

Formulation and characterization of advanced nanostructured lipid carriers (NLC) incorporating coenzyme Q10 and licorice extract to explore their synergistic anti-oxidant and antityrosinase effects

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ARTICLE INFO

Article type:

Original

Article history:

Received: May 10, 2026

Accepted: Aug 11, 2026

Keywords:

Anti-tyrosinase

Coenzyme Q10

Licorice extraction

NLC

sROS inhibition

Synergistic effect

ABSTRACT

Objective(s): The development of novel nanostructured lipid carrier (NLC) formulations incorporating coenzyme Q10 (Co-Q10) and licorice extract (LE) was investigated to evaluate their synergistic effects as an anti-oxidant and antityrosinase agent, respectively.

Materials and Methods: LE was obtained by maceration and quantified by HPLC. The NLCs F1-F8 were prepared and thoroughly characterized for particle size, entrapment efficiency (EE) %, and long-term stability. The cytotoxicity and protective effects of the NLC formulations on B16F10 murine melanoma cells were evaluated by MTT assay, ROS levels by DCFH-DA assay, and anti-tyrosinase activity. Melanin content was determined spectrophotometrically.

Results: The ethyl acetate fraction of LE, containing 6.59 mg/g of liquiritin and 31.71 mg/g of glabridin, was successfully encapsulated with Co-Q10 in the NLC matrix. Characterization of the formulations (F1 to F8) demonstrated favorable surface properties, with Z-average sizes ranging from 146 to 248 nm, polydispersity indices (PDI) between 0.21 and 0.27, and zeta potentials from -5 to -15 mV, indicating acceptable physicochemical stability over six months and EE% exceeding 69%. Furthermore, these formulations significantly reduced reactive oxygen species (ROS) levels and tyrosinase activity in cell assays compared to controls, without exhibiting cytotoxic effects. Notably, NLCs with 1:1, 1:0.5, and 0.5:1 Co-Q10/LE ratios showed greater efficacy than the individual components, with formulation F8 (Co-Q10/LE: 0.5/1) displaying the most pronounced synergistic effect.

Conclusion: The Co-Q10/LE-NLC formulations (F1-F8) demonstrate stable physicochemical characteristics, indicating their suitability for topical use in anti-aging and skin-lightening treatments.

► Please cite this article as

Atapour-Mashhad H, Hoseinsalari A, Tayarani-Najaran Z, Asili J, Sedighidarijani A, Golmohammadzadeh Sh. Formulation and characterization of advanced nanostructured lipid carriers (NLC) incorporating coenzyme Q10 and licorice extract to explore their synergistic anti-oxidant and antityrosinase effects. Iran J Basic Med Sci 2027; 30:

Introduction

Skin aging is a complex process influenced by both internal and external factors (1). Among these, ultraviolet (UV) radiation plays a significant role in accelerating aging; UV rays, including UVA and UVB, penetrate the skin and generate reactive oxygen species (ROS) (2), leading to oxidative stress and damaging essential cellular components such as DNA, proteins, and lipids (3). As a result, collagen breaks down, and procollagen and hyaluronic acid production decreases, contributing to wrinkles and

reduced skin elasticity (4). Another consequence of UV-induced damage is hyperpigmentation. UV rays serve as the primary physiological trigger for melanogenesis, influencing melanocytes directly or indirectly through the release of factors such as α -MSH (α -melanocyte-stimulating hormone) (5). Although melanin offers protection against UV rays (6), excessive production can lead to pigmentation issues (7). Tyrosinase, a copper-dependent enzyme, is crucial for melanin synthesis and catalyzes several key steps in this process (8). Inhibiting tyrosinase activity can reduce

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melanin synthesis and prevent pigmentation (9).

Anti-oxidants such as niacinamide (vitamin B3), vitamin E, vitamin C, resveratrol, glutathione, curcumin, and coenzyme Q10 (Co-Q10) are known to enhance collagen production, protect against UV damage, and provide anti-inflammatory benefits (10). These compounds improve skin elasticity and help reduce signs of aging (10). Among anti-oxidants, Co-Q10 is unique because cells synthesize it naturally (11). It is essential for energy production and helps safeguard against oxidative damage (12), inhibits lipid peroxidation, and shields tissues from ROS (12). Regrettably, Co-Q10 levels in the skin decrease with age, and using Co-Q10-containing products with high skin penetration could meet the skin's needs (13). Co-Q10's limited water solubility (0.193 µg/ml (14), high lipophilicity (logP > 10), substantial molecular weight (863.36 g/mol), and susceptibility to degradation from light exposure hinder its effectiveness in medicine and its ability to penetrate the skin (15).

Licorice root from *Glycyrrhiza glabra* L. is valued for its medicinal properties and is native to Asian regions, such as Iran, and some Mediterranean countries (16). It contains various beneficial compounds like triterpenoid saponins, flavanones, chalcones, flavones, isoflavones, isoflavanes, and coumarins, which exhibit antibacterial, antiviral, anti-inflammatory, antidiabetic, and anti-oxidant properties (17). Among these licorice phytochemicals, glabridin and liquiritin are particularly noted for their skin-lightening properties (18). They have a logP value of less than 5 (19). Liquiritin, a flavonoid with a flavanone glycoside, disperses melanin and enhances its elimination from the epidermis (20). It also reduces inflammation and erythema caused by UV rays (21). Glabridin, another flavonoid with a pyranisoflavan structure, is a potent tyrosinase inhibitor and exhibits skin-whitening activity 16 times stronger than hydroquinone (22). However, glabridin has poor skin permeability and chemical stability (23). Therefore, a formulation containing Co-Q10 and licorice extract (LE) is needed to improve chemical stability and bioavailability.

Nanostructured lipid carriers (NLCs) are innovative lipid-based nanoparticles designed to enhance the delivery of active substances in topical formulations (10). They consist of a blend of solid lipids, liquid lipids, and surfactants that collaborate to enhance the stability and effectiveness of the encapsulated substances (10). NLC formulations in topical products can significantly increase the chemical stability of active ingredients, lower toxicity levels, and boost skin hydration thanks to their occlusive properties (24).

This study explores the creation of innovative skincare products by formulating new NLCs that incorporate a combination of Co-Q10 (an anti-aging agent) and LE (a skin-lightening agent) in ratios of 1/1, 1/0.5, and 0.5/1. The research investigates their synergistic effects through surface characterization, long-term stability assessments, entrapment efficiency measurements, modulation of ROS levels, inhibition of melanin production, and antityrosinase activities.

Materials and Methods

Materials

Materials used in this study include glyceryl behenate (Compritol® 888 ATO) and glyceryl palmitostearate (Precirol® ATO 5) generously provided by Gattefossé (Pvt. Ltd., France). Glyceryl tripalmitate (tripalmitin) was purchased from Fluka (Germany), while glycerol monostearate (GMS), cetyl palmitate, dimethyl sulfoxide

(DMSO), poloxamer 188, Span® 80, N-acetylcysteine (NAC), kojic acid, phenylmethanesulfonyl fluoride (PMSF), L-3,4-dihydroxyphenylalanine (L-DOPA), and 2',7'-dichlorofluorescein diacetate (DCFH-DA) from Sigma-Aldrich Co. (Deisenhofen Germany); stearic acid and cetyl alcohol from Merck (Germany), and HPLC grade coenzyme Q10 (purity: >98%) was obtained from Tinab Shimi Khavarmianeh (Mashhad, Iran); HPLC grade ridin glabridin and liquiritin (purity: >98%) were acquired from Nanjing Dilger Medical Technology (Nanjing, China), and Tween 80 was acquired from Kimyagaran Emrooz (Tehran, Iran), oleic acid and medium-chain triglyceride (MCT) were obtained from Oxin Chemistry Company (Kh. Razavi, Iran); methanol (analytical grade) and ethyl acetate were sourced from Dr. Mojallali Industrial Chemical Complex Co. (Tehran, Iran), HPLC grade solvents, methanol and acetonitrile were acquired from Tedia (Fairfield, OH, USA); tetrahydrofuran (THF) was sourced from CARLO ERBA (Emmendingen, Germany). All chemicals utilized were of analytical grade, with a purity greater than 98%.

The fetal calf serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Gibco, located in Grand Island, New York, USA. Resazurin (AlamarBlue®) was sourced from BioSource Invitrogen, while doxorubicin (DOX) at a concentration of 10 mg/5 ml was acquired from Ebewe.

Plant material collection and authentication

The roots of *G. glabra* were acquired from Shirin Darou Company in Shiraz, Fars, Iran, on May 9, 2021. After authentication and assignment of the collection number (PMP-1255), voucher specimens were lodged in the Herbarium of the Department of Pharmacognosy at Tehran University of Medical Sciences, Tehran, Iran, for documentation and verification.

Preparation of licorice extract

Subsequently, the dried roots were finely crushed using an electric mill before extraction. About 200 g of the ground roots were then placed in an Erlenmeyer flask and macerated three times with methanol at 24-, 48-, and 72-hour intervals (25). Following maceration, the concentrated methanolic extract was fractionated using ethyl acetate three times. The solvent was separated with a rotary evaporator. The residual solvent was eliminated using a Laminar Flow Hood and a freeze-dryer. Finally, the ethyl acetate fraction was prepared for encapsulation in NLC formulations.

HPLC analysis

Glabridin and liquiritin, used as quantitative standards for LE, together with Co-Q10, were analyzed using high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). The HPLC system was supplied with a C18 reversed-phase column (5 µm, 250×4.6 mm; Perfect Sil Target ODS-3), a solvent delivery unit (LC-20A pump), and a UV-VIS detector (SPD-20A), following the guidelines outlined in USP35. All samples were injected using a fixed 20 µl loop with a Rheodyne® Model 3725i valve. For the analysis of glabridin and liquiritin, a mobile phase of acetonitrile and water was used at a flow rate of 1 ml/min, considering the polarity of these compounds (19). A gradient solvent system ranging from 10:90 to 90:10 (v/v) acetonitrile and water was implemented, as detailed in Table S1 of the supplementary file. The UV wavelength for glabridin detection was 280 nm (26), and for liquiritin (27), it was 276 nm. The retention

times for glabridin and liquiritin were approximately 38-39 min and 16-17 min, respectively. The standard curves were constructed at six points with 0.03-1 mg/ml concentration ranges.

To quantify Co-Q10, a Lichrospher C8 column (5 μ m, 4.6 mm ID, 25 cm; Hanbag, Dalian, China) was employed. The mobile phase was an isocratic solvent system of 95:5 (v/v) methanol and tetrahydrofuran. UV detection was set to 275 nm, with Co-Q10 exhibiting a retention time of 5 to 6 min. A standard six-point calibration curve was established at concentrations ranging from 0.06 to 2 mg/ml (28). This procedure was carried out in triplicate for each compound.

Standard and sample solution preparation procedures

Stock solutions of glabridin and liquiritin were prepared at 1 mg/ml in methanol. A Co-Q10 stock solution was prepared at 2 mg/ml in a mixture of methanol and THF (50:50). These solutions served as the basis for constructing the calibration curves.

Various aliquot concentrations were obtained by diluting the initial stock solutions. The resulting concentrations were as follows: glabridin and liquiritin ranged from 1 to 0.0312 mg/ml; Co-Q10 ranged from 2 mg/ml to 0.0625 mg/ml. Before each injection, a 0.22-micron syringe filter was used for standard and sample solutions. Samples were injected at constant volume using a Rheodyne[®] injector Model 3725i valve equipped with a 20 μ l fixed loop.

Solubility test of active ingredients

To develop an effective formulation of NLC containing Co-Q10 and LE, the solubility of the active ingredients in several solid lipids, including Precirol, Tripalmitin, Compritol, cetyl palmitate, stearic acid, and GMS, as well as in liquid lipids like oleic acid and MCT oil, was assessed. Additionally, surfactants such as Tween 80, Poloxamer 188, and Span 80 were evaluated. The solubility tests were conducted based on the NLC formulation; for instance, 50 mg of LE was dissolved in 350 mg of each solid lipid.

The solutions were heated in a water bath to around 10 °C above the melting point of the lipids and maintained at this temperature while being stirred continuously for 10 min. The criteria for determining dissolution of the active ingredients within the lipid and surfactant matrix were the clarity of the resulting solution and the absence of sedimentation.

Formulation of Co-Q10/LE-NLCs

Initially, 50 mg of LE was combined with 100 μ L of an equal ratio of ethanol/propylene glycol, then heated to 75 °C in a water bath. Following this, the solid and liquid lipids and surfactant (as specified in Table 1) were added and melted at a temperature about 10 °C above the melting point of the solid lipid. Co-Q10 was then incorporated into the lipid phase and agitated until dissolved (approximately 1 min). Simultaneously, double-distilled water was subjected to identical thermal conditions as the lipid mixture. The phases were combined and homogenized using an Ultra-Turrex (Heidolph DIAK) in three sequential steps: 1.5 min at 18,000 rpm, followed by 1.5 min at 23,000 rpm, and finally 1 min at 28,000 rpm. During homogenization, the mixture temperature was maintained approximately 5 °C above the melting point of the solid lipid (approximately 75 °C). The mixture underwent sonication for seven cycles, each lasting 60 sec, with a 15-second resting period between cycles, using

a Bransonic sonicator probe (USA) (28). Subsequently, the solution was gradually cooled to ambient temperature to form a nanoemulsion (28).

The exact amounts of active ingredients in the NLCs (equivalent amounts in percentage, moles, and μ g/ml) are given in Table S3 in the supplementary file. During NLC preparation, the LE's high melting point (140 $\pm$ 2 °C) reduces the risk of degradation at the process temperature (70 °C). Earlier research has investigated the compatibility of Co-Q10 in NLC and SLN preparations, confirming its suitability for the process (29). This suggests that the NLC preparation process for LE and Co-Q10 is likely to be stable and effective.

Physicochemical characterization of the NLC formulations

The Z-average size, polydispersity index (PDI), and zeta potential were assessed using dynamic light scattering (DLS) with a Zetasizer/Nano-ZS90 instrument from Malvern, UK. Measurements were taken at a 90° angle in 10 mm diameter cells at 25 °C with each measurement conducted in triplicate. Each measurement was repeated three times. Before analyzing particle size, the aqueous mixture of the NLC formulation was adjusted with distilled water to achieve the appropriate concentration (30).

Transmission electron microscopy (TEM) analysis

The selected Co-Q10/LE-NLC (F6) feature was observed using TEM (LEO 912 AB, ZEISS, Germany). Before experimentation, the specimen was diluted 1:50 (v/v) in deionized water. The diluted solution was dropped onto the carbon-coated copper grid and then stained with 2% uranyl acetate solution. After staining, the grid was left to dry at room temperature (31).

Thermal analysis of materials using differential scanning calorimetry

Thermal analysis of freeze-dried Co-Q10, solid lipid, blank NLC (drug-free), and Co-Q10/LE-NLC formulations was performed using a Differential Scanning Calorimeter (METTLER TOLEDO DSC822e, Gießen, Germany). Each sample (3 mg) was loaded into a 40 μ L aluminum DSC pan, sealed, and placed in the DSC instrument (32).

Key parameters such as melting point, onset temperature, and enthalpy were evaluated using STARe software. The DSC analysis was conducted over a temperature range of 0-200 °C at a heating rate of 10 °C/min under a nitrogen flow of 50 ml/min (33).

Analysis using Fourier transform infrared spectroscopy

Fourier transform infrared (FT-IR) spectra were obtained using a Thermo Nicolet AVATAR 370 FTIR. Some freeze-dried NLC was mixed with KBr salt and compressed into a transparent disk, which was then scanned in the region of 400-4000 cm^{-1} (34).

Entrapment efficiency (EE)%

To measure the EE%, an ultrafiltration Falcon (Amicon Ultra 30 kDa MWCO, Millipore, USA) was utilized to separate free active ingredients from the NLC lipid matrix (28). Approximately 2 ml of NLC formulation was placed into a filter tube and centrifuged for 10 min at 13,000 rpm and 4 °C using a Remi Elektrotechnik Ltd. centrifuge from Maharashtra, India. We applied direct and indirect methods to assess EE%. The indirect method involved analyzing

the filtrate that passed through the filter membrane using HPLC. In the direct method, 50 µl of the sample retained on the filter was dissolved in a 50:50 mixture of methanol and tetrahydrofuran at 50 °C for one minute. The solution was subsequently permitted to sit at room temperature for two hours to facilitate lipid sedimentation. The sample was then centrifuged for 10 min at 13,000 rpm and 4 °C, and the clear supernatant was analyzed using the HPLC system. The EE% was calculated using the formula, where C_T represents the total amount of Co-Q10/LE present in the system, and C_E indicates the amount of Co-Q10/LE found in the prepared sample.

$$EE\% = \frac{C_E}{C_T} \times 100$$

The analysis methods for Co-Q10 and LE are detailed in the HPLC analysis section.

Investigating stability over time

All formulated NLCs were stored in siliconized glass vials at 4 °C, 25 °C, and 40 °C to evaluate stability parameters, including color variation, uniformity, and phase separation, at the 1st, 3rd, and 6th months. Concurrently, the Z-average size, PDI, and zeta potential were measured (Table 3) (35).

Cellular growth and maintenance

The murine melanoma cell line B16F10 was sourced from the Pasteur Institute located in Tehran, Iran. These cells were cultured in DMEM supplemented with 10% (v/v) FBS, along with 100 units/ml penicillin and 100 µg/ml streptomycin. Culture conditions were maintained in a humidified environment at 37 °C with 5% CO₂ and 90% humidity.

All formulations were freshly prepared before each experiment, ensuring that the concentration of NLC in the culture media remained below 1% (v/v) (36). Given the relative solubility of Co-Q10 in alcohols, a stock solution was prepared in THF before each test and then diluted in the culture medium to provide free Co-Q10.

Assessment of cell viability

The cytotoxic effect of NLC formulations and their protective roles against H₂O₂ were evaluated using a modified MTT assay. In this procedure, B16F10 cells (10⁴ cells/well) were plated in a 96-well format and incubated for 24 hr. After this, the cells were treated with 1% (v/v) NLC formulations in cell culture medium for 24 hr. After removing the culture medium, a solution containing 10% MTT (5 mg/ml) was added to the wells, and the cells were incubated for a further four hours. The MTT solution was then discarded, and 100 µl of DMSO was introduced to dissolve the formed formazan crystals. Cell viability for each treatment concentration was assessed in triplicate at 570 nm, using DOX at 5 µg/ml as a positive control and untreated cells as a negative control.

Cellular ROS level determination

2', 7'-dichlorofluorescein diacetate (DCFH-DA) is a prominent reagent utilized for assessing oxidative stress levels (37). This compound can permeate cell membranes and is hydrolyzed to the DCFH carboxylate anion. Upon undergoing two-electron oxidation, DCFH is converted into dichlorofluorescein (DCF), which exhibits fluorescence (37). To measure reactive oxygen species (ROS) levels, DCFH-DA was employed in this study. Approximately 10⁴ of

B16F10 cells were plated in a 96-well format and pretreated for 24 hr with various treatments, including free Co-Q10, lipid carrier blank, Co-Q10/LE-loaded NLC formulations (ranging from 6 to 50 µg/ml), and N-acetylcysteine (NAC) as a positive control. Following this, 10 µM DCFH-DA was introduced to the cells, which were then exposed to H₂O₂ (10 mM) at 37 °C for 90 min. The fluorescence intensity of DCF was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm employing a fluorescent microplate reader (VICTOR X5, PerkinElmer, USA) (38).

Quantitative analysis of melanin

B16F10 cells (10⁴ cells/well) were seeded in a 12-well plate and treated with varying concentrations of NLC formulations alongside kojic acid as a positive control, followed by a 24-hr incubation. After treatment, cells were detached by trypsinization and washed with PBS. A 2 M NaOH solution was then added, and the plate was incubated at 100 °C for 30 min. Melanin levels in the treated cells were quantified at 405 nm using a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, USA) (39).

Tyrosinase activity assessment in cellular models

Cellular tyrosinase activity was assessed using a spectrophotometric method that measures the oxidation of DOPA to dopachrome (40). This assay helps determine the effectiveness of compounds in inhibiting the melanogenesis pathway by preventing this conversion. B16F10 cells were cultured and treated for melanin quantification, then washed with PBS and detached using trypsin. The cell contents were centrifuged (1100 rpm for 7 min), washed again, and lysed in a buffer containing 0.1 mM PMSF, 1% Triton X-100, and 100 mM sodium phosphate buffer adjusted to pH 6.8, using a freeze-thaw method for effective cell disruption. PMSF was replenished every 30 min due to its limited half-life. The resulting lysate underwent centrifugation at 10,000 rpm for 20 min at a temperature of 4 °C. After this, 100 µl of the supernatant was combined with an equal volume of 5 mM DOPA in a 96-well plate and incubated at 37 °C for two hours. The absorbance was then measured at 475 nm using a Synergy H4 Hybrid Multi-Mode Microplate Reader from BioTek, located in Winooski, USA.

Data analysis

Statistical analysis was performed using One-way ANOVA (GraphPad Prism 9.0; GraphPad Software, Inc., USA). A significance level of $P < 0.05$ was established for determining statistical significance. Isobolographic analysis was conducted with CalcuSyn software v. 2.1.

Results

Evaluation using HPLC techniques

Figure 1 displays the HPLC chromatograms for glabridin, liquiritin, and Co-Q10. The linear equations, limit of detection (LOD), limit of quantification (LOQ), and the squared correlation coefficients (r^2) for each compound are reported in Table S2 (supplementary file). The calibration curves were generated using Microsoft Excel 2016, and the mentioned parameters were obtained by the LINEST function.

Quantitative HPLC analysis indicated that the methanolic extract contained 8.21 mg/g of liquiritin and 11.51 mg/g of glabridin, and the ethyl acetate fraction contained 6.59 mg/g of liquiritin and 31.71 mg/g of glabridin.

The HPLC chromatogram of the ethyl acetate fraction is

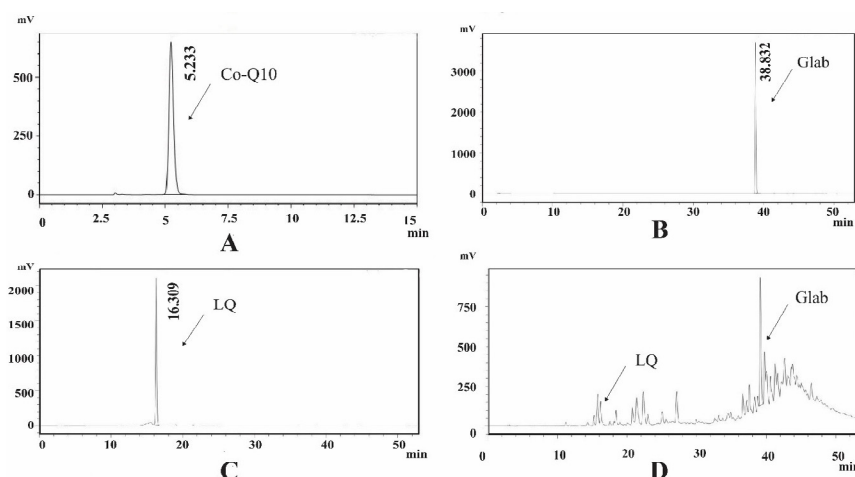


Figure 1. High-performance liquid chromatography (HPLC) chromatogram of the standard solution of coenzyme Q10 (Co-Q10) (1 mg/ml) (A), standard solution of glabridin (Glab) (1 mg/ml) (B), standard solution of liquiritin (LQ) (1 mg/ml) (C), and licorice extract (LE) (ethyl acetate fraction, 10 mg/ml) (D)

shown in Figure 1; liquiritin exhibited a shorter retention time than glabridin due to its more polar structure.

Optimization of Co-Q10/LE-NLCs formula

Based on the solubility test results, Compritol® 888 ATO (as the solid lipid), MCT oil (as the liquid lipid), and Tween 80 (as the surfactant) were chosen for the formulation of the desired NLCs.

Formulation F6, containing 5 mg/ml Co-Q10 and 5 mg/ml LE (highest level of ingredients), was selected for TEM analysis, along with F2 (containing 5 mg/ml Co-Q10) and F3 (containing 5 mg/ml LE), which were selected for thermal analysis and FT-IR evaluation. All prepared formulations were further used to investigate the combined effects of Co-Q10 and LE on ROS levels, melanin content, and anti-tyrosinase activity.

Physicochemical characterization of the Co-Q10/LE NLCs

Particle size is a critical surface property for nanocarriers, as it significantly impacts stability, EE%, drug release behavior, and biodistribution (41). Table 1 presents the Z-average size measurements for freshly prepared NLC suspensions. The Z-average size for the NLC formulations was observed to be less than 248 nm and ranged from 146 to 248 nm. In addition, an increase in particle size was observed in formulations containing a higher ratio of LE to Co-Q10.

The polydispersity index reflects the uniformity of particle size distribution within a given sample, with values ranging from 0.0, indicating a uniform particle size, to 1.0, which signifies a sample with a wide variety of particle sizes (41). Formulations F1 to F8 showed relatively low PDI values (0.21 to 0.27), indicating a narrow particle-size distribution.

Zeta potential indicates surface charge and plays an important role in determining physical stability and interactions with surrounding ions (42). All NLC formulations exhibited a zeta potential ranging from -5 to -15 mV. In formulations with different Co-Q10/LE ratios, the zeta potential values were lower than those of the blank (drug-free) NLC.

TEM measurement

The microscopic morphology of F6 is shown in Figure 2. TEM analysis of the surface modification revealed a rounded shape and excellent dispersibility of the nano-sized particles. The mean size of the selected Co-Q10/LE-NLC was about 100 nm.

Differential scanning calorimetry (DSC)

The DSC analyzer is widely used to assess the melting and recrystallization characteristics of NLC (43). Evaluating the extent of lipid crystallization and modification is crucial for determining the quality and stability of NLC/SLN formulations (44). The melting peaks of Co-Q10 and

Table 1. Surface characteristics and the percentage of the components of coenzyme Q10 (Co-Q10)/licorice extract (LE)-loaded nanostructured lipid carrier (NLC) (equivalent amounts in moles and µg/ml; refer to Table S2 in the supplementary file)

Formulation	Compritol (%)	MCT Oil (%)	Tween 80 (%)	Coenzyme-Q10 (%)	Licorice extract (%)	Z-Average (nm)±SD	Polydispersity Index (PDI)±SD	Zeta potential (mV)±SD	Color
F1				-	-	244.5±3.34	0.259±0.003	-5.00±0.70	White
F2				0.5	-	201.5±1.08	0.222±0.004	-12.70±2.36	Yellow
F3				-	0.5	241.7±4.50	0.230±0.008	-14.71±1.03	Light brown
F4	3.5	1.5	5	0.25	-	155.7±5.11	0.166±0.010	-10.80±1.78	Yellow
F5				-	0.25	226.5±2.31	0.278±0.012	-6.92±1.89	Light brown
F6				0.5	0.5	162.7±2.19	0.273±0.001	-13.20±1.59	Mustard yellow
F7				0.5	0.25	146.3±3.42	0.210±0.007	-15.75±1.02	Mustard yellow
F8				0.25	0.5	248.1±1.08	0.279±0.004	-10.70±2.36	Mustard yellow

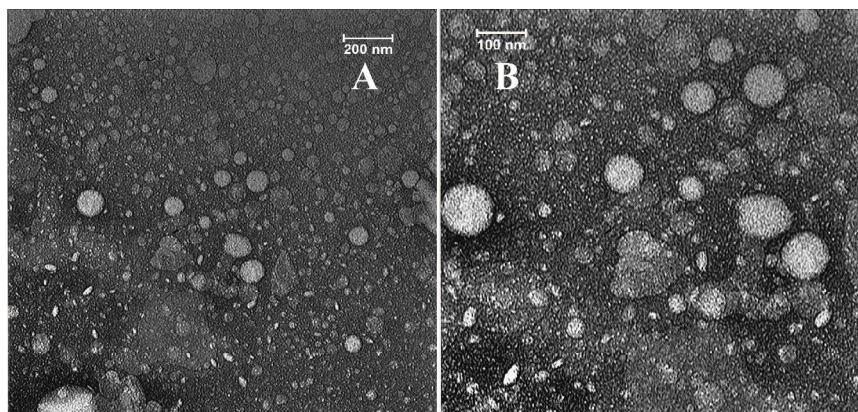


Figure 2. TEM image (scale bar: (A) 200 nm; (B) 100 nm) of coenzyme Q10 (Co-Q10)/licorice extract (LE)-loaded nanostructured lipid carrier (NLC) (F6)

Compritol were obtained at 51 °C and 70 °C, respectively, which is consistent with their expected melting temperatures (Figure 3A). In NLC formulations, the melting point of Compritol decreased from 73 °C to 65 °C, attributed to imperfections in the lipid structure introduced by the NLC system. In formulations containing Co-Q10, the melting peak of Co-Q10 at 51 °C disappeared.

The enthalpy value of Compritol was significantly greater than the value for drug-free lyophilized NLC (112.03 J/g in the lipid bulk compared to 51.41 J/g in the drug-free lyophilized NLC).

Fourier transform infrared (FT-IR) spectra

The FT-IR spectra of lipids, LE, Co-Q10, and NLC formulations F1, F2, F3, and F6 were recorded in the range of 400–4000 cm^{-1} (Figure 3B and Figure S1 in the supplementary file). Compritol and MCT oil showed characteristic carbonyl stretching vibrations at 1739 and 1745 cm^{-1} , respectively. No peak shifts were observed in NLC formulations.

The characteristic peaks of Co-Q10 at 1648 cm^{-1} (C=O stretching) and 1610 cm^{-1} (C=C stretching) were retained in F2 and F6. LE showed hydroxyl stretching at 3374.6 cm^{-1} and flavonoid-associated C=O (1710.3 cm^{-1}) and C=C (1618 cm^{-1}) vibrations in F3 and F6 without noticeable shifts. No new peaks were detected in NLC formulations containing Co-Q10 and LE.

Entrapment efficiency (EE)%

Due to the high lipophilicity of Co-Q10 ($\log P > 10$),

its detectable concentration in the aqueous medium after filtration was below the LOQ; therefore, its EE% was determined using the direct method. Liquiritin and glabridin EE% values were determined using indirect and direct methods, respectively.

The EE% values of fresh NLC formulations F2, F3, and F6 are reported in Table 2. High Co-Q10 encapsulation efficiency was observed in F2 (5 mg/ml Co-Q10) and F6 (5 mg Co-Q10/ml + 5 mg/ml LE), with F2 showing the highest value. Liquiritin and glabridin loading in F3 was higher than in F6. Formulation F7 (5 mg/ml Co-Q10 + 2.5 mg/ml LE) showed slightly higher Co-Q10 EE% than F6, while F8 (2.5 mg/ml Co-Q10 + 5 mg/ml LE) exhibited a slightly higher glabridin EE% compared to F6 and F7. Liquiritin EE% decreased slightly in F7 but remained comparable between F6 and F8.

After six months, formulations F2, F3, and F6 showed only a minor reduction in EE%, as shown in Table 2.

Physicochemical stability

The surface characteristics of blank NLC and Co-Q10/LE-NLC (F1–F8) formulations were evaluated for stability over time. Particle size, PDI, and zeta potential of blank NLC and Co-Q10/LE-NLC formulations (F1–F8) were measured after 1, 3, and 6 months at different storage temperatures (Table 3). No visible signs of instability, such as color change or phase separation, were observed. No significant changes in Z-average size or PDI were detected during storage. Zeta potential remained stable over six months for all

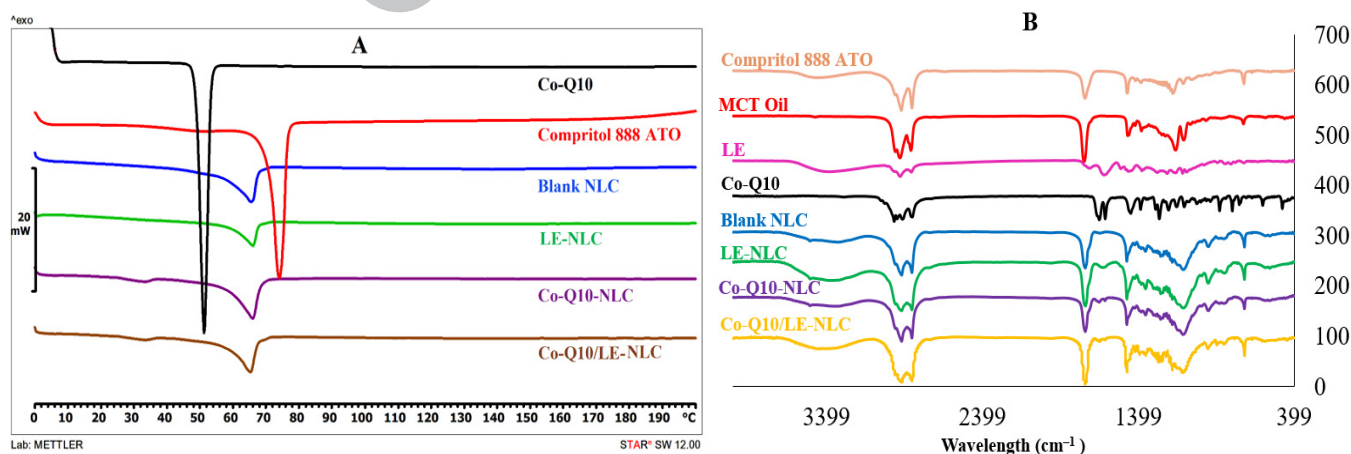


Figure 3. (A) Differential scanning calorimetry (DSC) curves of coenzyme Q10 (Co-Q10), solid lipid, freeze-dried blank nanostructured lipid carrier (NLC) (F1) and Co-Q10/licorice extract (LE)-loaded NLC (F2, F3, and F6); (B) The FT-IR spectra of Co-Q10, LE, freeze-dried blank NLC (F1), and Co-Q10/LE-loaded NLCs (F2, F3, and F6), and at 400–4000 cm^{-1} range

Table 2. Encapsulation efficiency (EE%) of nanostructured lipid carrier (NLC) formulations F2, F3, F6, F7, and F8 was determined both immediately after preparation and after 6 months of storage

Formulation	Encapsulation efficiency (EE%)					
	Fresh			After 6 months		
	Coenzyme Q10	Liquiritin	Glabridin	Coenzyme Q10	Liquiritin	Glabridin
F2	87.21±1.42	-	-	82.45±2.4	-	-
F3	-	66.78±3.12	73.25±2.57	-	59.52±4.12	68.56±3.59
F6	76.56±2.42	60.21±2.97	69.57±1.68	74.75±4.21	58.18±3.22	63.52±4.09
F7	79.28±3.75	58.46±2.63	68.05±3.42	-	-	-
F8	78.60±2.75	60.37±4.21	71.97±2.48	-	-	-

Table 3. Physicochemical stability of coenzyme Q10 (Co-Q10)/licorice extract (LE)-loaded nanostructured lipid carriers (NLCs) over time and temperature

Code	Temperature (°C)	Time (month)	Z-Average size (nm)±SD	Polydispersity index (PDI)±SD	Zeta potential (mV)±SD	Homogeneity/Color
F1	4	0	244.5±3.34	0.259±0.003	-5.0±0.70	Homogenous/white
		1	242.3±4.23	0.329±0.002	-7.5±0.23	Homogenous/white
		3	251.4±0.89	0.331±0.009	-7.1±0.22	Homogenous/white
		6	278.3±2.45	0.346±0.008	-6.2±0.31	Homogenous/white
		1	217.4±1.56	0.463±0.003	-6.7±0.31	Homogenous/white
		3	192.2±1.23	0.512±0.013	-6.0±0.24	Homogenous/white
	25	6	208.2±2.31	0.586±0.018	-5.8±0.34	Homogenous/white
		1	248.8±1.59	0.479±0.003	-3.39±0.40	Homogenous/white
		3	237.2±1.37	0.461±0.009	-3.86±0.24	Homogenous/white
		6	249.4±2.24	0.481±0.007	-3.92±0.22	Homogenous/white
		0	201.5±1.08	0.222±0.004	-12.70±0.36	Homogenous/yellow
		1	209.7±2.95	0.263±0.012	-19.7±0.09	Homogenous/yellow
F2	4	3	207.2±1.87	0.245±0.017	-6.7±0.24	Homogenous/yellow
		6	221.3±2.50	0.289±0.008	-10.8±0.17	Homogenous/yellow
		1	211.3±1.86	0.25±0.003	-13.0±0.19	Homogenous/yellow
		3	220.0±1.75	0.262±0.007	-13.6±0.24	Homogenous/yellow
		6	234.1±3.18	0.275±0.024	-16.7±0.25	Homogenous/yellow
		1	208.8±1.95	0.257±0.008	-12.9±0.38	Homogenous/yellow
	25	3	187.3±1.02	0.253±0.026	-7.48±0.18	Homogenous/yellow
		6	213.2±1.89	0.245±0.008	-11.2±0.24	Homogenous/yellow
		0	241.7±1.00	0.230±0.008	-14.71±1.03	Homogenous/light brown
		1	286.2±1.05	0.299±0.012	-17.5±0.09	Homogenous/light brown
		3	294.5±2.12	0.298±0.015	-16.0±0.15	Homogenous/light brown
		6	325.1±3.7	0.301±0.008	-17.2±0.24	Homogenous/light brown
F3	25	1	270.6±1.86	0.303±0.003	-12.4±0.19	Homogenous/light brown
		3	446.4±1.75	0.310±0.017	-12.5±0.24	Homogenous/light brown
		6	-	-	-	Sediment formation/light brown
		1	251.6±1.95	0.29±0.008	-12.7±0.38	Homogenous/light brown
		3	324.5±1.02	0.267±0.026	-9.6±0.18	Homogenous/light brown
		6	-	-	-	Sediment formation/light brown
	40	0	155.7±5.11	0.166±0.010	-10.80±1.78	Homogenous/yellow
		1	154.1±0.76	0.168±0.003	-10.87±1.33	Homogenous/yellow
		3	155.5±2.40	0.173±0.006	-11.21±0.98	Homogenous/yellow
		6	158.1±3.27	0.175±0.004	-11.05±1.03	Homogenous/yellow
		1	159.7±2.34	0.176±0.005	-11.71±1.34	Homogenous/yellow
		3	158.8±1.62	0.214±0.006	-9.83±0.38	Homogenous/yellow
F4	25	6	164.9±2.49	0.279±0.009	-9.30±1.08	Homogenous/yellow
		1	159.2±1.45	0.174±0.007	-10.21±0.78	Homogenous/yellow
	40	3	162.3±1.42	0.214±0.008	-9.62±1.74	Homogenous/yellow

Continued Table 3.

F5	4	6	166.4±2.89	0.226±0.008	-8.74±1.32	Homogenous/yellow	
		0	226.5±2.31	0.278±0.012	-6.92±1.89	Homogenous/light brown	
		1	235.2±2.16	0.272±0.013	-7.42±0.79	Homogenous/light brown	
		3	287.4±1.42	0.283±0.007	-6.47±1.25	Homogenous/light brown	
		6	295.1±3.35	0.308±0.019	-7.21±1.65	Homogenous/light brown	
		1	279.5±3.12	0.293±0.006	-6.91±0.78	Homogenous/light brown	
	25	3	338.3±2.74	0.310±0.015	-6.82±1.13	Homogenous/light brown	
		6	423.4±1.36	0.354±0.006	-5.43±1.46	Homogenous/light brown	
		1	269.3±1.79	0.302±0.008	-7.68±0.89	Homogenous/light brown	
	40	3	316.6±2.32	0.267±0.015	-9.6±0.18	Homogenous/light brown	
		6	-	-	-	Sediment formation/light brown	
		0	162.7±2.19	0.273±0.001	-13.2±1.59	Homogenous/mustard yellow	
F6	4	1	165.3±2.95	0.301±0.012	-17.6±0.09	Homogenous/mustard yellow	
		3	172.3±1.64	0.321±0.021	-14.6±0.48	Homogenous/mustard yellow	
		6	175.1±4.01	0.357±0.007	-14.9±0.36	Homogenous/mustard yellow	
		1	169.6±1.75	0.297±0.004	-10.4±0.26	Homogenous/mustard yellow	
		3	166.2±1.75	0.303±0.017	-6.2±0.24	Homogenous/mustard yellow	
		6	175.3±2.56	0.351±0.024	-7.7±0.1	Homogenous/mustard yellow	
	40	1	141.6±1.78	0.287±0.009	-8.1±0.27	Homogenous/mustard yellow	
		3	182.7±1.02	0.282±0.026	-5.8±0.18	Homogenous/mustard yellow	
		6	203.1±2.36	0.297±0.007	-6.1±0.24	Homogenous/mustard yellow	
	F7	4	0	146.3±3.42	0.21±0.007	-15.75±1.02	Homogenous/mustard yellow
			1	138.9±2.95	0.213±0.012	-17.6±0.09	Homogenous/mustard yellow
			3	148.1±2.31	0.228±0.026	-16.8±0.21	Homogenous/mustard yellow
6			153.1±3.21	0.211±0.008	-16.1±0.37	Homogenous/mustard yellow	
1			154.2±1.86	0.243±0.003	-11.0±0.19	Homogenous/mustard yellow	
3			145.6±1.75	0.232±0.017	-14.5±0.24	Homogenous/mustard yellow	
40		6	152.4±2.1	0.253±0.022	-14.6±0.37	Homogenous/mustard yellow	
		1	141.1±2.2	0.257±0.007	-9.8±0.41	Homogenous/mustard yellow	
		3	147.1±1.02	0.210±0.026	-10.1±0.18	Homogenous/mustard yellow	
F8		25	6	151.3±2.51	0.245±0.018	-9.8±0.23	Homogenous/mustard yellow
			0	146.1±1.08	0.279±0.004	-10.7±2.36	Homogenous/mustard yellow
			1	291.8±2.95	0.313±0.012	-14.8±0.09	Homogenous/mustard yellow
	3		304.4±1.75	0.321±0.024	-14.1±0.17	Homogenous/mustard yellow	
	6		321.1±3.74	0.350±0.007	-9.7±0.24	Homogenous/mustard yellow	
	1		257.2±1.86	0.287±0.003	-10.7±0.19	Homogenous/mustard yellow	
	40	3	257.9±1.75	0.330±0.017	-8.7±0.24	Homogenous/mustard yellow	
		6	261.2±2.54	0.381±0.018	-7.9±0.32	Homogenous/mustard yellow	
		1	237.3±1.95	0.312±0.008	-13.4±0.38	Homogenous/mustard yellow	
		3	298.1±1.02	0.286±0.026	-6.6±0.18	Homogenous/mustard yellow	
		6	324.7±2.54	0.324±0.012	-6.6±0.51	Homogenous/mustard yellow	

formulations except F3, which showed instability after three months.

Cell viability

MTT assay results showed no significant difference in the viability of B16F10 cells treated with 1% (v/v) NLC formulations (F1–F8) compared with the control group after 24 hr incubation (Figure 4A). H₂O₂ treatment induced cytotoxicity after 90 min, while pretreatment with NLC formulations partially protected cells against oxidative damage (Figure 4B). Formulations F6, F7, and F8, containing

a combination of Co-Q10 and LE, demonstrated enhanced cytoprotective effects compared to other formulations.

Cellular ROS level determination

Oxidative stress occurs when there is a disruption between the generation of ROS and the body's anti-oxidant defenses (45). H₂O₂ application is a commonly used method for the expression of oxidative damage/stress in cell models (45). Exposure to 10 mM H₂O₂ for 90 min reduced cell viability to approximately 70%. We entrapped coenzyme Q10 in NLC nanocarriers at three constant ratios with LE

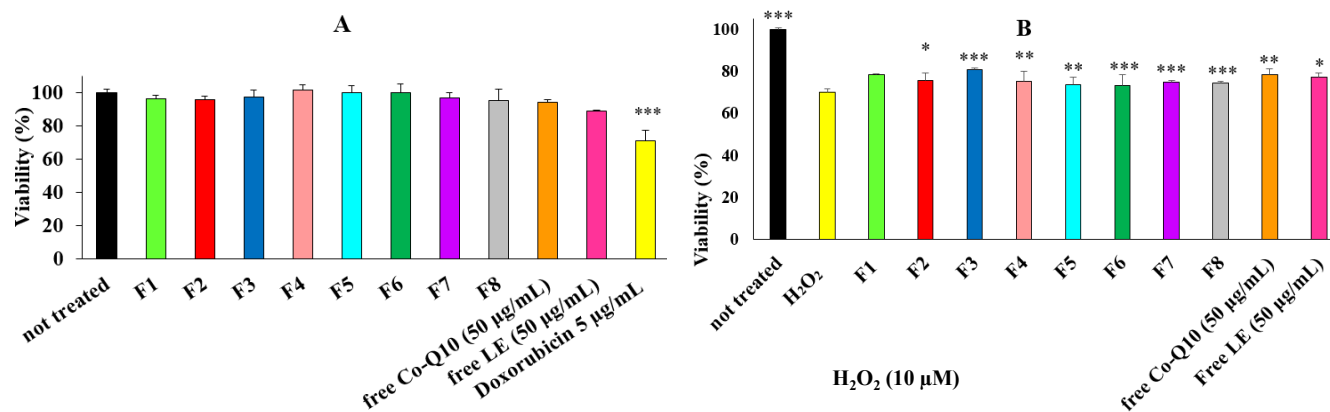


Figure 4. Comparative analysis of delivery performance between coenzyme Q10 (Co-Q10)/licorice extract (LE)-loaded nanostructured lipid carriers (NLC) (F6) and its equivalent Co-Q10/LE-loaded emulsion formulation (A) Cumulative release kinetics of the standardized marker compounds, glabridin (Glab) and liquiritin (LQ) (derived from licorice extract), and Co-Q10 over a 48-hour duration, comparing the performance of the Co-Q10/LE-NLC (F6) system with the equivalent Co-Q10/LE-emulsion formulation. (B) Evaluation of the skin penetration (S.P) and surface retention (S.S.) profiles for Glab and LQ serving as standard markers of the licorice extract within the Co-Q10-loaded NLC formulation. The chart contrasts the efficacy of the NLC system against the equivalent emulsion 12 hr post-application. Results are presented as mean±SEM (n=3).

and investigated its synergistic effect in suppressing ROS. We investigated the effect of NLC formulations on ROS scavenging at 0, 12, 25, and 50 µM of active ingredient at 1:1, 1:0.5, and 0.5:1 constant ratios.

The DCF absorbance results (Figure 5) showed that intracellular ROS levels increased after H₂O₂ treatment compared with the negative control group. Pretreatment with Co-Q10/LE-NLCs decreased ROS production in a concentration-dependent manner. Cell pretreatment with blank NLC at 1% had no significant effect on ROS production. All NLC formulations, including a combination of Co-Q10/LE, were more effective at ROS scavenging than

NLCs loaded with a single ingredient.

The effective dose (ED₅₀) refers to the amount of a drug required to produce a specified biological response in 50% of the population and serves as a benchmark for assessing the drug's effectiveness (46, 47). The ED₅₀ values for both active ingredients underwent isobolographic analysis to evaluate the nature of their interaction when Co-Q10 and LE were administered together (46, 47). The interaction index derived from the ED₅₀ values of the individual components in relation to their combined effects. An interaction index greater than 1 indicates antagonism, an index of 1 signifies an additive effect (no interaction), and

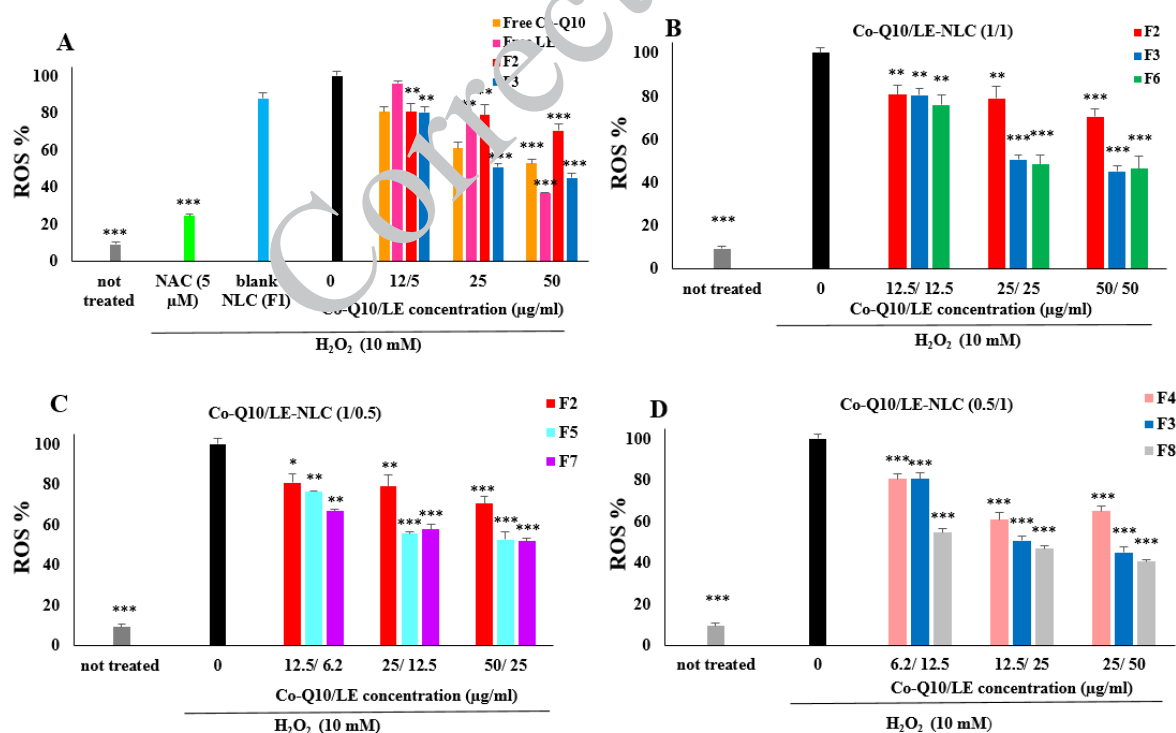


Figure 5. Cytotoxicity (A) and cytoprotective (B) effects of nanostructured lipid carrier (NLC) formulations (at a concentration of 1% in cell culture media) were evaluated using the MTT assay. Results are presented as mean±SEM (n=3). Asterisks indicate statistically significant differences compared to the non-treated (A) and H₂O₂ (B) groups: *P<0.05, **P<0.01, ***P<0.001.

Table 4. Effective dose (ED₅₀) and interaction index values for F6, F7, and F8 in reactive oxygen species (ROS), tyrosinase, and melanin levels

Formulation	Coenzyme Q10/licorice extract ratio	Reactive oxygen species (ROS) inhibition		Anti-tyrosinase activity		Melanin synthesis inhibition	
		Interaction index	Effective dose (ED ₅₀)	Interaction index	Effective dose (ED ₅₀)	Interaction index	Effective dose (ED ₅₀)
		values	values (μg/ml)	values	values (μg/ml)	values	values (μg/ml)
F6	1:1	1.02	40.86±0.16	0.78	167.04±0.53	2.46	45.03±0.26
F7	1:0.5	0.98	49.44±0.22	0.56	358.08±1.61	0.70	41.36±0.41
F8	0.5:1	0.62	21.72±0.19	0.48	101.33±0.62	1.78	27.34±0.15

an index less than 1 suggests synergism. (47, 48). According to the CalcuSyn analysis, the interaction index values for F6, F7, and F8 were 1.0, 0.9, and 0.6, respectively (Table 4). Therefore, F6, containing an equal amount of Co-Q10 and LE, had an additive effect on inhibiting ROS production. F7, containing Co-Q10/LE at a 1/0.5 ratio, showed a synergistic effect. A stronger synergistic effect was also observed for F8, which contains a 0.5:1 ratio of Co-Q10/LE.

Melanin production study

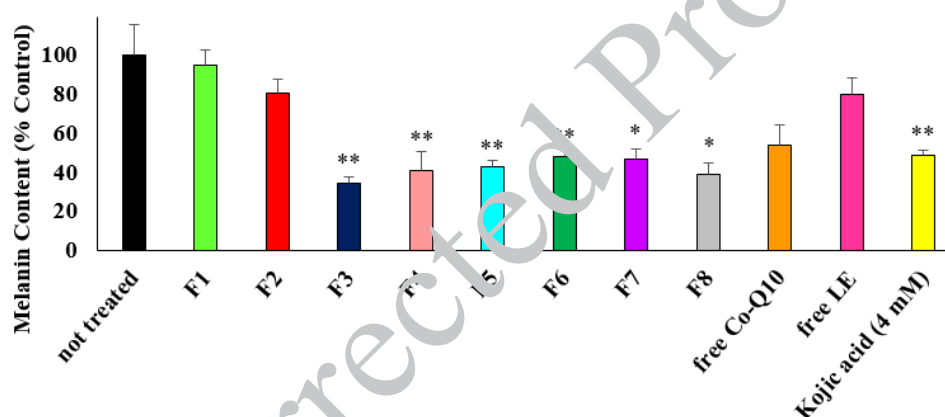
The melanin level graphs (Figure 6 and Figure S1 in the Supplementary data) showed that melanin production was reduced in melanoma cells treated with Co-Q10/LE-NLCs compared with untreated cells, blank NLC, free Co-Q10,

free LE, and kojic acid (positive control).

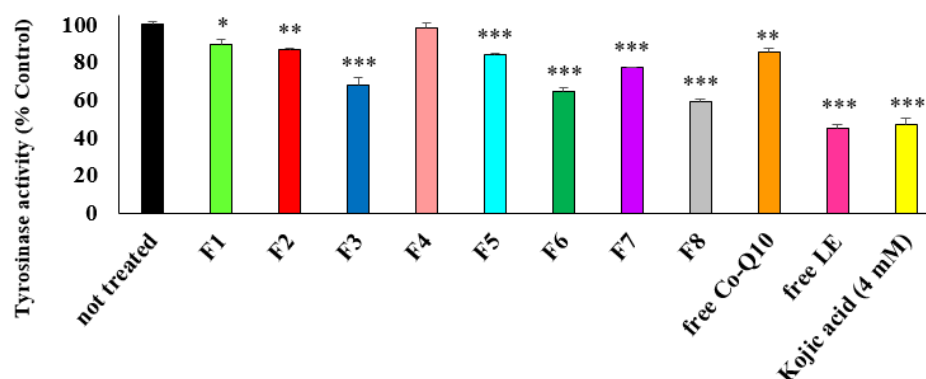
Cellular tyrosinase activity assay

DOPA assay is a spectrophotometric assay indicating tyrosinase activity (49). The tyrosinase activity of B16F10 cells treated with blank and Co-Q10/LE-loaded NLC formulations was evaluated and compared with their respective control groups (Figure 7 and Figure S2). Neither free Co-Q10 nor Co-Q10-loaded NLC (F4) showed a significant effect on tyrosinase activity, similar to the blank NLC.

The interaction index values for F6, F7, and F8 were calculated as 0.78, 0.56, and 0.48, respectively (Table 4). All NLC formulations containing a combination of Co-Q10

**Figure 6.** Enhanced reactive oxygen species (ROS) production in the presence of coenzyme Q10 (Co-Q10)/licorice extract (LE)-loaded nanostructured lipid carrier (NLC)

(A) Effect of free vs encapsulated single Co-Q10 and free N-acetylcysteine (NAC) is a positive control. (B) Effect of 1:1 ratio Co-Q10/LE formulation vs single-ingredient formulations. (C) Effect of 1:0.5 ratio. (D) Effect of 0.5:1 ratio. Results are expressed as mean±SEM (n=3). Asterisks denote statistical significance compared to control (H₂O₂ treatment only): *P<0.05, **P<0.01, ***P<0.001.

**Figure 7.** (A) Effect of coenzyme Q10 (Co-Q10)/licorice extract (LE)- nanostructured lipid carrier (NLC) formulations (at a concentration of 50 μg/ml; ratio 1/1, 1/0.5, 0.5/1) on melanin content in B16F10 cells after 24 hr. (B) Effect of Co-Q10/LE-NLC formulations (at a concentration of 50 μg/ml; ratios 1/1, 1/0.5, 0.5/1) on tyrosinase activity in B16F10 cells after 24 hr

Results are mean±SEM (n=3). Asterisks indicate statistical differences compared with the untreated group: *P<0.05, **P<0.01, and ***P<0.001.

and LE showed a synergistic inhibitory effect on tyrosinase activity, similar to the ROS-inhibitory effect. The order was: F8 (Co-Q10/LE: 1/1) > F7 (Co-Q10/LE: 1/0.5) > F6 (Co-Q10/LE: 0.5/1).

Discussion

Extraction strategy and lipid matrix optimization

Co-Q10 is the sole lipophilic anti-oxidant produced by human cells (11). The root extracts, specifically methanolic and ethyl acetate, contain polyphenols, terpenoids, flavonoids, saponins, and carbohydrate sugars (50). Compounds such as glycyrrhizic acid, glycyrrhizin, formononetin, glabridin, liquiritin, n-coumaric acid, luteolin-7-glucoside, acacetin, apigenin-7-O-glucoside, chicoric acid, and hesperetin have been reported in methanolic root extract. In contrast, glabrene, glabridin, hispaglabridin A and B, glabrone, and some derivatives have been found in ethyl acetate root extract (50). It has also been reported that non-polar solvents like ethyl acetate are ideal for extracting specific compounds such as glabridin from natural sources like licorice roots (51). The phytochemical profile of 25 licorice roots collected from various regions of Iran was investigated, and the levels of glycyrrhizic acid, glabridin, liquiritin, and liquiritigenin in the hydroalcoholic (80% methanolic) extract were measured. The quantities of these compounds varied among roots from different areas, with glycyrrhizic acid generally being present in higher amounts than the other compounds (52). The active ingredients in the extract may produce synergistic effects in both anti-oxidant and antityrosinase activities.

According to the HPLC chromatogram, liquiritin exhibits a shorter retention time than glabridin, which can be attributed to its higher polarity in accordance with its chemical structure. Following extract preparation, methanol was initially used as the extraction solvent, and an ethyl acetate fraction was subsequently obtained to concentrate the target compounds. Given the significantly higher glabridin content in the ethyl acetate fraction, and considering its role as an effective tyrosinase inhibitor, this fraction was selected for encapsulation in NLCs.

Compritol® 888 ATO is recognized as a highly valued ingredient for preparing NLC/SLN formulations due to its low cytotoxicity (53). It effectively enhances drug entrapment efficiency and facilitates the delivery of both hydrophilic and lipophilic compounds (53). MCT oil, characterized by its shorter carbon chain, offers improved drug loading capacity and accelerates release rates (54). Tween 80 is a non-ionic surfactant that ensures stable, consistent lipid dispersion in the aqueous phase, promoting effective drug release from the lipid matrix (55). Additionally, increasing its concentration in formulations may reduce particle size by decreasing surface rigidity between the aqueous medium and the lipid. The increasing ratio of liquid lipids to solid lipids lowers the possibility of nanoparticle aggregation despite the increase in their particle size (56). Therefore, solid-to-liquid lipids were used in a 70:30 ratio (57).

Physicochemical characterization

The literature has shown that lipid-based nanocarriers with a Z-average diameter of 600 nm or greater cannot deliver encapsulated active compounds into the deeper layers of the stratum corneum (41). Therefore, we produced nanocarriers with a Z-average diameter of less than 243 nm, which can transport their active ingredients into deeper skin

layers (58). PDI values below 0.2 are typically associated with enhanced long-term stability of NLC formulations (42). The NLC formulations F1-F8 exhibited an acceptable zeta potential ranging from -5 to -15 mV, which is a guarantor of the relative stability of the particles (42).

TEM analysis confirmed the spherical shape of the nanoparticles, which closely matches the particle size measurements obtained by DLS. The disappearance of the Co-Q10 melting peak in DSC curves indicates successful encapsulation and alteration of its crystalline structure, consistent with previous studies (28). The enthalpy value was another indicator of the crystal order (43). As observed, higher melting enthalpy values in pure lipid indicate a higher-ordered network arrangement and vice versa, suggesting a disruption in crystal structure within the NLC formulations (59). According to the FT-IR spectroscopy, the absence of peak shifts in lipid matrices indicates preservation of lipid structural integrity (60). Retention of the characteristic functional group vibrations of Co-Q10 and LE confirms their chemical stability during nanoparticle preparation (36, 60). The lack of new absorption bands suggests that no chemical interactions occurred between active compounds and lipid components, indicating physical encapsulation of Co-Q10 and LE within the lipid matrix.

The logP value quantifies a compound's distribution between organic and aqueous phases, indicating its lipophilicity or hydrophilicity (61). Higher logP values suggest greater affinity for lipophilic environments, while lower values indicate a preference for aqueous environments. This parameter is vital in drug development, influencing pharmacokinetic properties such as absorption, distribution, metabolism, and excretion (ADME) (61). The high lipophilicity of Co-Q10 explains its strong affinity for the lipid matrix and the need to apply the direct EE% measurement method. The results support the ability to simultaneously load the LE and Co-Q10 to an acceptable level in prepared NLC formulations. The minimal reduction in EE% over six months suggests good chemical stability of Co-Q10 and LE within NLC systems, supporting the suitability of NLCs as nanocarriers for these active compounds (62).

The stability of surface characteristics over time indicates good physicochemical stability of the developed NLC formulations, which is essential for long-term storage and performance.

Synergistic anti-oxidant and antityrosinase activity

The absence of cytotoxicity confirms the biocompatibility of the developed NLCs. The improved protection observed with combined Co-Q10/LE formulations suggests a beneficial interaction between the two anti-oxidants in mitigating oxidative stress.

Coenzyme Q10, as an electron transport agent, is capable of suppressing intracellular ROS (63). Within cells, the benzoquinone structure of Coenzyme Q10 can exist in three interconvertible oxidation states: the reduced form ubiquinol (Co-Q10H₂), the ubisemiquinone intermediate (Co-Q10H[•]), and the fully oxidized ubiquinone (Co-Q10). This redox state is supported by additional anti-oxidant sources, like α-tocopherol and vitamin C (64). The synergistic effects of Co-Q10 in combination with other anti-oxidants, such as beta-carotene (65) and astaxanthin (66), have been reported previously. Co-Q10 and licorice extracts, as anti-oxidants, can reduce cellular imbalance caused by increased ROS

levels (63). They were encapsulated in NLCs as lipophilic ingredients to improve their distribution in an aqueous medium (49).

The results show that, compared with individual NLCs, the combined Co-Q10/LE formulation was more effective at scavenging ROS. Among the combination formulations, F6 (Co-Q10/LE, 1:1) produced an additive effect on ROS suppression, whereas F7 (1:0.5) and F8 (0.5:1) exerted synergistic effects, with the greatest synergy observed for F8. Due to its high flavonoid content, LE exhibits notable anti-oxidant and anti-inflammatory properties, which may contribute to its interaction with Co-Q10 in reducing oxidative stress (67).

Compared with untreated cells, blank NLC, free Co-Q10, and free LE, all Co-Q10/LE-loaded NLCs reduced melanin content in treated cells, with F3 (0.5% LE) showing the highest inhibition.

Recently, Co-Q10 was found to inhibit tyrosinase activity (68). Co-Q10 down-regulates melanin synthesis by suppressing MITF-expressing and cAMP-mediated CREB signaling cascades (68). Liquiritin and glabridin are promising depigmenting agent compounds in LE. The synergistic effect of Co-Q10 and glabridin on tyrosinase activity has been reported previously (64). Co-Q10 and LE are natural tyrosinase inhibitors with no side effects, such as dermatitis, compared with other inhibitors of melanin synthesis, such as arbutin, kojic acid, hydroquinone, corticosteroids, and mercury (64). All Co-Q10/LE combination NLCs synergistically inhibited tyrosinase, mirroring their anti-oxidant activity. F8 (1:1 ratio) showed the greatest anti-melanogenic effect, followed by F7 (1:0.5) and F6 (0.5:1).

Conclusion

We developed novel NLC formulations containing Co-Q10 and LE, both individually and in combination, to improve the efficacy of natural products. In conclusion, all NLCs demonstrated favorable surface properties and good physicochemical stability, with particle sizes ranging from 146 to 248 nm, polydispersity indices (PDI) between 0.21 and 0.27, and zeta potentials from -5 to -15 mV. The EE% for active ingredients was consistently above 60%. Our formulations demonstrated robust stability over six months. Key parameters like particle size, polydispersity index (PDI), and zeta potential remained stable under various storage conditions. No visible signs of degradation, such as phase separation or color changes, were detected. Although some formulations (F2, F3, and F6) showed minor decreases in EE%, the NLC system effectively preserved the stability of Co-Q10 and LE over time. The synergistic anti-oxidant and antityrosinase activity of Co-Q10-NLC, LE-NLC, and Co-Q10/LE-NLC confirmed that F8 (Co-Q10/LE: 0.5/1 loaded NLC) showed the strongest synergistic effect on ROS suppression and tyrosinase inhibition. F7 (Co-Q10/LE: 1/0.5 loaded NLC) also exerted a synergistic effect in both experiments. F6 (Co-Q10/LE: 1/1 loaded NLC) had an additive effect on ROS scavenging and a synergistic effect on decreasing tyrosinase activity. Therefore, a combination of Co-Q10/LE-loaded NLC at a 0.5/1 ratio is recommended as a topical agent for anti-aging and skin-lightening. Future research will involve *in vivo* trials to validate the formulations' safety and efficacy in clinical settings. Additionally, optimizing these formulations for large-scale production and exploring other bioactive compounds

within the NLC framework could expand their potential in dermatology and cosmetics.

Acknowledgment

Mashhad University of Medical Sciences, Iran, supported this research (grant number 4021191).

Authors' Contributions

H AM, A H, and S G conceived the study; H AM, A H, and JA developed the methodology; H AM and A H conducted the investigation; H AM curated the data; A H performed the formal analysis; J A acquired resources; H A M prepared the original draft; A H, Z TN, and A S reviewed and edited the manuscript; Z TN and S G supervised; S G administered the project. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration

We acknowledge the use of Grammarly to improve the manuscript's English grammar and language style.

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