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Dual delivery of ginger oil and hexylresorcinol with lipid nanoparticles for the effective treatment of cutaneous hyperpigmentation

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ABSTRACT

Cutaneous hyperpigmentation is a widespread pathology affecting a large slice of the world population. In this work, we propose a treatment approach based on the co-delivery of 4-hexylresorcinol (HR) and ginger oil (GO), using lipid nanoparticles. In specific, HR and GO were encapsulated within nanostructured lipid carriers (NLCs), produced with the high-shear homogenization technique, and using Precirol ATO 5 as solid lipid component. Different NLCs and solid lipid nanoparticles (SLNs) formulations were prepared in order to evaluate any additive or synergistic effect between HR and GO. All the formulations were properly optimized on the basis of the mean size and polydispersity of the nanoparticles population, determined by Dynamic Light Scattering (DLS) technique, and on the basis of stability studies. The rheological behavior of both SLNs and NLCs formulations was evaluated through flow sweep analyses and opportunely adjusted to allow proper topical application. These formulations were then submitted to a cosmetic clinical test on a panel of women presenting cutaneous stains. To this end, CIE L*a*b* and melanin index colorimetrical analyses were carried out to verify the efficacy of the formulations in treating cutaneous hyperpigmentation. Hence, SLNs and especially NLCs have proven to be suitable carriers for a novel treatment approach as an adjuvant in skin concerns in the pharmaceutical and cosmetic fields.

1. Introduction

Hyperpigmentation is a skin disorder affecting millions of people worldwide and is characterized by uneven skin coloration, mainly in the regions of the facial skin, that can cause significant negative psychological and social impacts [1]. The cause of skin hyperpigmentation resides in excessive melanin production by melanocytes and increased transportation of the melanosome, where the melanin is stored, from melanocytes to the keratinocytes of the epidermal melanin unit [2,3]. This hyperactivity has a significant relation with excessive UV exposure and can also be determined by endocrine and autocrine factors [4]. Melanin's characteristic colour is given by blending two different pigments, eumelanin and pheomelanin. Three enzymes carry out the synthesis of these two pigments: tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2) [5]. TYR is necessary for the reaction to occur. Thanks to its monophenolase and diphenolase activity, it catalyses the rate-limiting step of the reaction, the conversion of tyrosine to dopaquinone [6–9]. The biosynthesis of these three enzymes is triggered by the presence of MITF (Microftalmia Transcription Factor). Several pathways turn on or shut down MITF expression, thus regulating the synthesis of enzymes essential to melanin production [6].

One rational approach to treat hyperpigmentation is to target the first step of the reaction. TYR affinity to tyrosine can be outclassed by 4-hexylresorcinol (HR), an enzyme's alternative substrate. HR catalysis does not produce precursors for melanin production, arresting the earliest step of the reaction [10,11]. Another approach is to shut down MITF production. It has been found that Ginger Oil (GO), an oily extract obtained from *Zingiber officinale* rhizomes, can inhibit MITF expression thanks to the presence of 6-shogaols and 6-gingerols [12–14]. Ginger is a well-known plant used in traditional medicines. It is used in the treatment of rheumatic disease [15] and increases immunity [16]. Nowadays, this phytonutrient with countless beneficial properties [17] is rediscovering a new light as an active cosmetic ingredient due to its high

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Table 1
Composition of NLC and SLN formulations prepared modifying type and amount of surfactant and co-surfactant, in order to modify the HLB of the resulting mixture (HLB_{MIX}). The amount of Precirol® ATO 5 used to formulate NLCs and SLNs was 8% *w/w* and 9% *w/w* respectively.

SAMPLE	P188 (% w/w)	SP30 (% w/w)	SPAN85 (% w/w)	HLB_{MIX}	GO (% w/w)	Labrafac (% w/w)	HR (% w/w)	Nanoparticles formation
NLC_1	5.0	_	_	29	_	1.0	_	No
NLC_2	4.5	0.5	_	26.7	_	1.0	_	Yes
NLC_3	4.6	_	0.4	26.7	_	1.0	_	No
NLC_4	6.0	_	0.5	26.7	_	1.0	_	No
NLC_5	4.0	1.0	_	24.4	_	1.0	_	Yes
NLC_6	4.2	_	0.8	24.4	_	1.0	_	No
NLC_7	5.4	_	1.1	24.4	_	1.0	_	Yes
NLC_8	3.0	2.0		19.8	_	1.0	_	Yes
NLC_9	3.3	_	1.7	19.8	_	1.0	_	No
NLC_{10}	4.3	_	2.2	19.8	_	1.0	_	No
NLC_{11}	2.5	2.5	_	17.5	_	1.0	_	Yes
NLC_{12}	2.9	_	2.1	17.5	_	1.0	_	No
NLC_{13}	3.7	_	2.8	17.5	_	1.0	_	No
NLC_{14}	2.0	3.0	_	15.2	_	1.0	_	No
NLC_{15}	2.5	_	2.5	15.2	_	1.0	_	No
NLC_{16}	3.2	_	3.3	15.2	_	1.0	_	No
NLC_{17}	1.0	4.0	_	10.6	_	1.0	_	No
NLC_{18}	1.7	_	3.3	10.6	_	1.0	_	No
NLC_{19}	2.2	_	4.3	10.6	_	1.0	_	No
NLC_{20}	4.0	1.0	_	n.c.	_	1.0	0.5	Yes
NLC_{21}	4.2	_	0.8	n.c.	_	1.0	0.5	Yes
NLC_{22}	5.4	_	1.1	n.c.	_	1.0	0.5	Yes
NLC_{23}	4.0	_	_	n.c.	_	1.0	1.0	Yes
NLC_{24}	4.5	_	_	n.c.	_	1.0	1.0	Yes
NLC_{25}	5.0	_	_	n.c.	_	1.0	1.0	Yes
NLC_{26}	4.5	_	_	n.c.	1.0	_	1.0	Yes
NLC_{27}	4.5	_	_	n.c.	1.0	_	_	Yes
NLC_{28}	4.5	1.0	_	n.c.	_	1.0	_	Yes
SLN_1	4.5	_	_	n.c.	_	_	1.0	Yes

content in powerful compounds and it is a likely candidate as a nutricosmetic, in line with the growing demand for natural, plant-sourced actives [18–21]. The essential oil obtained from ginger rhizome contains many active compounds, including α-zingiberene, camphene, ß-sesquiphyllandrene and bisabolene, which have antioxidant activity [22], whereas 6-shogaols can reduce UVB-induced wrinkling and aging in experimental rats [23] and inhibit leukocyte infiltration into inflamed tissue accompanied by reduction of edema swelling [24]. Gingerols, instead, are under investigation for their immunomodulatory, anti-inflammatory and antioxidant activity [25,26]. Moreover, GO has proven to possess inhibitory effect on tyrosinase activity [27]. Therefore, the dual delivery of HR and GO may result in an interesting additive or synergistic effect in the treatment of cutaneous hyperpigmentation.

To be effective, the whitening agents have to reach and target the innermost skin layers, where melanocytes are localized [2,3,14,28]. In recent years, lipid nanoparticles (LNPs) have been proposed as an alternative to more conventional formulations, such as emulsions. They consist of a solid lipophilic matrix, which can incorporate and protect active molecules from degradation, ensuring efficient delivery and a modified release in the innermost skin layers [29–36]. Two primary lipid nanoparticles have been developed over the years: SLNs (solid lipid nanoparticles) and NLCs (nanostructured lipid carriers). The main difference resides in the physical state of the nanoparticles. Indeed, SLNs are characterized by a solid lipid matrix, in which the active principle is dispersed in organized spaces of lipid crystals [34]. In contrast, the dispersed phase of NLCs is constituted of a blend of solid and liquid lipids, which creates multiple imperfections within the particle structure. This feature allows achieving higher drug loading and more efficient entrapment, avoiding drug expulsion issues [37–40].

All that considered, this study aimed to develop stable lipid nanoparticles able to co-deliver HR and GO. The efficacy of the formulations was evaluated on Caucasian women with localized hyperpigmentation on their skin.

2. Materials and methods

2.1. Materials

Precirol® ATO 5 (glyceryl palmitostearate, melting temperature 50–60 °C) and labrafac™ lipophile WL 1349 were kindly given by GATTEFOSSÉ SAS (Cadex, France). Lutrol® F68 (Poloxamer 188, P188) was obtained by BASF (Ludwigshafen, Germany). 4-Hexylresorcinol (Synovea® HR) was a gift from SYTHEON (Boulogne Billancourt, France); Ginger Oil (GO) was gifted by INDENA (Milano, Italia); sepimax zen (polyacrylate cross polymer-6) was a gift from SEPPIC (Milano, Italia). Microstabil (phenethyl alcohol and caprylyl glycol) was a kind gift from AKEMA Srl (Coriano di Rimini, Italy). Span 85 was purchased from Honeywell Fluka™ (Milano, Italy). Sisterna SP30–C (sucrose distearate) was obtained from CHIMAB s.p.a (Padova, Italy); bidistilled water, formic acid 98–100% (HCOOH), methanol RS (MeOH) for HPLC, acetic acid (CH_3COOH), and sodium acetate (CH_3COONa) were obtained from Carlo Erba (Milano, Italia), while acetonitrile (CH_3CN), dialysis membranes made of regenerated cellulose (cut-off 12–14 kDa), xanthan gum and Sephadex® G-75 were purchased at Sigma Aldrich (Milano, Italia). Airless bottles purchased from Eurovetrocap (Milano, Italia) were used for the primary packaging of the formulations.

2.2. Preparation of SLNs and NLCs

SLNs were prepared using the high-shear homogenization technique [41]. The solid lipid (Precirol® ATO 5) was melted at 70.0 ± 0.5 °C. Surfactant and co-surfactant were dispersed in water and heated at the same temperature of the lipid phase. Then, the aqueous phase was slowly poured in the melted lipids maintained under high shear stress conditions with an Ultraturrax® T18 (IKA, Staufen, Germany). The homogenization process was carried out at 22,000 rpm for 10 min. HR-loaded SLNs were obtained dissolving HR in the melted lipids before homogenization.

NLCs were prepared following the same procedure, but blending

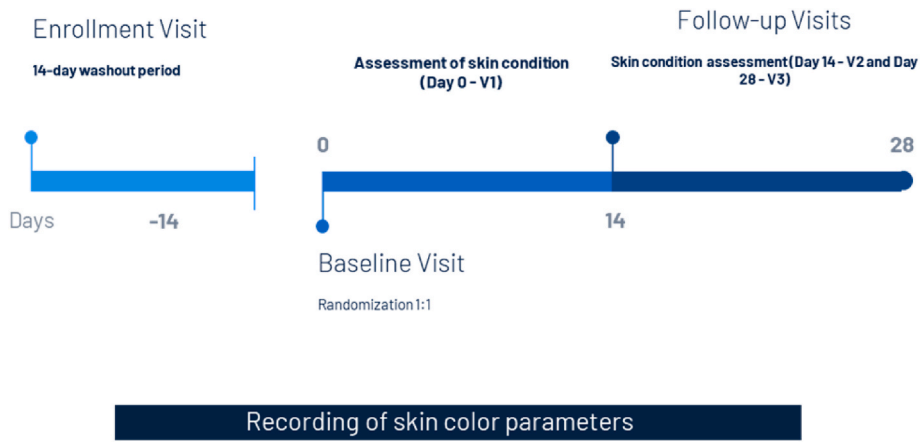

Fig. 1. Timeline scheme of the study.

solid lipids with labrafac or GO. All the SLNs and NLCs formulations were allowed to rest 24 h before analysis.

Different SLNs and NLCs formulations were prepared modifying type and amount of surfactant and co-surfactant, in order to modify the HLB of the resulting mixture (HLB_{MIX}). More specifically, poloxamer P188 (P188), sucrose distearate (SP30) and span 85 were used for the scope. The composition of the NLCs and SLNs has been reported in Table 1.

The preservative microstabil (0.5% *w/w*) was added to the formulations NLC_{24}, NLC_{26}, NLC_{27} and SLN_1 tested for clinical efficacy, in order to avoid microbial proliferation.

2.3. Particle size, PDI, and Z-potential analysis

The mean hydrodynamic diameter and PDI of the nanoparticles were determined by a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). Before measurement, all the samples were diluted with distilled water to gain an appropriate scattering intensity, and measurements were performed at 25.0 ± 0.5 °C. The same diluted suspensions were introduced in a polycarbonate cuvette with gold electrodes to measure the Z-potential (ζ) value of the nanoparticles. Each formulation was measured in triplicate, and the results are reported as the mean values ± the standard deviation.

2.4. Evaluation of HR entrapment efficiency

The amount of HR entrapped into the lipid nanoparticles was directly evaluated by a solid-liquid extraction process. To this end, aliquots (1 mL) of NLC_{24}, NLC_{26} and SLN_1 were purified by size-exclusion chromatography (SEC) on Sephadex® G75, to remove all the non-entrapped HR. The recovered nanoparticles were freeze-dried, then 0.1 g of the solid samples were extracted with acetonitrile (8 mL) at 37.0 ± 0.5 °C for 20 min and vortexed for 10 min. The suspension was centrifuged at 6,000 rpm for 10 min to achieve clear phase separation and allow the recovery of the supernatant. The extraction process was repeated five times, and then all the organic fractions were collected and filtered through 0.20 μm Millex® filters. The amount of HR was quantified by HPLC analysis using a Perkin Elmer HPLC system equipped with a Series 200 LC pump, a 235C Diode Array Detector and a RP-18 column [42]. The analyses were carried out using a mobile phase constituted by a 60:40 *v/v* mixture of CH_3CN/HCOOH (HCOOH 0.075% *v/v* in bidistilled water) and a flow rate of 1 mL/min. The concentration of HR was monitored at the wavelength of 280 nm and determined through the calibration curve y=0.0774x-0.0006; R^2=0.9991.

Aliquots (1 mL) of crude NLC_{24}, NLC_{26} and SLN_1 (not purified by SEC) were freeze-dried and submitted to the same solid-liquid extraction, in order to evaluate the efficiency of the extraction procedure. Each experiment was performed in triplicate, and the results are reported as the mean values ± the standard deviation.

The Entrapment Efficiency (EE%) was expressed as:

$$EE(\%) = \frac{mg\ of\ entrapped\ HR}{mg\ of\ theoretical\ HR} \times 100$$

2.5. Release studies in vertical Franz diffusion cell

The release profile of HR was determined with a vertical Franz diffusion cell having a cylindrical acceptor chamber of 4 mL. A semi-permeable membrane with a cut-off of 12–14 kDa, having an area of 1 cm^2 and thickness of 48 μm, was used to separate the donor and the acceptor compartment.

All the release studies in the Franz cell were performed by placing 0.1 mL of NLC_{24}, NLC_{26} and SLN_1 in the donor chamber, whereas the acceptor chamber was loaded with 4 mL of 2:3 *v/v* EtOH/acetate buffer solution (0.1 M CH_3COOH/CH_3COONa; pH = 5), capable of simulating the physiological pH of the skin. EtOH was added to the release medium to allow achieving sink conditions, considering that HR is very slightly soluble in water (<1 mg/ml at 25 °C). The acetate buffer solution was previously sonicated for 5 min using a SONICA® Ultrasonic Cleaner (SOLTEC®, Milano Italia) and subsequently blown with N_2 for additional 5 min to reduce air bubbles formation during the release studies. The acceptor compartment was kept at 37.0 ± 0.1 °C under constant magnetic stirring (700 rpm). Aliquots of 250 μL of the release medium were withdrawn at fixed time points, refilling it with an equal volume of fresh release medium preheated at 37.0 ± 0.1 °C. The release medium was sampled every hour for the first 8 h and after 24, 27, 30, and 48 h. The concentration of HR was determined by HPLC analysis as described in section 2.4.

Each experiment was performed in triplicate, and the results were reported as the mean values ± the standard deviation.

2.6. Rheological characterization

Flow curves were measured on NLC_{24}, NLC_{26}, NLC_{27}, NLC_{28} and SLN_1 formulations using a Discovery TA HR-1 stress-control rheometer. In order to optimize the rheological properties of the different SLN and NLC formulations, two types of thickening agents were evaluated, namely sepimax zen (0.2% *w/w*) and xanthan gum (1.0% *w/w*). The thickening agents were dispersed in the selected formulations at 25 ± 1 °C with a mechanic stirrer RZR1 (120 rpm). Sepimax zen required 8 h for complete dispersion, whereas xanthan gum needed 5 h.

Flow curves of the selected SNL and NLC formulations were recorded on 0.3 mL aliquots, using a cone-plate geometry with a diameter of 40 mm (α 1.005°, gap 27 μm) and working in the range of 0.01–300 Pa at 37.00 ± 0.01 °C. All the experiments were carried out at least in

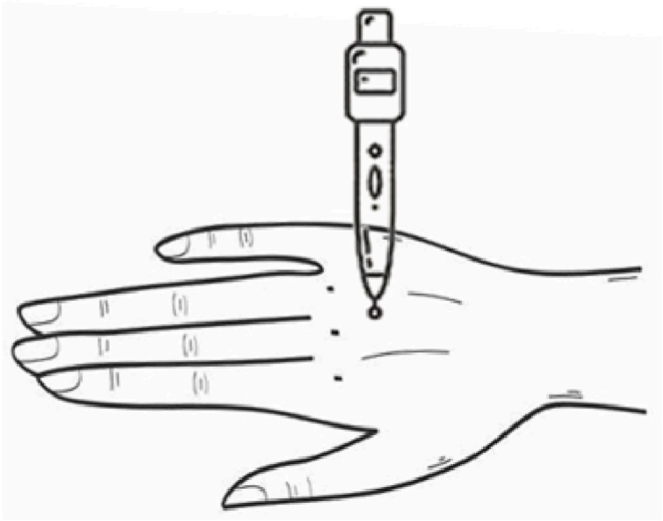

Fig. 2. Measuring position on the back of the hand.

Table 2
Dimensional analyses, zeta potential and HR entrapment efficiency (EE) of the optimized NLC and SLN formulations. The results are the mean ± S.D. of three different batches.

Sample	Mean hydrodynamic diameter (nm)	PDI	Zeta potential (mV)	EE (%)
NLC_{24}	113.0 ± 1.2	0.219 ± 0.010	−24.2 ± 1.0	92.0 ± 1.0
NLC_{26}	119.0 ± 1.3	0.209 ± 0.018	−20.9 ± 0.5	90.3 ± 0.9
NLC_{27}	99.3 ± 2.2	0.144 ± 0.015	−20.5 ± 0.8	—
NLC_{28}	114.0 ± 1.7	0.142 ± 0,026	−23.5 ± 0.2	—
SLN_1	96.0 ± 1.1	0.192 ± 0.015	−21.7 ± 1.8	99.0 ± 0.7

n.c. not calculated

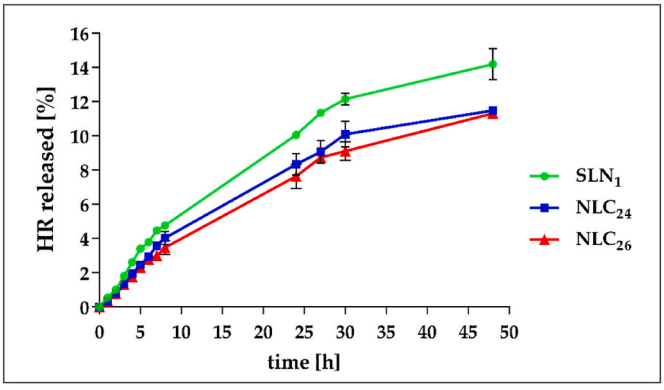

Fig. 3. Cumulative release profiles of HR from NLC and SLN formulations, obtained in a vertical Franz diffusion cell. The results are reported as mean values ± SD (n = 3).

triplicate.

2.7. Clinical skin lightening efficacy

This work was designed as a double-blind, single-center comparative study on NLC and SLN formulations efficacy, employing a cosmetic-clinical trial on 100 healthy Caucasian women (phototype Fitzpatrick II to III, 30–60 years old) in treatment for one month. The study was conducted from January 15, 2019, to November 7, 2021.

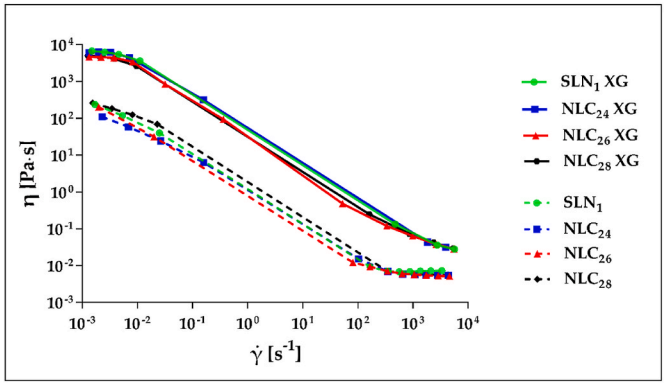

Fig. 4. Flow curves of optimized SLN and NLC formulations as prepared (dashed lines) and after addition of 1% *w/w* of xanthan gum (solid lines).

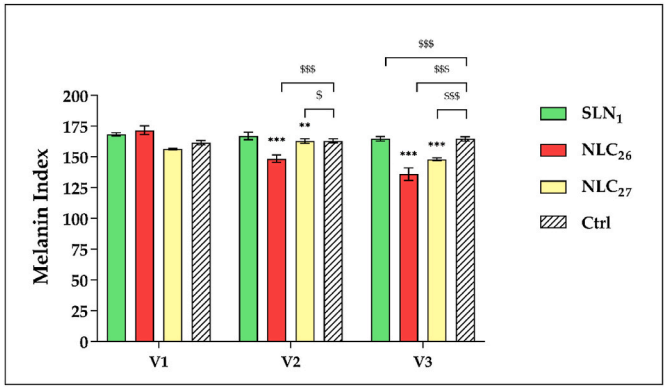

Fig. 5. Average skin melanin content ± SEM detected with Mexameter® MX 18 at every visit day in all groups treated. Measurements were carried out before treatment (V1) and after 14 days (V2) and 28 days (V3) of treatment with SLN and NLC formulations. Ctrl group was treated with formulation NLC_{28} prepared using labrafac and not containing HR. (Difference *vs* baseline ***p < .001, **p < .01, *p < .05; difference *vs* ctrl $^{\$\$\$}$p < .001, $^{\$\$}$p < .01, $^{\$}$p < .05).

Participants were randomized in a 1:1 ratio to either the active treatment groups (NLC_{24}, NLC_{26}, NLC_{27} and SLN_1) or the inactive control group (ctrl – NLC_{28}) using the statistical software SPSS 21 (IBM Corp.). The study protocol adhered to the Declaration of Helsinki and Good Clinical Practice guidelines. The mandatory requirements for the participants were as follows: no evidence of skin disorders, no history of chronicle system diseases, no exposure to UV rays, and no employment of any topical medications of glucocorticoids or retinoids. Exclusion criteria were as follows, too: subjects with phototype Fitzpatrick I, who were using any replacement hormones, undergoing an aesthetic procedure, such as acid peeling or laser, and pregnant or lactating females. Participants were selected according to the criteria mentioned above. All subjects voluntarily participated in the study and signed a written informed consent after a full explanation of the risks and benefits of the treatment.

An overview of the study timeline is illustrated in Fig. 1. After the initial screening visit, a mandatory washout period of 14 days was required before study entry. During the baseline and each follow-up visit (14 and 28 days from the start of treatment), participants were assessed for transepidermal water loss (TEWL), corneometry, colorimetry, and melanin content.

2.7.1. Protocol

Panelists applied the test products on their cleansed and dry hands

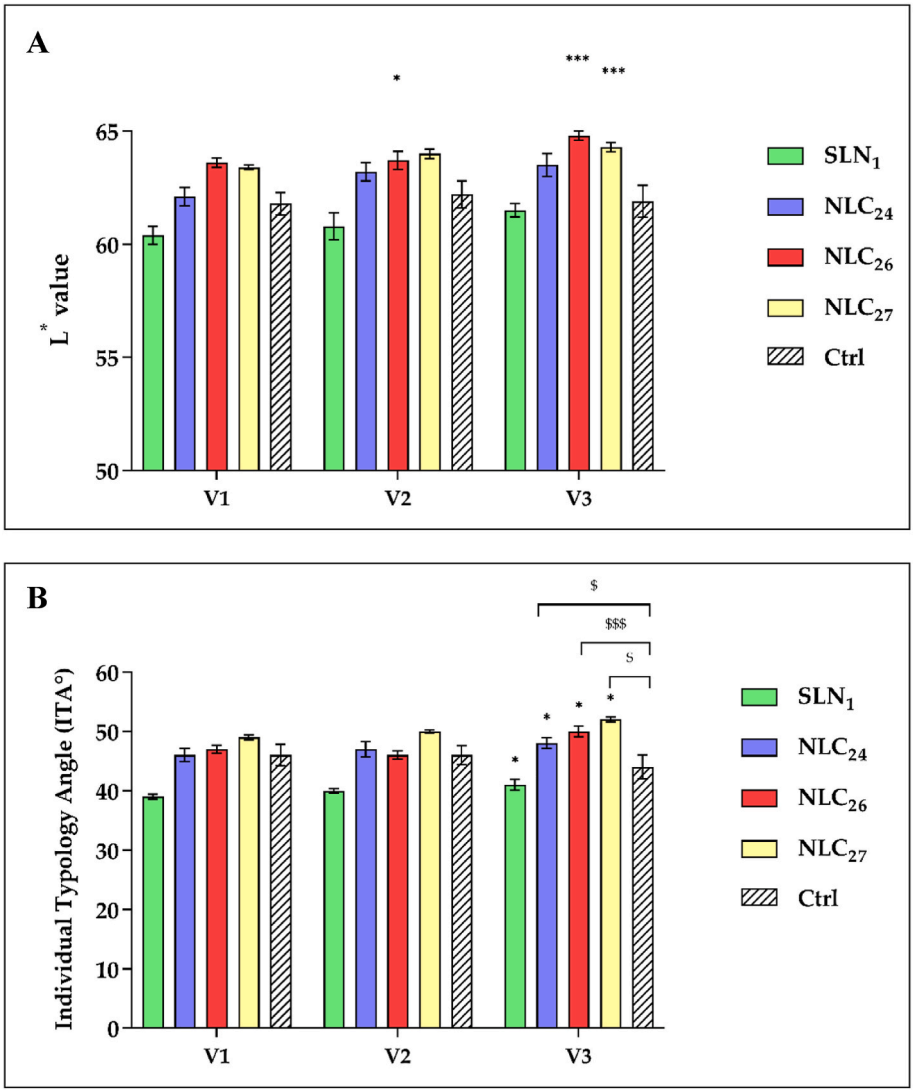

Fig. 6. A) Average L* values ± SEM, and B) sverage ITA° values ± SEM detected with Skin-Colorimeter® CL 400 at every follow-up visit in all groups treated. Measurements were carried out before treatment (V1) and after 14 days (V2) and 28 days (V3) of treatment with SLN and NLC formulations. Ctrl group was treated with formulation NLC_{28} prepared using labrafac and not containing HR. (Difference *vs* baseline ***p < .001, **p < .01, *p < .05; difference *vs* ctrl $^{\$\$}$p < .001, $^{\$\$}$p < .01, $^{\$}$p < .05).

twice daily (morning and evening), gently massaging until completely absorbed.

Subjects were evaluated for the final estimation on the last day before the study as a baseline, named visit 1 (V1). During the study, the exact measurements were conducted on subjects on days 14 (V2) and 28 (V3), respectively. The subjects were not allowed to use other cosmetic products on their hands during the whole study period.

2.7.2. Evaluations of skin color and skin physiological condition

At every visit day, the subject's skin color and melanin level were measured after 30 min of stationing. Skin readings were performed through devices for non-invasive clinical research, compatible with human subjects' safety requirements, as described previously [43]. The subject's hand color and melanin levels were measured on three back-hand positions using reflectance spectrophotometry with Mexameter® MX 18 and Skin-Colorimeter® CL 400 (Fig. 2). The panelists' skin hydration and transepidermal water loss were also quantitatively analyzed on the same three hand positions with Corneometer® CM 825 and Tewameter® TM 300 (Courage + Khazaka electronic GmbH, Germany) to assess overall skin improvement and formulations' skin compatibility. In this study, test subjects' hands were photographed using a digital camera under a unique light to ascertain measured parameters.

2.7.3. Safety

Observances and evaluations for adverse effects experienced by any subjects were tracked throughout the study.

2.8. Statistical analysis

All data were presented as mean percentage variation in each evaluation category of each visit date. The comparison of measurement results between groups (V2, V3) and baseline (V1) was done by ANOVA and Student's t-test, respectively, by using GraphPad Prism, with p significant at <.05.

3. Results and discussion

3.1. Optimization of NLC and SLN formulations

The main goal of this study was to exploit the synergistic effect between HR and GO for the treatment of hyperpigmentation skin disorders. Therefore, in order to obtain the dual delivery of these compounds, NLC formulations were developed using Precirol ATO 5 as solid lipid phase.

This lipid was chosen considering the melting point of HR (mp = 65–67 °C). Indeed, it is reported that the use of a lipid phase having a lower melting point than the molecule to be encapsulated, leads to the

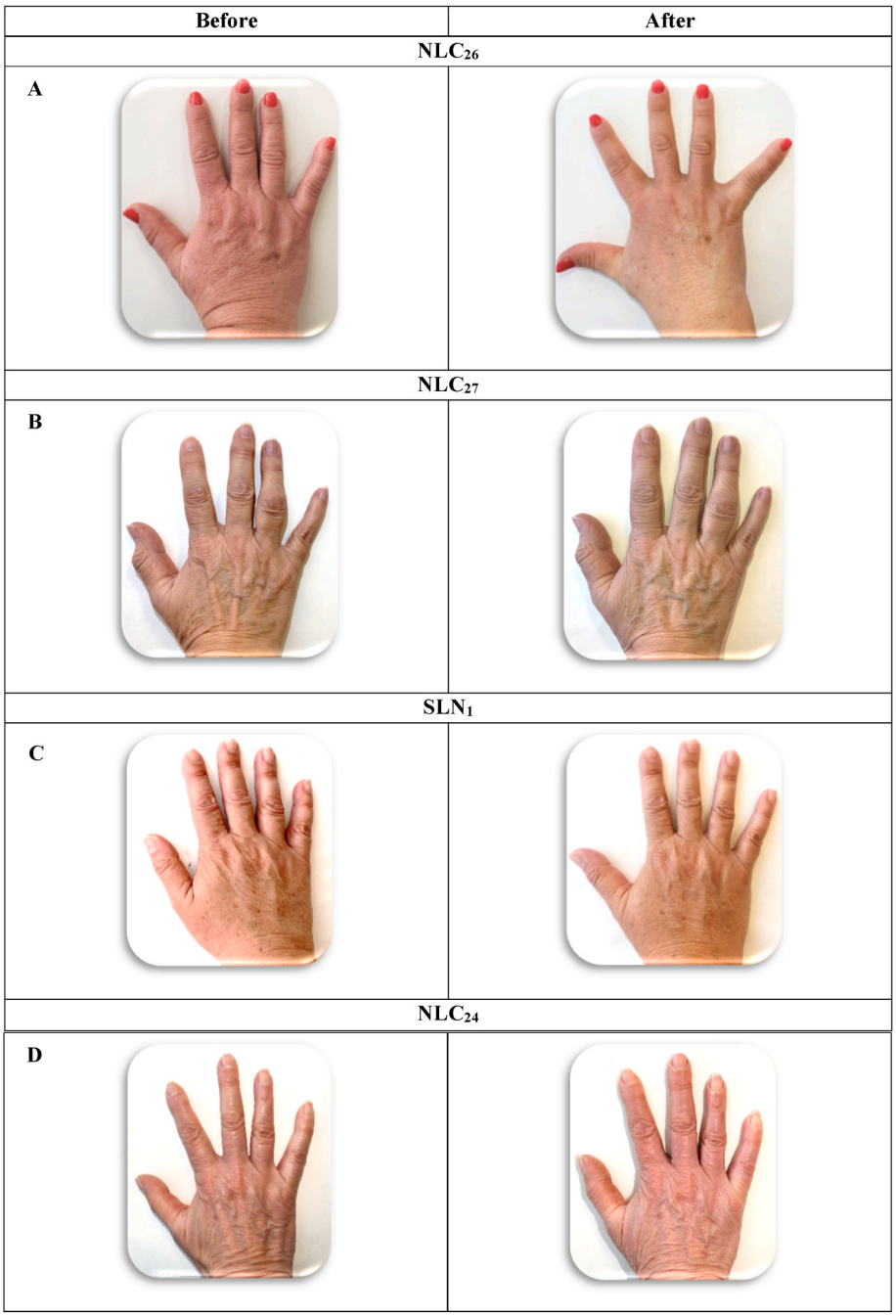

Fig. 7. Images of the backhands obtained before and after topical treatment with A) NLC_{26}, B) NLC_{27}, C) SLN_1, D) NLC_{24}.

formation of particles having a core enriched with the active principle, due to the different kinetics of solidification of the two components [44]. This feature should bring to a better encapsulation of HR within the lipid nanoparticles. In view of previous studies [45,46], two surfactants at different HLB were used. Previously reported formulations were prepared using a non-ionic surfactant (Poloxamer 188) and an ionic one (sodium cholate), however the use of ionic surfactants in cosmetic products should be avoided because of their irritating effect. Indeed, several reports on interactions between anionic surfactants and the skin, particularly the stratum corneum, have been reported in the literature [47–51]. Possible consequences of such interactions include impairment of the skin barrier function, increase in TEWL and decrease in skin hydration. As a result, the skin may become irritated, dry or red. Therefore, the first part of this study was focused on the substitution of sodium cholate with a hydrophobic non-ionic surfactant for the preparation of NLC formulations, while Poloxamer 188 (P188, HLB = 29) was maintained as the hydrophilic surfactant. Sucrose distearate (SP30, HLB = 6) and SPAN85 (HLB = 1.8) were selected for the scope and were respectively combined with P188 in different ratios, in order to find the best conditions for the formation of stable nanoparticle dispersions. The choice for these fatty acid esters was driven by environmental concerns about the effects of conventional tensides [52]. The optimization study of NLC formulations was carried out blending Precirol ATO 5 with labrafac, an oil composed of triglycerides of caprylic and capric acid, which was used as liquid lipid of the NLC systems. All the prepared formulations are reported in Table 1. It can be observed that the presence of the co-surfactant is needed to allow the formation of NLC nanoparticles, because the use of P188 alone determined the formation of a semisolid emulsion (NLC_1). Between the two co-surfactants tested, SP30 was able to stabilize the formation of NLC nanoparticles over a

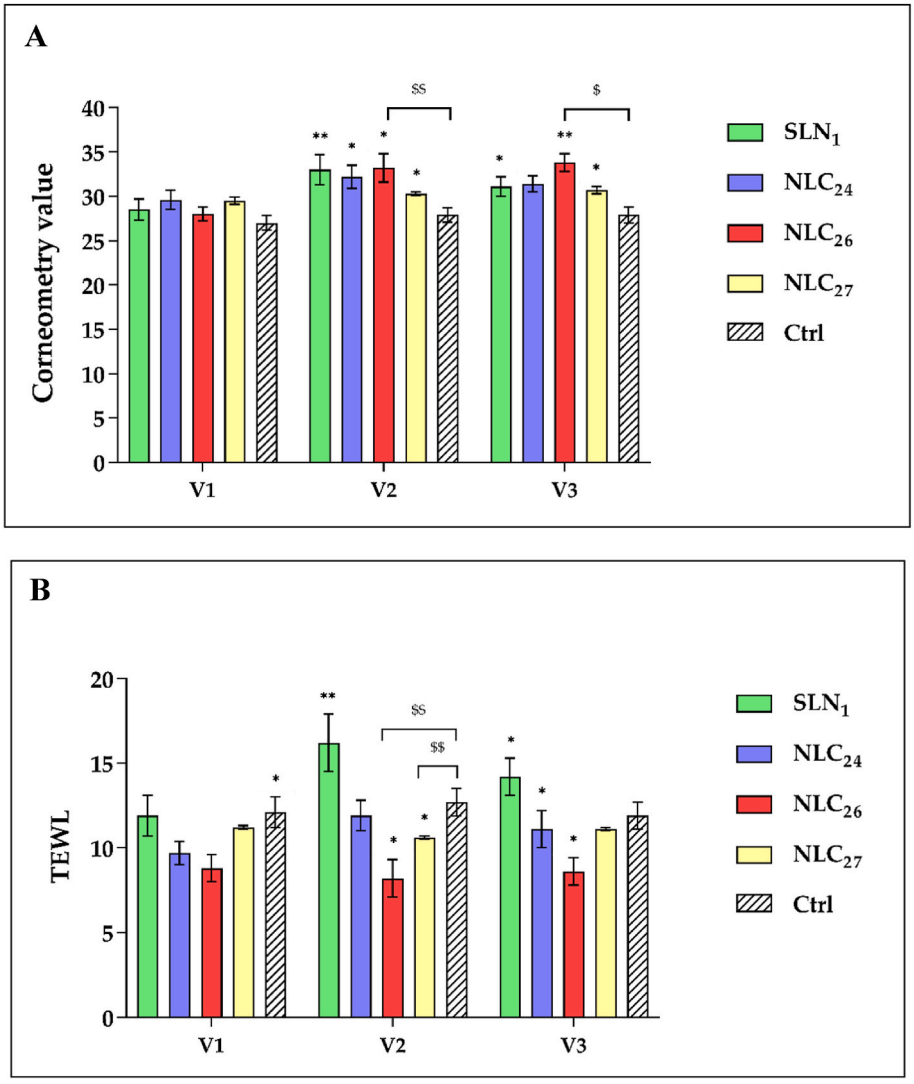

Fig. 8. A) Average corneometry values ± SEM, and B) average TEWL values ± SEM detected with Tewameter® TM Hex at every follow-up visit in all groups treated. Measurements were carried out before treatment (V1) and after 14 days (V2) and 28 days (V3) of treatment with SLN and NLC formulations. Ctrl group was treated with formulation NLC_{28} prepared using labrafac and not containing HR. (Difference *vs* baseline $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$; difference *vs* ctrl $^{\$\$\$}p < .001$, $^{\$\$}p < .01$, $^{\$}p < .05$).

wider HLB_{MIX} range (from 17.5 to 26.7), while SPAN85 allowed the NLCs formation only at the specific HLB_{MIX} value of 24.4, whereas all the other ponderal ratios tested brought to thickened emulsions or phase separation. The different behaviour may be dependent on the difference in the specific HLB of SP30 and SPAN85, which determines a different arrangement at the o/w interface. Therefore, formulations NLC_5, NLC_6 and NLC_7 were selected for the encapsulation of HR, thus obtaining the corresponding NLC_{20}, NLC_{21} and NLC_{22} samples. In all the cases, the maximum HR loading capacity within these NLC structures was 0.5% *w/w*. Indeed, higher amounts of HR brought to phase separation or to the formation of inhomogeneous dispersions with macroscopic aggregates. Given the chemical structure of HR and considering its affinity for the lipid phase (logP = 3.45), it was supposed that it could have altered the optimal hydro-lipophilicity ratio obtained with the two surfactants. In specific, it could have been placed near to the nanoparticles surface, thus playing a surfactant-like role and competing with the co-surfactant for the stabilization of the NLC dispersion. To prove this hypothesis, further formulations were prepared using only the most hydrophilic surfactant (P188), while the use of the co-surfactant was completely avoided. In specific, these formulations were prepared using 4.0, 4.5 and 5.0% *w/w* of P188 (NLC_{23}, NLC_{24} and NLC_{25}). All the compositions resulted in the formation of homogeneous nanoparticles dispersions. Moreover, under these conditions, it was possible to increase the loading capacity up to 1.0% *w/w*, which corresponds to the concentration usually employed in commercially available cosmetic products used for hyperpigmentation purposes [53,54]. These results, joined with the observation that P188 alone is unable to stabilize NLC dispersions, effectively confirm the co-surfactant role played by HR within these nanoparticles. So, in summary, it was possible to increase the loading capacity of the NLC delivery system, substituting the most lipophilic surfactant with HR in the formulation. Among the three different samples (NLC_{23}, NLC_{24} and NLC_{25}) obtained using different amounts of P188, the NLC_{24} one resulted the most stable and homogeneous formulation, therefore, this composition was used for the preparation of an NLC formulation containing ginger oil in place of labrafac (sample NLC_{26}). Moreover, to better evaluate the synergistic effect between GO and HR, HR-loaded SLN and GO-based NLCs (not containing HR) were also prepared. Stable and homogeneous HR-loaded SLN (formulation SLN_1) and GO-based NLC (formulation NLC_{27}) were obtained using P188 alone (4.5% *w/w*). While the formation of a stable SLN dispersion can be explained by the surfactant-like effect of HR, GO-based NLCs may have been stabilized by the phenolic constituents of GO [23,55].

3.2. Physical-chemical characterization

The optimized formulations (NLC_{24}, NLC_{26}, NLC_{27}, NLC_{28} and SLN_1) were characterized for the hydrodynamic diameter, PDI and zeta potential and the results are reported in Table 2.

All the formulations showed a monomodal distribution of nanoparticles with values of mean hydrodynamic diameter in the range from 96 to 119 nm and PDI values in the range from 0.144 to 0.219. It can be observed that the presence of HR causes a small increase in the polydispersity of the nanoparticles distributions (NLC_{24}, NLC_{26} and SLN_1). Instead, NLCs containing GO (NLC_{27}) show slightly smaller mean hydrodynamic diameter, than NLCs formulated with labrafac (NLC_{24}, NLC_{26} and NLC_{28}). This difference can be explained considering the different chemical composition and interfacial tension of labrafac compared to the more hydrophilic GO.

The EE of HR in the different formulations (SLN_1, NLC_{24} and NLC_{26}) was evaluated in a direct way, by extraction of the active molecule from the nanoparticles. To this end, it was first necessary to separate the unencapsulated HR. First attempts were made by ultracentrifugation, which did not allow reaching an adequate separation. Therefore, the separation was accomplished by SEC carried out on a Sephadex G75 column. The eluted nanoparticles were freeze-dried and then submitted to solid-liquid extraction, and the amount of HR determined by HPLC-DAD analysis. The obtained values were corrected considering the efficiency of the extraction process, which was determined carrying out the extraction procedure on complete SLN and NLC formulations, not submitted to SEC purification. The final EE results are reported in Table 2. All the analyzed formulations were able to efficiently entrap high percentages of HR, in accordance with the lipophilic nature of the molecule (logP = 3.45).

3.3. Release profiles of HR from SLN and NLC formulations

The release profile of HR from SLN_1, NLC_{24} and NLC_{26} formulations was studied in a vertical Franz diffusion cell using a mixture EtOH/acetate buffer (pH 5) in the acceptor chamber. The obtained results are reported in Fig. 3 and show, in all the cases, a sustained release of HR. It can be observed that the release profile does not change significantly by varying the composition of the NLC formulations, whereas SLNs release higher percentages of HR compared to NLC ones.

These results suggest that HR tends to settle better within the irregular lipid matrix of NLCs, compared to the more ordered matrix characteristic of SLNs [56]. However, no recrystallization and HR expulsion occurred from the SLN and NLC formulations over time. Indeed, release studies carried on SLN and NLC formulations at time zero and after 3 and 4 weeks from the preparation showed superimposed release profiles (data not shown). Furthermore, no signs of nanoparticles aggregation, flocculation or creaming were observed up to 4 weeks from the preparation.

3.4. Rheological characterization of the NLC and SLN formulations

Viscosity represents a primary requisite to provide the proper dose of a topical formulation from the container and allow its spreadability on the skin. Therefore, rheological studies were conducted on the NLC_{24}, NLC_{26}, NLC_{27}, NLC_{28} and SLN_1 formulations. In all the cases, as expected, a pseudoplastic behaviour was obtained, scarcely influenced by the characteristics and nature of the nanoparticles. The pseudoplastic trend is ideal for a pharmaceutical or cosmetic formulation, but, unfortunately, the viscosity values of the developed formulations were too low to allow proper application on the skin (Fig. 4). This feature could limit the efficacy of the NLCs and SLNs, for this reason, a thickening agent was added to all the formulations. In particular, two different viscosifying agents were evaluated, namely sepimax zen and xanthan gum. They were able to produce the same increase in the viscosity values when used at 0.2% *w/w* and 1.0% *w/w* respectively (data not shown). However, shorter dispersion and hydration times were needed by xanthan gum, and, for this reason, it was chosen to thicken all the optimized NLC and SLN formulations.

3.5. Whitening and depigmenting action

The optimized NLC and SLN formulations were evaluated for their whitening and depigmentation activity. Hyperpigmentations were monitored on darker areas of the test subjects' backhand to assess and compare the test products whitening action. For this purpose, Mexameter® MX 18 was employed; the probe detects melanin content on the skin surface through an absorption/reflection method. More precisely, two light wavelengths (660 nm-red and 880 nm-near-infrared) were emitted to specifically detect melanin. Skin instrumental readings suggest a decreasing trend of the melanin content throughout the 4-week treatment with the tested NLC and SLN formulations compared to baseline (V1). Whitening and depigmenting actions are evident for the NLC_{26} preparation, which is the unique formulation showing the activity as early as two weeks of application (V2). After four weeks, all preparations depleted the pigmented treated area, but NLC ones were more effective than SLN_1, indicating higher percentages of melanic index change (Fig. 5).

These results can be related to the nanostructures releasing profiles. More precisely, kinetics analysis shows a massive, albeit prolonged, release of HR by SLNs, whereas NLCs shows a more controlled release profile (Fig. 3). This behavior of NLCs may allow the skin guarding the activity over time, leading to increased efficacy, probably due to receptor and enzyme non-saturation.

Lastly, the outcomes suggest that the encapsulation of GO in NLC_{26} and NLC_{27} formulations contributes to area whitening, influencing the extent of brightening and the onset time.

3.5.1. Skin lightening and brightening

Skin color was measured (Skin-Colorimeter® CL 400) through the CIELab system, defined by the International Commission on Illumination (CIE), in cooperation with Technical Committee ISO/TC 274 [57].

In the system mentioned above (also known as L*a*b*), each letter represents an axis: L* refers to the black-white axis, a* is the green-red axis, and b* is the blue-yellow one [58,59].

The Individual Typology Angle ITA° was automatically calculated from L* (luminance) and b* (yellow chromatic) to objectify skin color [60]. Since L* refers to the white-black axis, where 100 represents the maximum value, which is precisely white, its increase is desirable to demonstrate the lightening effectiveness of a topical treatment. Similarly, ITA° associates a very light complexion with values greater than 55°. Therefore, a rising variation of its basal value indicates a lighter phototype.

Topical parameters about the extent of the pigmentation of the test subjects' backhands, such as L* and ITA°, showed a similar increasing trend for all tested formulations, confirming their lightening property, with a slightly higher degree for the NLC_{26} formulation (Fig. 6A–B).

Fig. 7A–D presents representative before and after treatment images.

3.5.2. Skin physiology assessment

All treatment and control groups showed higher water content on days 14 and 28, than their respective baseline values ($p < .05$ for all follow-up visits). The control group (treated with NLC_{28}) also showed higher values on days 14 and 28, suggesting the occlusion exerted by the topic preparations. Changes in TEWL values from baseline were not similar between groups during all follow-up visits. SLN formulations significantly altered the skin barrier leading to water loss increases. This action can be explained by the massive release of HR implemented by SLNs. As is known [61], HR is an irritant, and its concentrated solutions can cause burns on the skin and mucous membranes. Notably, lipophilic compounds may accumulate at the stratum corneum's level, resulting in less site-specific direction and less penetration into deeper layers of a more polar nature. Similarly, NLCs formulated with labrafac (NLC_{24}) resulted in more significant water loss than NLC_{26}. Evidently, in NLC_{26}, the gradual release of the two substances means that high concentrations of neither HR nor GO are reached (Fig. 8A–B).

4. Conclusions

GO and HR were successfully encapsulated within lipid-based nanoparticles of both SLN and NLC type. GO was primarily used for its inhibitory effect on tyrosinase activity, anyway when blended with the solid lipid Precirol ATO it played the dual role of active ingredient and excipient of the corresponding NLC formulations. The inclusion of GO within the lipid matrix of Precirol-based SLNs allowed for a better encapsulation and a slower release of HR, which resulted in more efficacious depigmenting effect. Indeed, both SLNs and NLCs were evaluated for their ability to modify skin pigmentation. When applied twice-daily for 28 days, both SLNs and NLCs showed decreased skin pigmentation and augmented skin lightness, compared to the control group. However, the NLC formulation containing both HR and GO was able to achieve a greater lightening effect. Moreover, the NLC treatment group showed improvements related to the skin barrier when compared to both the control and the SLN group. In summary, the current analysis demonstrates the efficacy of the NLCs and SLNs as delivery systems suitable to reach the deepest skin layer and to perform a prolonged release of depigmenting and whitening active ingredients. Moreover, NLCs present themselves as more appropriate vehicles for cosmetic use since they can act without altering skin physiology. However, further development of the NLC formulations is needed and it could consider the use of other dermo-compatible lipid components, which, in addition to contributing to the delivery of the active principles, can efficiently restore the skin hydrolipidic film and exert a moisturizing and emollient effect.

Author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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