells (negative control). Ten fields of view were counted to each coverslip (four coverslips for each sample).

Inhibition of Anthralin-Induced Ear Swelling by Topical Application of Lycopene Formulations. Female NMRI (22 g) mice (Charles River) were used to examine the relationship between anthralin-induced oxidative stress and ear swelling. The ear swelling assay was performed according to the method of Lange et al.[36] Anthralin was suspended in a 70% ethanol/olive oil mixture (4/1). After the formulation had been administered, each animal was kept individually in a separate cage. A freshly prepared 10 mM anthralin suspension (10 μL) was applied on the inside of both ears and left to

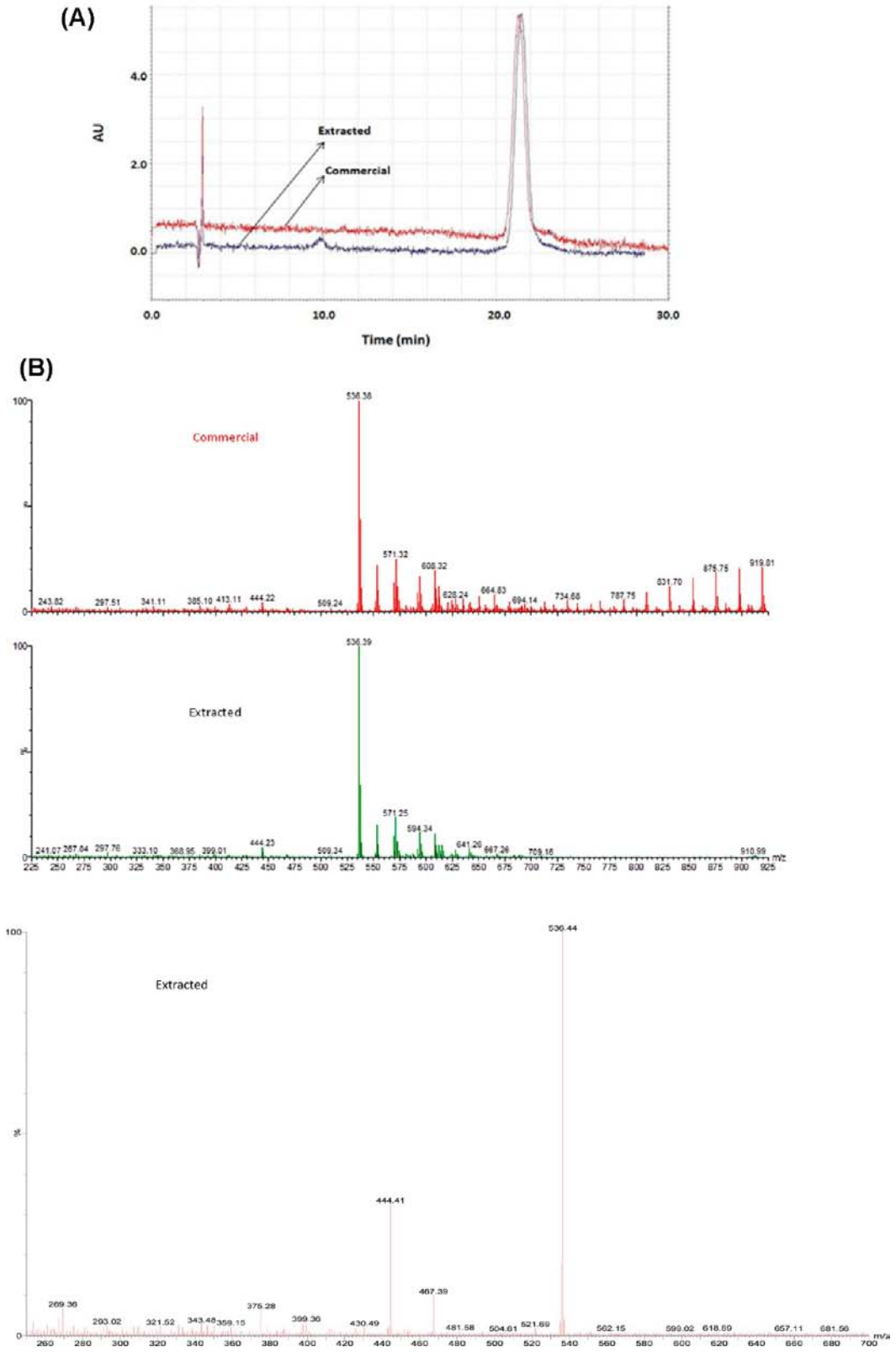

Figure 1. (A) HPLC chromatograms of lycopene extracted from tomato and commercially available lycopene. (B) Mass spectra (m/z 250−700) of commercial and extracted lycopene and MS/MS spectrum of a percursor ion at m/z 536 (collision energy of 10 eV).

dry out. One hour after being challenged with anthralin, animals received in one ear 10 μL of lycopene transfersomes, lycopene ethosomes, or a commercial cutaneous solution of 1 mg of betamethasone per gram (positive control). Ear thickness measurements were obtained before and 24 h after the animals had been challenged with a Mitutoya micrometer (Ascona Tools, Redwood City, CA) with three readings per ear. The degree of edema inhibition was calculated as a percentage of inhibition, determined by comparing the treated group with untreated controls. Four mice were used per group. At the end of the experiments, the animals were sacrificed. Skin

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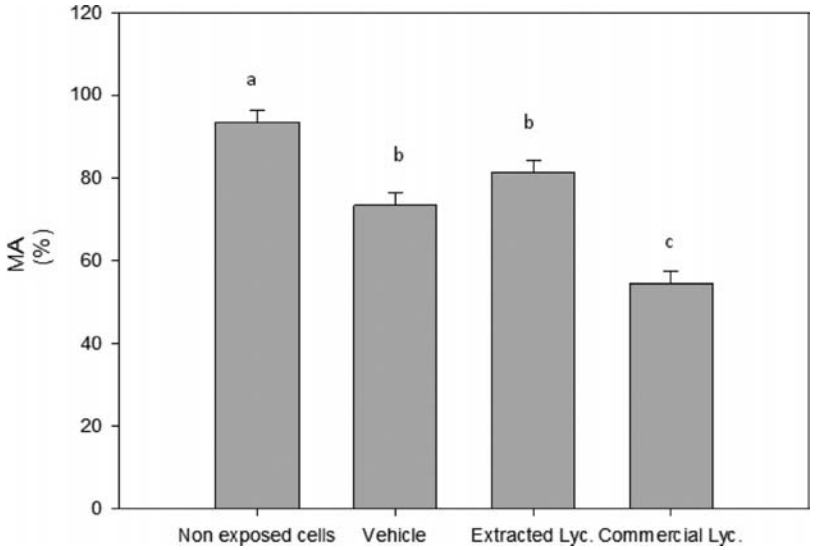

Figure 2. Effect of commercial (Commercial Lyc.) and extracted (Extracted Lyc.) lycopene (10 μM) and the respective vehicle (Vehicle) used (THF/BHT/FBS) on MA (%) measured by the MTT assay (means ± SD of three independent assays). In the multiple-comparison procedures (Holm−Sidak method), means with different letters are significantly different ($p < 0.05$).

ear biopsies were taken from treated areas and were preserved in a 10% formalin solution before being processed for histopathological studies. Tissue sections were stained with hematoxylin via an eosin standard procedure and finally examined under a light microscope (AH-2 Vanox, Olympus, Tokyo, Japan).

All animal experiments were conducted with the permission of the local animal ethical committee in accordance with the EU Directive (2010/63/UE), Portuguese law (DR 129/92, Portaria 1005/92), and all the applicable legislation. The experimental protocol was approved by Direcção Geral de Veterinária (DGV).

Statistical Analysis. The results are reported as means ± the standard deviation (SD) of at least three experiments. The results of all these experiments were statistically analyzed by analysis of variance (ANOVA) with all pairwise/nonpairwise multiple-comparison procedures using SigmaPlot version 11.0. The differences were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Lycopene Extraction and Quantification. In this work, we used a simplified, inexpensive, and rapid method of extraction to obtain lycopene from tomatoes and compare it with the commercially available form. Few published studies have used comparable mild conditions.[37] We used dried tomato, a single solvent, gentle stirring, and room temperature as the platform developed for the advantageous extraction protocol. Lycopene has been assessed in food or biological samples by many analytical methods, such as UV−vis spectrophotometry and especially liquid chromatography with spectrophotometric detection. Solubilization of lycopene is quite difficult, and many strategies have been reported in the literature.[9,10] Here we solubilized both commercial and extracted lycopene in hexane for their quantification by HPLC. Figure 1 shows the HPLC chromatogram and mass spectra of extracted and commercially available lycopene for the described operational conditions. In fact, a high degree of similarity between the chromatograms and spectra of both lycopene samples was obtained. A high correlation coefficient was obtained (0.999), and HPLC curves of $y = 221.53x + 1942.7$ and $y = 252.31x + 2804.6$ were determined for commercial and extracted lycopene, respectively. The limits of detection and quantification (LOD and LOQ, respectively) were 88.7 and 268.8 nM, respectively, for the commercially available lycopene and 54.0 and 163.6 nM, respectively, for the extracted lycopene. The lycopene recovery was 27 mg of extracted lycopene per 100 g of dry tomato.

When analyzed by mass spectrometry, both lycopene samples (commercial and extracted) presented similar mass spectra with a molecular ion at m/z 536.4, which is according to data for lycopene.[38] MS/MS experiments with the ion at m/z 536.4 showed two main fragments for both samples: (1) $[M + H - 92]^+$ corresponding to the ion at m/z 444 and (2) $[M + H - 69]^+$ corresponding to the ion at m/z 467. The fragments detected at m/z 467 and 444 correspond to the loss of the isoprene unit and toluene, respectively.[39] When a higher collision energy was applied, smaller fragments were obtained, such as the ion at m/z 375 corresponding to $[M + H - 161]^+$ as expected on the basis of the linear chemical structure of lycopene. The *cis* and all-*trans* isomers of lycopene may be distinguished by the relative abundances of fragment ions at m/z 467, 521, 493 (*cis*-lycopene), and 444 (all-*trans*-lycopene).[40] In the spectra obtained, there is a higher abundance of all-*trans*-lycopene in tomato extract. In addition, according to the chromatograms obtained, two other peaks absorbing at 472 nm were detected in the tomato extract but in small amounts at 18.9 and 41.15 min (6.1 and 3.4%, respectively). These compounds present UV−vis spectra characteristic of this family of compounds, and the molecular ions at m/z 552 and 536 detected indicate they can correspond to β-carotene and β-cryptoxanthin, respectively.[38] The presence of these two compounds did not interfere with lycopene analysis.

Evaluation of Lycopene Extract Cytotoxicity. The results of the MTT assay are presented in Figure 2. The cells previously exposed to extracted lycopene presented an MA significantly higher than that of the group exposed to the commercial form, further encouraging the use of this raw material. However, no favorable effect on skin cells was obtained from this exposure.

Lycopene was vehiculated in the THF/BHT/FBS vehicle to improve its stability and cellular uptake,[41,42] and also considering the cytotoxicity of deformable vesicles components studied in a previous work because of the high lipid and

Table 1. Characterization of Lycopene-Loaded Transfersomes and Lycopene-Loaded Ethosomes

formulation	vesicle diameter (nm)	polydispersity index	ζ potential (mV)	incorporation efficiency (%)
transfersomes	124 ± 0	0.13 ± 0.01	−6.4 ± 0.3	94 ± 1
ethosomes	153 ± 11	0.29 ± 0.03	−19 ± 2.5	92 ± 18

surfactant concentrations. Even working at a very low concentration of THF (0.1%), the vehicle used (THF/BHT/FBS) resulted in 73.20% cell viability, and this contributed to the larger differences compared to the negative control (nonexposed cells), especially in the case of commercial lycopene, the result of which was lower than acceptable. However, such a significant difference was not obtained in previous MTT experiments; thus, it should not be emphasized.

Characterization of Vesicular Nanocarrier Formulations. Table 1 presents the particle size analysis of the studied formulations. Both lycopene-loaded transfersomes and lycopene-loaded ethosomes presented definite particle sizes and low polydispersity indices. The incorporation efficiency was high and very similar between formulations. It should be mentioned that empty vesicles did not interfere with the lycopene HPLC assay. As expected, ethosomes presented a negative ζ potential value because of the presence of ethanol in the formulation.[34] In the case of transfersomes, SPC (a neutral or zwitterionic phospholipid over a pH range from strongly acidic to strongly alkaline) and Tween 80 do not contribute to a very negative ζ potential. However, the different ζ potential did not influence the lycopene incorporation efficiency.

With regard to the chemical stability under stress light conditions already described, lycopene-loaded transfersomes presented a much higher stability (~1.2-fold ± 0.2) than lycopene-loaded ethosomes probably because of the higher lipid concentration of the former, and the lycopene localization in each formulation.

When using deformable vesicles, it is important to check the ability of loaded vesicles to deform because this skill is reported to be the decisive characteristic for enhanced penetration. The degree of deformability could be extrapolated from the flow of vesicles through the pores of a known size (30 nm) driven by an external pressure. The flow of lycopene-loaded vesicles was found to vary significantly ($p < 0.05$) between transfersomes and ethosomes (Figure 2), and it was observed that transfersomes were more deformable under the tested experimental conditions. One possible reason for this result could be the partial evaporation of ethanol from the formulation during experimental technical operations. In fact, ethosomes and transfersomes are in a more fluid state and less rigid than conventional liposomes. In the case of ethosomes, ethanol disturbs the lipid bilayer (an interdigitation effect) and increases the fluidity of phospholipid bilayers in the liquid crystalline state.[43] In the case of transfersomes, the creation of small Tween/phosphatidylcholine mixed micelles is typically preceded by presolubilization structures, first in the form of deformable, strongly fluctuating, bilayer vesicles.[44] Compared to ethanol, this surfactant can solubilize fatty molecules and bilayer membranes but needs quite a long time to accomplish this.[44] A comparative study between ethosomes and flexosomes (transfersomes prepared with Tween 20) has shown that ethosomes were much less deformable than flexosomes.[45] For the authors, the explanation for this finding resided in the fact that ethanol increases the membrane fluidity of ethosomes inefficiently enough for deformation to pass through the pores smaller than their own particle size. In fact, this ethosomal formulation was larger (including PdI) than the transfersome formulation. In addition, assuming that lycopene is associated with the vesicle lipid bilayer, the degree of interaction between lycopene and ethosomes might be higher than that between lycopene and the transfersome bilayer, according to the results presented in Figure 3. The flow of loaded ethosomes is weaker

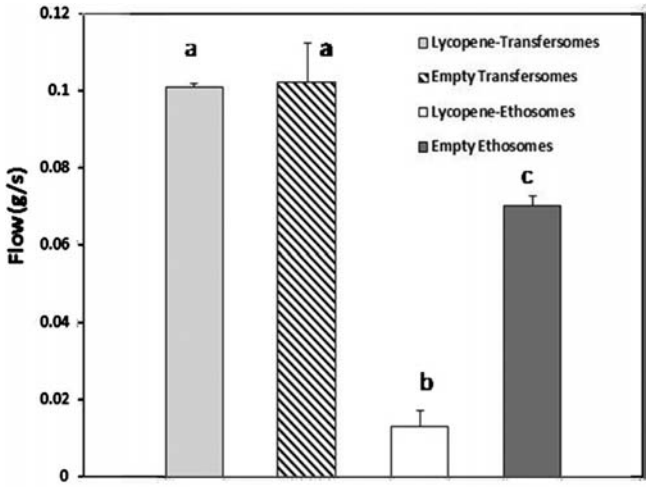

Figure 3. Flow of lycopene transfersomes and lycopene ethosomes, both prepared with extracted lycopene, and the respective empty formulations, through 30 nm pores driven by an external pressure, measured as a function of time. Means ± SD with different letters are significantly different ($p < 0.05$).

than that of the empty vesicles. For transfersomes, the incorporation of lycopene did not change the biomechanical properties of the carrier.

***In Vitro* Skin Permeation and Retention Study.** The concept of dermal delivery involves the accumulation of active molecules in the epidermis and dermis layers with a reduced rate of systemic delivery. In the particular case of lycopene, its strong lipophilicity and its ability to interact with SC components make its penetration into and across the skin difficult. Because deformable liposomes possess skin penetration enhancement capacity, the use of these systems for topical application and dermal delivery of lycopene was tested. Both systems present soybean phosphatidylcholine as the main vesicular component and a membrane-softening agent. Its presence makes the resulting lipid bilayers more flexible. Transfersomes contain a polysorbate, Tween 80, as the surface-active agent that has a tendency to adsorb at surfaces. The distribution of the detergent between the aqueous and lipid environments makes these systems dynamic structures.[46] In the case of ethosomes, ethanol is distributed inside and outside the vesicles.[34] Surfactants and ethanol present in this type of delivery system could also facilitate lycopene solubilization.

No lycopene was detected in the receptor solution 24 h after the *in vitro* skin permeation study, as already observed in previous similar experiments with the same vehicle (transfersomes with a lipid concentration of 20%). With regard to the skin retention study, it was possible to recover ~64% of

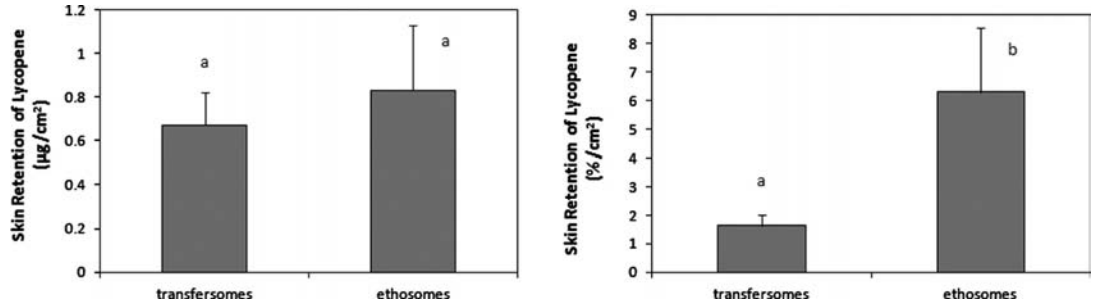

Figure 4. Skin retention study results 24 h after the *in vitro* skin permeation study of lycopene-loaded transfersome and ethosome formulations. Data are expressed as the amount of lycopene (micrograms per square centimeter or percent per square centimeter) retained in the skin (SC, epidermis, and dermis), considering the total amount of lycopene applied in each formulation. Means ± SD (n = 3) with different letters are significantly different (p < 0.05; t test).

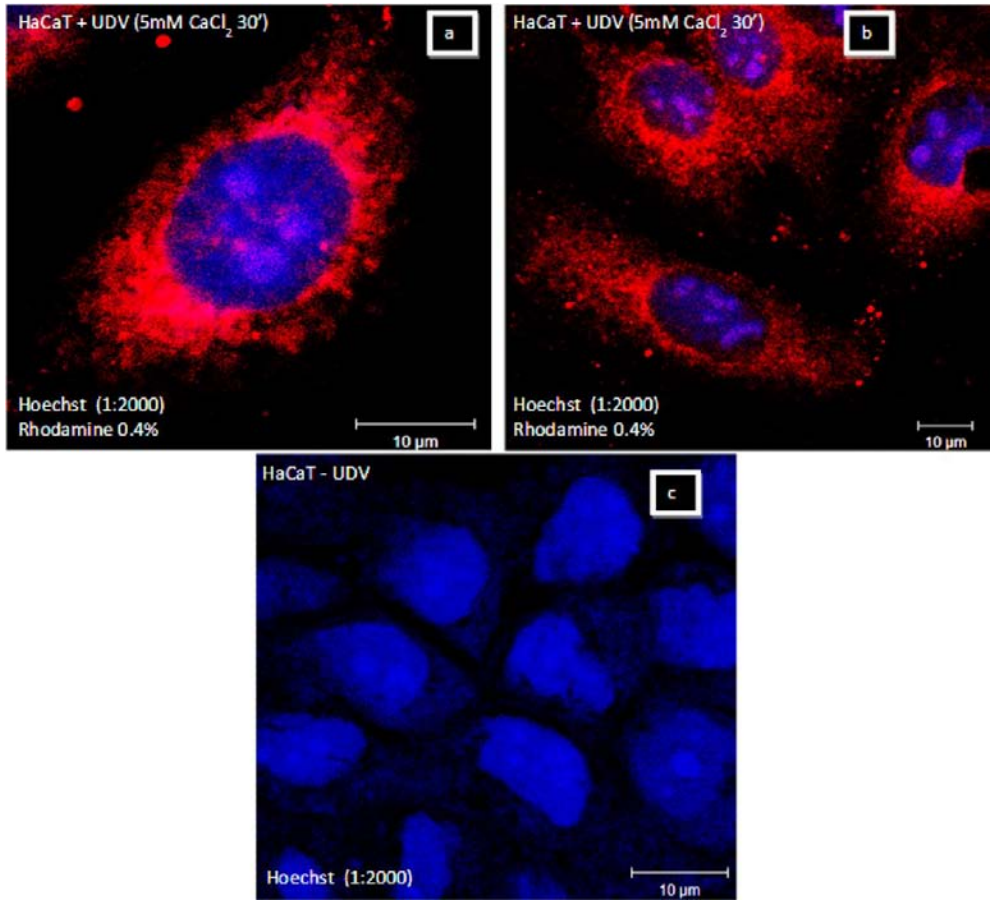

Figure 5. Confocal microscopy of rhodamine UDV uptake by cells (a and b) and a negative control (c). The fluorescent filters used to observe the Hoechst and rhodamine fluorescence correspond to excitation wavelengths of 330−380 and 510−560 nm, respectively (total magnification of 10×100×).

lycopene from skin using the HPLC mobile phase as the extraction solvent. Unfortunately, it was not possible to quantify the amount of lycopene in each skin layer because it was smaller than the equipment LOD. Thus, results are expressed as the total amount of lycopene retained in *stratum corneum* (SC), epidermis, and dermis layers (Figure 4). However, theoretically, there should be a higher concentration of lycopene in the upper skin layers because of the transportation of carotenoids to the skin surface via eccrine sweat glands and/or sebaceous glands and migration of epidermal keratinocytes in which carotenoids have been thought to be loaded.[47−49] In fact, dermal or topical delivery permits the extension of antioxidant skin deposition and promotion of skin antioxidant enrichment.[24]

Unexpectedly, lycopene skin retention (expressed in percent per square centimeter) was significantly lower (p = 0.024) for transfersomes than for ethosomes. On one hand, although transfersomes are more deformable vesicles because of the presence of Tween 80, this formulation also has a higher lipid content, which contributes to its greater retention on the skin surface. On the other hand, ethanol evaporates from the ethosomal formulation topically applied under non-occlusive conditions, and consequently, there is an increase in the lycopene concentration in the skin layers. Although the

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influence of the total ethosomal composition on the bilayer structure of SC is not yet fully understood,[24] it is known that ethanol may extract some lipids of the SC,[50] compromising the skin barrier function until the skin rapidly restores itself.

UDV Cellular Uptake. To observe the cellular uptake of UDVs, we prepared a transfersome formulation with 0.4 mol % rhodamine PE. This formulation presented a vesicle size and ζ potential of 121 nm (PdI = 0.082) and −6.92 mV, respectively. Twenty-four hours after incubation (≈50% cell confluence), calcium was added to the cell culture medium before the addition of UDVs to increase the cell membrane permeability and further observe the intracellular localization of UDV.

Two fluorescent dyes were used to stain cells and UDVs (Hoechst-33258 and rhodamine PE, respectively). Hoechst is a fluorescent stain that can pass through an intact cell membrane and bind strongly to DNA. Therefore, it can be used to stain both live and fixed cells. When bound to double-stranded DNA, this dye is excited by ultraviolet light around 350 nm and emits blue/cyan fluorescence light around an emission maximum at 461 nm. On the other hand, rhodamine is excited at 510−560 nm (green light).

The confocal microscopy images of rhodamine UDV cellular uptake by cells previously exposed to 5 mM $CaCl_2$ are presented in Figure 5. The smaller vesicles were taken up by the cells as confirmed by the different sections of confocal microscopy. The cell's nucleus was clearly visualized with Hoechst staining, including the nucleus division. In Figure 5a, intracellular localization of UDV stained with rhodamine can be observed around the nucleus. In fact, Sharoni et al.[51] suggested that lycopene or one of its derivatives might interact with members of the nuclear receptor superfamily according to the synergistic inhibition of cancer cell proliferation by lycopene and retinoic acid, two members of this receptor family. Nuclear receptors (RAR and RXR) are ligand-activated transcription factors. Upon binding to their ligand, these receptors bind as homo- or heterodimers to specific DNA sequences in the promoter region of target genes and activate (or repress) their transcription.

The cellular uptake and fluorescence microscopy of antigen-loaded transfersomes has already been conducted *ex vivo* in macrophages, and the study revealed an enhanced uptake of vesicles.[52] The mechanism of cellular uptake of liposomes proposed by Mastrobattista et al.[53] may be extended to UDV. (1) Liposomes may, after specific cell binding, release their contents in the proximity of the target cells, with subsequent cellular uptake of released molecules. (2) Liposomes may fuse with the cell membrane, thereby releasing their contents into the cytosol. (3) Cell-bound liposomes may be internalized by the target cells via receptor-mediated endocytosis, followed by the intracellular release of encapsulated compounds. Whether liposomes will be internalized is dependent on a variety of factors, such as the liposome size,[54] the type of cell, and the type of target receptor.[55] White et al.[56] also refers to the importance of cell confluence, concluding that optimized conditions for the uptake of oligodeoxynucleotides in cultured keratinocytes consisted of subconfluent populations and utilization of nontoxic concentrations of an appropriate liposome formulation.

***In Vivo* Effect of Lycopene Formulations on an Anthralin-Induced Ear Edema Model.** Oxidative stress plays an important role in chemically induced inflammation. This is the case with contact irritants such as anthralin, which is known to generate reactive oxygen species (ROS) within the skin.[36] Therapeutic use of lycopene, an active scavenger of ROS, was tested in an animal model, anthralin-induced ear swelling; i.e., the penetration behavior of lycopene-loaded deformable carriers applied to inflamed skin was studied. The model was adapted from Lange et al.,[36] and it was chosen because of some advantages, namely, the simplicity and rapidity of the results.[57] Betamethasone was reported to reduce mice ear edema in this cutaneous inflammation model[57] and was used in this study as a positive control. Application of 10 mM anthralin induced edema and erythema on the ears, which were remarkably attenuated by the epicutaneous application of both tested formulations containing 0.05% lycopene (~0.005 mg/ear) and a 1 mg/g betamethasone cutaneous solution (0.01 mg/ear). The results presented in Figure 6 show that there

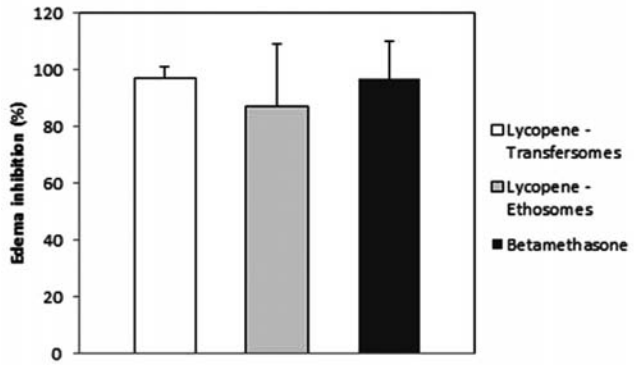

Figure 6. Effect of the treatment with lycopene transfersomes, lycopene ethosomes, and a commercial betamethasone formulation, 1 h after challenge with 10 mM anthralin, on anthralin-induced ear swelling, evaluated 24 h after challenge (mean ± SD; n = 4).

were no statistically significant differences between the inibition of anthralin-induced ear swelling of tested lycopene formulations (and between them) and a cutaneous solution of betamethasone.

The experimental inflammation evoked by the application of anthralin involves the enlargement or hyperplasia of sebaceous glands (which are involved in the immunomodulation process), as can be observed in histological images of mice ear sections [Figure 7A,D (black arrows)].[57] Topical application of lycopene remarkably reduced the level of inflammatory infiltrate (Figure 7D). Levels of epidermal hyperplasia and inflammatory cell infiltration were even more reduced when lycopene was carried by means of transfersomes (Figure 7B) versus ethosomes (Figure 7C). Carrier-mediated skin delivery of lycopene might have contributed to the enhancement of skin retention, which may have successfully contributed to the therapeutic effect.

In summary, among the many antioxidants that can be used topically, lycopene appears to be a powerful agent to be delivered at the cutaneous level. Above all, an antioxidant for topical use must be free of local toxicity and simultaneously must reach the skin structures to exert its beneficial properties, which was achieved in this study. In particular, the results of this study show that a method for extracting lycopene from tomato based on a low-cost and non-time-consuming protocol was successfully developed, and ultradeformable lipid vesicles were good carriers for its incorporation. Furthermore, this extraction was quite selective and safe because the extracted lycopene showed low or no cytotoxicity in skin cells. Transfersomal and ethosomal formulations presented high

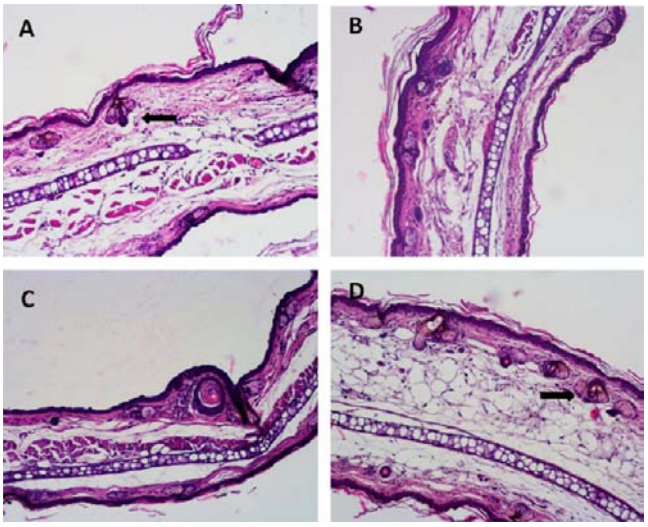

Figure 7. Mice ear skin sections stained with hematoxylin and eosin. (A) Ears challenged with anthralin. (B) Ears challenged with anthralin and treated with lycopene transfersomes. (C) Ears challenged with anthralin and treated with lycopene ethosomes. (D) Ears challenged with anthralin and treated with a betamethasone solution (total magnification of 10×10×).

incorporation efficiencies, meaning that both are good candidates for improving the topical bioavailability of lycopene. In the case of ethosomes, the deformability was more affected by lycopene−vesicle interaction. In addition, we have demonstrated that ultradeformable lipid vesicle formulations were efficiently taken up by human keratinocytes previously exposed to $CaCl_2$. Finally, the therapeutic significance of the use of extracted lycopene-loaded deformable carriers was demonstrated *in vivo* by the inhibition of edema formation in an anthralin-induced ear swelling model.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

UV−vis spectra of lycopene extracted from tomato and commercially available lycopene that show the characteristic absorption peaks at 504, 472, and 446 nm and TLC analysis that showed extracted and commercial standard lycopene exhibited similar R_f values in silica plates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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