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Research paper

Co-delivering of oleuropein and lentisk oil in phospholipid vesicles as an effective approach to modulate oxidative stress, cytokine secretion and promote skin regeneration

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ABSTRACT

In this work oleuropein and lentisk oil have been co-loaded in different phospholipid vesicles (i.e., liposomes, transfersomes, hyalurosomes and hyalutransfersomes), to obtain a formulation capable of both inhibiting the production of different markers connected with inflammation and oxidative stress and promoting the skin repair.

Liposomes were prepared using a mixture of phospholipids, oleuropein and lentisk oil. Tween 80, sodium hyaluronate or their combination have been added to the mixture to obtain transfersomes, hyalurosomes and hyalutransfersomes. Size, polydispersity index, surface charge and stability on storage was evaluated. The biocompatibility, anti-inflammatory activity and wound healing effect were tested using normal human dermal fibroblasts.

Vesicles were small (mean diameter ~ 130 nm) and homogeneously dispersed (polydispersity index ~ 0.14), highly negatively charged (zeta potential 02053–64 mV) and capable of loading 20 mg/mL of oleuropein and 75 mg/mL of lentisk oil. The freeze-drying of dispersions with a cryoprotectant permitted to improve their stability on storage. The co-loading of oleuropein and lentisk oil in vesicles inhibited the overproduction of inflammatory markers, especially MMP-1 and IL-6, counteracted the oxidative stress induced in cells using hydrogen peroxide, and promoted the healing of a wounded area performed *in vitro* in a cell monolayer of fibroblasts.

The proposed co-loading of oleuropein and lentisk oil in natural-based phospholipid vesicles may hold promising therapeutic value especially for the treatment of a wide variety of skin disorders.

1. Introduction

Oleuropein is a phenolic compound naturally occurring in Oleaceae, Cornales, and Gentianales family [1], while from the chemical point of view, it is an ester of elenolic acid and hydroxytyrosol (3, 4-dihydroxyphenylethanol), Fig. 1 [2].

It is present in high amount (up to 14 %) in the stem of olive tree and it is responsible for the bitter and spicy taste of extra virgin olive oil [1]. However, rather than in the fruit it is particularly concentrated in the leaves, reaching 60–90 mg/g of dried source [3]. Several studies confirmed the biological properties of oleuropein such as anti-inflammatory, antibacterial, antifungal, analgesic, antiallergic, radioprotective and anticarcinogenic [4,5]. Like most of the polyphenols, oleuropein is a strong antioxidant, capable of counteracting reactive oxygen species (ROS), thanks to its *ortho*-diphenol group, which neutralizes ROS through the donation of hydrogen atoms, and stabilizes oxygen radicals by means of an intramolecular hydrogen bond [6]. Thanks to this activity, it inhibits the cascade of processes connected with oxidative stress and inflammation thus promoting skin homeostasis and repair when topically applied [7]. Indeed, the daily application, in *in vivo* models, of a cream containing oleuropein favoured the wound contraction and increased hydroxyproline and glutathione level over

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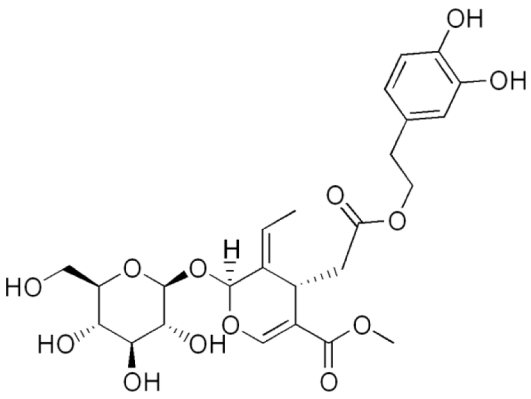

Fig. 1. Chemical formula of oleuropein.

time [8–10]. Its beneficial properties can be improved by the synergic activity of other molecules with complementary chemical properties and biological activity. Indeed, in a previous study, the extract obtained from olive oil leaves and rich in oleuropein has been associated to tocopherol aiming at obtaining an effective system capable of alleviating the cardiac toxicity induced by doxorubicin in rats [11]. It was also associated with doxorubicin to potentiate its activity in the treatment of breast cancer [12]. An extract rich in oleuropein has been added to other phytocomplexes as well, to synergically attenuate hypertension and insulin resistance in rats [13].

In general, the association of different phytochemicals, to synergically or complementarily improve the efficacy, is a valuable strategy to design new and effective formulations. The same strategy can be applied to oleuropein, aiming at potentiating its effectiveness in the treatment of skin lesions, and the oil obtained from the berries of *Pistacia lentiscus* appears to be a good candidate considering its synergic ability to promote the repair of skin lesions and inhibit lipid oxidation and depletion of antioxidant defence enzymes [14]. This oil was commonly used in folk medicine for the treatment of scabies or topically to treat sores, wounds and burns [15,16]. Its regenerative effect at skin level is especially related to its unsaponifiable fraction, predominantly hydrocarbons, monoterpenes and sesquiterpenes, associate with polyunsaturated fatty acids and polyphenols. The total amount of unsaturated fatty acid contained in the lentisk oil is 74 %, mainly represented by oleic acid (51 %), linoleic acid (21 %) and palmitic acid (24 %), while the most abundant terpenes are α-pinene, β-pinene, limonene, terpinen-4-ol and α-terpineol [17]. These monoterpenes act as cyclooxygenases, as they exert anti-inflammatory activity when orally and topically administered and reduce the levels of cytokines and proinflammatory proteins through the inhibition of the nuclear factor kB (NF-kB) pathway [18].

The association of oleuropein and lentisk oil in a unique formulation can be achieved by their simultaneous loading in phospholipid vesicles, as previously confirmed using other molecules [19]. In addition, these carriers have themselves anti-inflammatory effect and can stimulate cell proliferation, and, above all, they can simultaneously deliver several molecules with different physiochemical properties, improving their bioavailability, especially when topically used [20].

In the present work, to obtain an antioxidant, anti-inflammatory and regenerative effect, oleuropein and lentisk oil were combined and co-loaded in phospholipid vesicles tailored for the treatment of skin lesions. Liposomes were prepared and then modified by the addition of tween 80 or hyaluronic acid. Hyaluronic acid has been chosen thanks to its ability to interact with phosphatidylcholine on the bilayer surface leading the formation of immobilized vesicles [21,22], while the choice of Tween 80 is mainly connected with its ability to improve the deformability of the vesicular bilayer, thus facilitating the deposition and passage of the bioactives into and through the skin [23–25].

The main physico-chemical properties of the vesicles (i.e., mean size, surface charge, entrapment efficiency) were evaluated. Formulations were freeze-dried with or without cryoprotectant to improve their stability on storage. Finally, a deep *in vitro* evaluation of the biological properties of these formulations was performed measuring their biocompatibility and efficacy to inhibit the production of proinflammatory markers, along with their ability to improve cell proliferation and migration in a wounded cell monolayer.

2. Materials and methods

2.1. Materials

Phospholipids (S75), a mixture of phospholipids from soy (70 % phosphatidylcholine and 9 % phosphatidylethanolamine and 3 % of lysophosphatidylcholine) was a gift from Lipoid GmbH (Ludwigshafen, Germany). Fixed lentisk oil was kindly provided by SSA Mediflora (Pula, Italy). Phosphate buffer solution (PBS, pH 7) was purchased from Carlo Erba Reagents (Rodano, Milan, Italy). Oleuropein (≥80 % by HPLC), ethanol, tween 80, and all the other reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Oleuropein standard was purchased from Carbosynth (Compton, U.K.). Heat-inactivated fetal bovine serum (FBS, HyCylone™) was purchased by Thermo Fisher Scientific (Waltham, USA). Dulbecco's modified Eagle's medium high glucose 4.5 g/L (DMEM), penicillin–streptomycin solution, trypsin-EDTA (0.25 %), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS substrate), 3,3′,5,5′-Tetramethylbenzidine (TMB substrate) were purchased by Merck (Waltham, Germany). ELISA Human IL-6 Kit (900-K16) and ELISA Human IL-8 Kit (900-K18) were purchased by Pepro-Tech (London, United Kingdom). Human Total MMP-1 DuoSet ELISA (DY901B-05) and Human MMP-2 Duo-Set ELISA (DY902) were purchased by R&D Systems (Minneapolis, USA).

2.2. Vesicle preparation

Liposomes were prepared using S75 (180 mg/ml), oleuropein (20 mg/mL) and lentisk oil (75 mg/mL); Tween 80 (7.5 mg/mL) was added to obtain transfersomes, hyaluronic acid (0.1 %) for the preparation of hyalurosomes and the mixture of Tween 80 and hyaluronic acid was added to obtain hyalutransfersomes (Table 1). Empty vesicles without both oleuropein and lentisk oil, were prepared as well and used as reference.

All components were weighted in a glass vial and hydrated with distilled water. Then, the dispersions were sonicated (5 s on and 2 s off, 10 cycles; 13 μm of probe amplitude) with a high intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, UK), until clear opalescent dispersions were obtained. Samples were purified from the non-incorporated phytochemicals by dialysis. Briefly, 1 mL of dispersion was loaded into dialysis tubing (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands) and dialysed against distilled water (1000 mL) for 2 h at 25 °C, by changing the water after one hour, which was appropriate to favour the dissolution and consequent removal of the non-incorporated substances.

2.3. Vesicle characterization

Vesicle formation and morphology were checked and confirmed by cryogenic transmission electron microscopy (cryo-TEM), using a Tecnai G2 20 twin (FEI) microscope, operating at an accelerating voltage of 200 KeV in a bright-field image mode and low-dose image mode. An aliquot of the sample (3 μL) was applied to glow-discharged 300 mesh TEM grid and used for plunge freezing into liquid ethane on a FEI Vitrobot Mark IV (Eindhoven, The Netherlands). The frozen grid was then transferred to a 626 DH Single Tilt Cryo-Holder (Gatan, France), maintained below −170 °C (liquid nitrogen temperature) and then transferred to the TEM for visualization.

Table 1
Composition of prepared vesicles.

Sample	S75 (mg/mL)	Oleuropein (mg/mL)	Lentisk oil (mg/mL)	Tween (mg/mL)	Hyaluronan (mg/mL)	Water (mL)
Liposomes	180	20	75	–	–	1
Transfersomes	180	20	75	7.5	–	1
Hyalurosomes	180	20	75	–	1	1
Hyalutransfersomes	180	20	75	7.5	1	1

The average diameter and polydispersity index of the samples were measured by means of Photon Correlation Spectroscopy technique using a Zetasizer Ultra (Malvern Panalytical ltd, Worcestershire, UK). Samples were backscattered by a helium–neon laser (633 nm) at an angle of 173° and a constant temperature of 25 °C. The polydispersity index was used as a measure of the width of the size distribution of vesicular dispersions. Zeta potential was measured using the Zetasizer Ultra as well, by the M3-PALS (Mixed Mode Measurement-Phase Analysis Light Scattering) technique, which measures the particle electrophoretic mobility in a thermostated cell. All samples were analysed 24 h after their preparation.

The entrapment efficiency, expressed as the percentage of phytochemicals before and after dialysis, was determined by DPPH colorimetric assay. Samples (20 μL) were dissolved in 1980 μL of DPPH methanolic solution (40 μg/mL) and incubated for 30 min at room temperature in the dark. At the end of the experiment, the absorbance (ABS) was measured at 517 nm against the blank. All the experiments were performed in triplicate. The antioxidant activity (%) was calculated as follows: $[(ABS_{DPPH} - ABS_{sample})/ABS_{DPPH}] \times 100$.

2.4. Lyophilization and rehydration

To improve their stability, vesicle dispersions, with or without mannitol as cryoprotectant, were frozen at −80 °C and freeze-dried using a freeze-drier Operon (fdb-8603, Operon Advantech Korea), thus obtaining a dried porous and not hygroscopic powder. Before the use, samples were simply rehydrated with the original volume of water, by manual shaking and equilibrated at room temperature for 30 min. Samples were stored for 18 months, at scheduled time-points were rehydrated and their main physico-chemical properties (mean diameter, polydispersity index and surface charge) were measured. As comparison, the average size, polydispersity index and zeta potential of the liquid dispersions of vesicles, stored at 25 ± 1 °C, were measured.

2.5. Oleuropein stability during preparation and freeze-drying processes

Stability of oleuropein during the preparation and lyophilization processes, was evaluated by its quantification using an UPLC (Ultra-Performance Liquid Chromatography, Acquity H-class plus (CH-A), Waters, Milano, Italy), equipped with a photodiode array UV detector and a C18 reverse-phase column (Waters 1.7 μm, 2.1 mm × 50 mm) at 25 °C. The injected volume was 5 μL. The mobile phase was a mixture of methanol (5 %), acetonitrile (50 %) and distilled water (45 %), delivered at a flow rate of 200 μL/min. The detection wavelength was set at 280 nm, the time of drug retention was 1.2 min and run time was 6 min. Vesicles were disrupted by dilution (1:1000) with methanol before the analysis. A calibration curve was build using decreasing concentrations of oleuropein solution (19.500, 39.060, 78.125, 156.250, 312.500, 625.000 μg/mL) (Supplementary materials, Figure S1).

2.6. Drug release

The release of phytochemicals from the vesicle dispersions was measured. The dispersions (2 mL) were transferred into dialysis tubes of regenerated cellulose (Spectra/Por 137® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories 138 Inc., DG Breda, Netherlands) and were dialyzed against phosphate buffer (25 mL) at pH 7.4, maintained at 37 °C and stirred at 100 rpm for 24 h. At scheduled time-points, the medium of the receptor compartment was removed and replaced with an equal volume of pre-thermostated phosphate buffer (pH 7.4). The released phytochemicals have been quantified measuring the antioxidant activity of the solution removed from the receptor compartment by means of the DPPH colorimetric assay and the concentrations were expressed as Trolox equivalents by building a calibration curve. The amount of phytochemicals released were expressed as a percentage of the initial antioxidant activity.

2.7. Cell culture

Normal human dermal fibroblasts were isolated from skin fragments. The skin specimens were obtained from healthy tissue taken from patients which have had a plastic surgery from Faculty Hospital Olomouc after the signature of the informed consent. The study was performed according to the Code of Ethics of the World Medical Association (ref. number 41/09). These cells were chosen to mimic the real conditions that can undergo in vivo and to lead the prediction of the effective potential of selected substances in wound healing [26,27].

Normal human dermal fibroblasts were cultured in DMEM medium supplemented with 10 % FBS and antibiotics (1 % penicillin and streptomycin) at 37 °C in 5 % CO_2 humidified atmosphere. The medium was changed every 72 h to promote the proliferation of cells. The cells between passages 2–4 were used for the experiments.

2.8. Cell viability

Primary normal human dermal fibroblasts were seeded in 96–well plates at the final density of 1×10^4 cells/well. The following day, the tested formulations (liposomes, transfersomes, hyalurosomes, hyalutransfersomes), appropriately diluted in serum-free DMEM medium to reach a concentration of oleuropein from 0.02 μg/mL to 20 μg/mL, were applied to the cells. Oleuropein in dispersion has been tested as well, at the same concentrations, and used as comparison. After 24 h, the cell viability was assessed by the MTT colorimetric assay, that measures the mitochondrial reduction of MTT (3-(4,5-dimethyltiazol-2–yl)–2,5–diphenyltetrazoliumbromide) to formazan, which has the maximum of absorbance at 540 nm.

2.9. In vitro scratch assay

2.9.1. Scratch assay

For scratch assay, the primary normal human dermal fibroblasts were seeded in 6-well plates at a cell density of 0.5×10^5 cells/well and cultivated until reaching confluence. The cell monolayer has been scratched by using a 5 mL pipette to produce a wounded area. After that, the cells were washed with phosphate buffer to remove detached cells and the tested dispersions were applied to each well at two different concentrations (0.2 μg/mL and 2 μg/mL of oleuropein). Untreated cells (only treated with the culture medium) were used as a control. The rate of cell migration leading to wound closure was observed under a light microscope with a 10 × objective, capturing images at scheduled intervals of time (0, 24 and 48 h). The captured images were quantified by ZEN 3.3 blue edition (Carl Zeiss) measuring the area of the wound. The

cells migration was expressed as percentage of wound closure: wound closure % = $[(a_0 - a\Delta / a_0] \times 100$,

where a_0 is the wounded area immediately after scratching, and $a\Delta$ is the wounded area measured at 24 and 48 h after scratching [28].

2.9.2. Scratch assay in cells stimulated with lipopolysaccharide

Primary normal human dermal fibroblasts were cultured, until the confluence was reached, in 6-well plates. The lipopolysaccharide (*Pseudomonas aeruginosa*, final concentration 10 μg/mL) was added to half of the plates for 6 h to stimulate the pathological inflammatory response. Next, the cell monolayers of all plates (included plates with lipopolysaccharide) were scratched, and each well was gently washed with phosphate buffer to remove detached cells. Finally, the cells were treated with the vesicle dispersions at two different dilutions (corresponding to 0.2 μg/mL and 2 μg/mL of oleuropein) for 24 h. At the end of the incubation period, the culture medium was collected and stored at –80 °C [26,29]. Untreated, unscratched, unstimulated cells were used as positive control; untreated, scratched, unstimulated cells were used as negative control 1; untreated, scratched, stimulated cells were used as negative control 2.

2.10. Enzyme-Linked immunosorbent assay (ELISA)

Levels of IL-6, IL-8, MMP-1 and MMP-2 in the supernatant of cells after the treatment with both lipopolysaccharide and vesicles were analysed using ELISA Human IL-6 Kit (900-K16), ELISA Human IL-8 Kit (900-K18) (PeproTech), Human Total MMP-1 DuoSet ELISA (DY901B-05) and Human MMP-2 Duo-Set ELISA (DY902) (R&D Systems) according to the manufacturer's instructions.

2.11. Statistical analysis of data

Results are expressed as the mean ± standard deviation. Analysis of variance (ANOVA) and Bartlet's test for homogeneity of variance were performed using SPSS version 17.0 for Windows (SPSS Inc., USA). Post hoc testing ($p < 0.05$) of the multiple comparisons was performed by the Scheffe test.

3. Results

3.1. Vesicle characterization

Liposomes, transfersomes, hyalurosomes and hyalutransfersomes, empty or loaded with oleuropein and lentisk oil, were prepared and their structure and morphology were observed (Fig. 2). Liposomes and transfersomes were mostly uni- or bi-lamellar, while the addition of sodium hyaluronate led the formation of oligo-lamellar and sometime multi-compartment vesicles. The combination of sodium hyaluronate and tween 80 did not significantly modify the morphology of vesicles as they were similar to both liposomes and hyalurosomes.

The size, polydispersity index, surface charge and entrapment efficiency of vesicles were measured and compared (Table 2). Empty liposomes, transferosomes and hyalutransfersomes were small (~108 nm) and slightly polydispersed (polydispersity index ~ 0.29), while hyalurosomes were smaller, ~90 nm ($p < 0.05$ versus values of other empty vesicles) but still slightly polydispersed (~0.31). The loading of oleuropein and lentisk oil caused simultaneously an increase of mean diameter (~145 nm, $p > 0.05$ among the size of loaded vesicles, $p > 0.05$ versus the size of empty vesicles) and a reduction of polydispersity index (~0.15), leading the formation of monodisperse samples. The zeta potential was always strongly negative (~64 mV) irrespective of the composition of the vesicles. Moreover, all tested formulations incorporated ≥ 87 % of the phytochemicals used during the preparation, regardless of their composition.

The stability of vesicle dispersions on storage has been evaluated monitoring the mean diameter, and polydispersity index for 6 months (Fig. 3). The tested parameters slightly increased up to 6 months, then a significant increase of size and size distribution occurred, and the samples became unmeasurable, probably because of hydrolysis and oxidative process, and/or aggregation and fusion phenomena, which in turn may cause loss of the entrapped phytochemicals and formation of aggregates.

To extend the stability of the vesicles, they were freeze-dried aiming at obtaining a stable powder easily redispersible just before the use. Due to the damaging effect of the freeze-drying process in lamellar vesicles, a suitable cryoprotectant was added to protect and preserve the structure of vesicles. Among others, the polysaccharide mannitol was chosen since its ability to replace the water and avoid the break of vesicle membrane during the freezing process. Mannitol was added prior to the sonication process aiming at favouring its distribution inside the vesicles and in the inter-vesicle spaces. The size, polydispersity index and zeta potential of vesicles, with or without mannitol, were measured after freeze-drying and rehydration and results were compared with those of unfreeze-dried ones (Table 3).

The mean diameter of vesicles prepared without mannitol did not significantly change after rehydration except for hyalutransfersomes, which increased up to ~ 307 nm. The size of the vesicles, freeze-dried

Table 2
Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) of empty and phytochemicals loaded vesicles after preparation. Mean values ± standard deviations are reported (n ≥ 3). The same symbol (¤, *) indicates the same value.

Fresh samples	DM (nm)	PI	ZP (mV)	EE (%)
Empty liposomes	*107 ± 14	0.31 ± 0.03	−79 ± 6	- -
Empty transfersomes	*110 ± 6	0.27 ± 0.01	−77 ± 3	- -
Empty hyalurosomes	90 ± 11	0.31 ± 0.02	−79 ± 2	- -
Empty hyalutransfersomes	*109 ± 11	0.27 ± 0.05	−75 ± 12	- -
Loaded liposomes	¤137 ± 24	0.12 ± 0.01	−64 ± 12	87 ± 1
Loaded transfersomes	¤141 ± 19	0.15 ± 0.02	−65 ± 5	88 ± 1
Loaded hyalurosomes	¤146 ± 25	0.14 ± 0.02	−64 ± 3	90 ± 2
Loaded hyalutransfersomes	¤153 ± 22	0.17 ± 0.02	−63 ± 6	89 ± 1

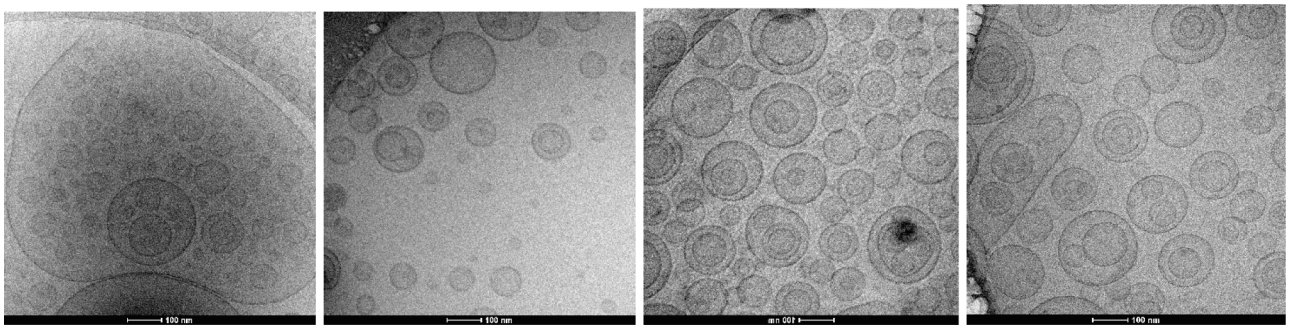

Fig. 2. Representative cryo-TEM images of liposomes (A), transfersomes (B), hyalurosomes (C) and hyalutransfersomes (D).

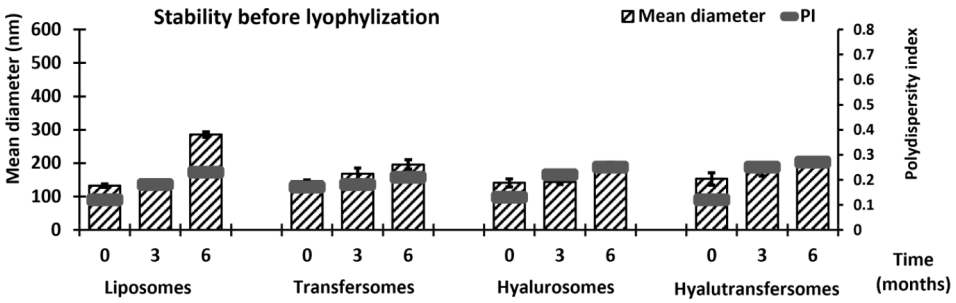

Fig. 3. Mean diameter (MD) and polydispersity index (PI) of phytochemicals loaded vesicles stored at 25 ± 1 °C for 6 months. Mean values (bars) ± standard deviations (error bars) are reported (n ≥ 3).

Table 3
Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (EE) of phytochemicals loaded vesicles with or without mannitol after freeze-drying and rehydration processes. Mean values ± standard deviations are reported (n ≥ 3). Symbol * indicates samples statistically different from that prepared with mannitol; Symbol # indicates samples statistically different from that measured before the lyophilization-rehydration process.

Rehydrated samples	DM (nm)		PI		ZP (mV)		EE (%)	
	Mannitol	No mannitol	Mannitol	No mannitol	Mannitol	No mannitol	Mannitol	No mannitol
Liposomes	146 ± 12	148 ± 14	0.12 ± 0.02	*0.17 ± 0.02	−62	−61	72 ± 3	68 ± 2
Transfersomes	149 ± 10	146 ± 14	0.10 ± 0.03	*0.16 ± 0.01	−63	−59	67 ± 2	68 ± 4
Hyalurosomes	#104 ± 6	*137 ± 10	0.13 ± 0.01	*0.17 ± 0.02	−59	−62	67 ± 5	69 ± 5
Hyalutransfersomes	#101 ± 11	*307 ± 23	#0.12 ± 0.02	*0.20 ± 0.02	−67	−63	68 ± 2	72 ± 2

with mannitol and rehydrated, was lower (~125 nm) than that of vesicles not freeze-dried (~140 nm), suggesting the key role of mannitol on facilitating the reconstruction of the dried vesicles. Moreover, the presence of mannitol caused a slightly decrease of the polydispersity index (~0.12) of vesicles in dispersion, while the polydispersity index of the vesicles freeze-dried without mannitol was like to that of not freeze-dried vesicles (~0.18). The zeta potential of all formulations remained constant and highly negative after freeze-drying and rehydration processes, without significant differences between all formulations. The freeze-dried dispersions were stored at 25 ± 1 °C under vacuum for 3, 6, and 18 months. At scheduled time-points dried samples, with or without mannitol, were rehydrated by a gentle handshaking and the mean diameter and polydispersity index were measured (Fig. 4).

The positive effect of mannitol was evident during the storge of freeze-dried vesicles. Indeed, those prepared with mannitol were more stable and their mean diameter and polydispersity index did not change during the 18 months of storage (Fig. 3). On the contrary, freeze-dried formulations prepared without mannitol, especially those containing hyaluronic acid, were less stable. Indeed, the mean diameter of freeze-dried liposomes increased up to ~ 175 nm at 18 months of storage,

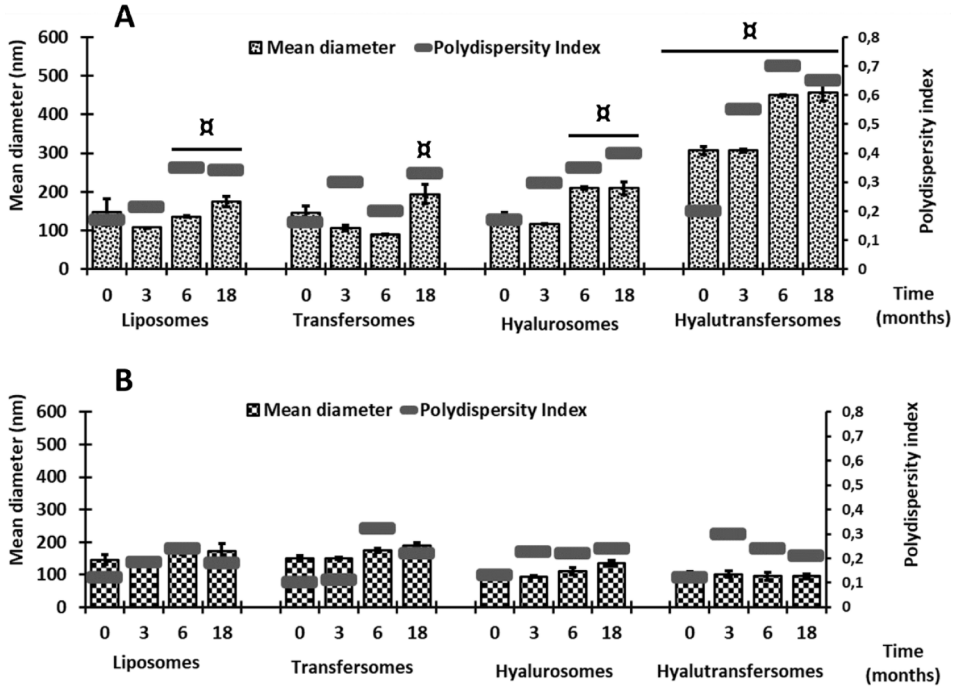

Fig. 4. Mean diameter and polydispersity index of phytochemicals loaded vesicles without mannitol (A) and with mannitol (B), freezer-dried and rehydrated at scheduled times. Mean values (bars) ± standard deviations (error bars) are reported (n ≥ 3). The symbol ¤ indicates values statistically different from that of vesicles prepared with mannitol (B).

that of hyalurosomes reached ~ 209 nm ($p < 0.05$ versus the values of liposomes) and that of hyalutransfersomes reached ~ 456 nm ($p < 0.05$ versus the values of others) (Fig. 3).

3.2. Stability of oleuropein after freezer-drying

The stability and integrity of oleuropein before and after preparation and freeze-drying processes were evaluated using an UPLC. The retention time of oleuropein standard was 1.2 min and that of oleuropein in dispersion or loaded in vesicles, before and after freeze-drying, with or without mannitol, was the same of the standard, confirming that the phytochemical was not modified by the dehydration processes. The quantification method demonstrates good linearity over the range of 0.02–0.6 mg/ml of oleuropein, with $r^2 > 0.999$ (Figure S1). The recovery of oleuropein in vesicles ranges from 94 to 101 %.

3.3. Release of bioactives from the rehydrated freeze-dried formulations

The amount of oleuropein and lentisk oil released from the rehydrated vesicles, prepared with or without cryoprotectant, was measured as a function of time at scheduled intervals of time (1, 2, 6, 8 and 24 h) (Fig. 5). The phytochemicals dispersed in water without vesicles was used as reference.

The amount of phytochemicals released from the water dispersion was the highest at all the times, while the vesicles controlled their release over time. Indeed, the 100 % of phytochemicals was released from the dispersion already at 8 h while any vesicles released 100 % of phytochemicals at 24 h. Hyalurosomes and hyalutransfersomes delayed the release of phytochemicals up to 8 h in a better extend than transfersomes. The release of phytochemicals provided from the vesicles prepared with mannitol was the same confirming that the addition of the cryoprotectant facilitated the rehydration of vesicles but did not affect their carrier ability and effectiveness in delaying the release of payloads.

3.4. Cell viability

Primary normal human dermal fibroblasts have been selected as the most representative cells of the skin, especially suitable for the evaluation of the cytotoxicity of formulations, which were added in their medium at different dilutions (Fig. 6). When cells were incubated with phytochemical loaded liposomes, transfersomes and hyalutransfersomes at the lower dilution (corresponding to 20 μg/mL of oleuropein) a decrease of cell viability was observed, as the viability was ~ 75 % using liposomes, ~46 % using transfersomes and ~ 50 % using hyalutransfersomes. The higher decrease of cell viability seemed to be related to the presence of tween 80, which can have a toxic effect. On the contrary, phytochemicals in dispersion or loaded in hyalurosomes at the same dilution did not have any toxicity as the cell viability was ~ 100 %. The phytochemicals in dispersion or loaded in vesicles at higher dilutions (corresponding to 2, 0.2 and 0.02 μg/mL of oleuropein) did not cause cell mortality, as the viability was ≥ 88 %. Based on the biocompatibility test, 0.2 and 2 μg/mL of oleuropein were selected as suitable dilutions for further experiments.

3.5. Scratch assay

In vitro scratch assay was carried out on a cell monolayer of normal human dermal fibroblasts. The ability of oleuropein and lentisk oil in dispersion or loaded in vesicles to stimulate cell proliferation and migration was evaluated measuring the rate of the closure of the wounded area (Fig. 7). At 24 h, the wound closure of untreated cells, used as positive control, was about 50 %. Similar results were obtained treating the wounded monolayer with the phytochemicals in dispersion as the percentage of wound closure was ~ 55 %, irrespective of the dilution used (corresponding to 0.2 and 2 μg/mL of oleuropein). When the scratched cell's monolayer was treated with liposomes, transfersomes, hyalurosomes and hyalutransfersomes at the highest dilution (corresponding to 0.2 μg/mL of oleuropein), wound closure was ~ 74 % at 24 h. The values increased up to ~ 80 % when vesicles were used at the lowest dilution (corresponding to 2 μg/ml of oleuropein). Using the vesicle formulations, at 48 h, the wounded area was completely closed, irrespective of both composition and dilution used (Fig. 7).

3.6. Enzyme-linked immunosorbent assay (ELISA) for quantification of MMP-1, MMP-2, IL–6 and IL-8

Matrix metalloproteinases (MMPs), cytokines and chemokines, especially the inflammatory ones (IL-6 and IL 8) are involved in the regulation of the healing processes and play an important role in immune response and tissue repair, thus their quantification in cells and tissues provides an estimation of the physio-pathological conditions. Given that, the monolayers of primary normal human dermal fibroblasts were stimulated with lipopolysaccharide, a damaging agent present in the membrane of Gram-negative bacteria, which triggers the abundant secretion of many cytokines and stimulates the inflammation cascade processes [30]. Subsequently, the cells were scratched, treated with the oleuropein and lentisk oil formulations diluted to reach 0.2 and 2 μg/mL of oleuropein and the levels of the matrix metalloproteinases MMP-1,

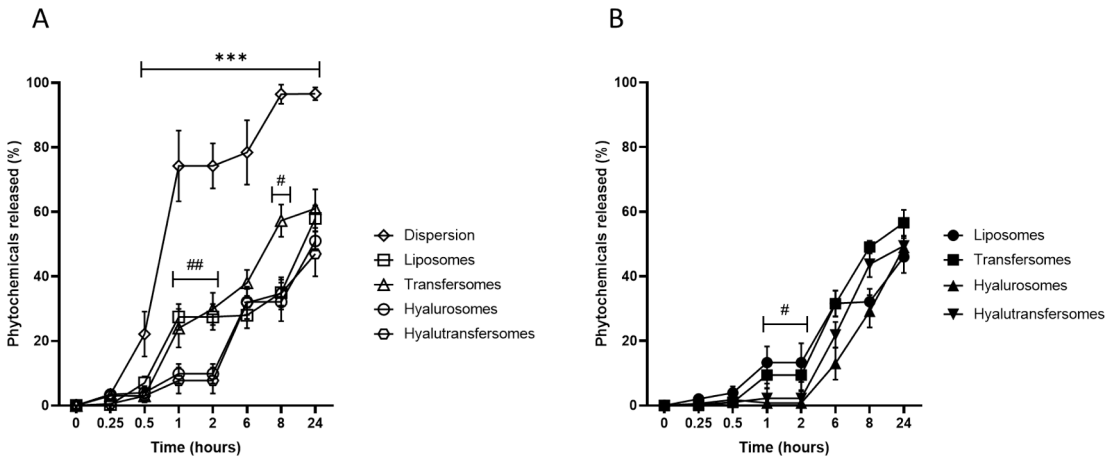

Fig. 5. Amount (%) of phytochemicals released from water dispersion or from vesicles prepared without (A) or with mannitol (B), as a function of the time. Symbol *** indicates values of dispersion statistically different from that of other formulations, $p < 0.0001$, symbol ## indicates values of hyalurosomes and hyalutransfersomes statistically different from that of other formulations ($p < 0.001$), symbol # indicates values of liposomes and transfersomes statistically different from that of other formulations ($p < 0.01$).

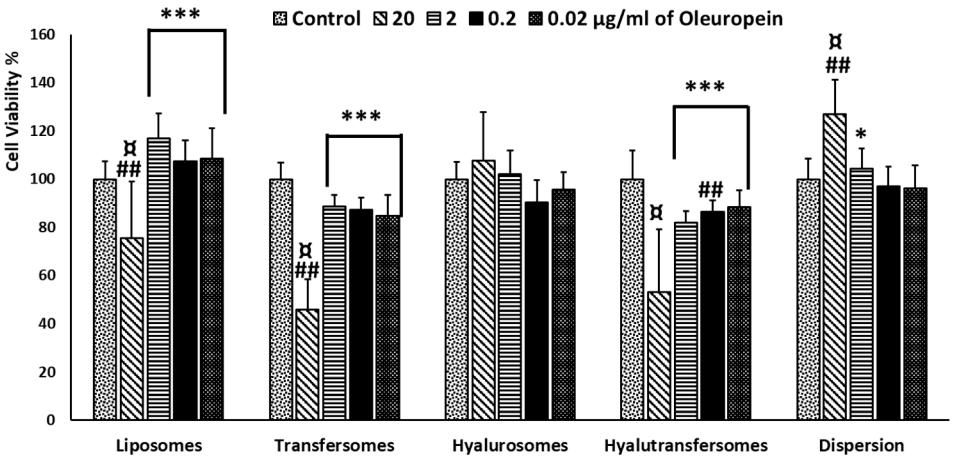

Fig. 6. Viability of normal human dermal fibroblasts treated with phytochemicals in dispersion or loaded in liposomes, transfersomes, hyalurosomes and hyalutransfersomes. Mean values (bars) ± standard deviations are reported, n = 3. Symbol ## indicates values statistically different from the control ($p < 0.005$). Symbol *** indicates values statistically different from the control ($p < 0.001$). Symbol * indicates values statistically different from the control ($p < 0.05$). Symbol ¤ indicates values statistically different from all the other formulations at the same concentration ($p < 0.05$).

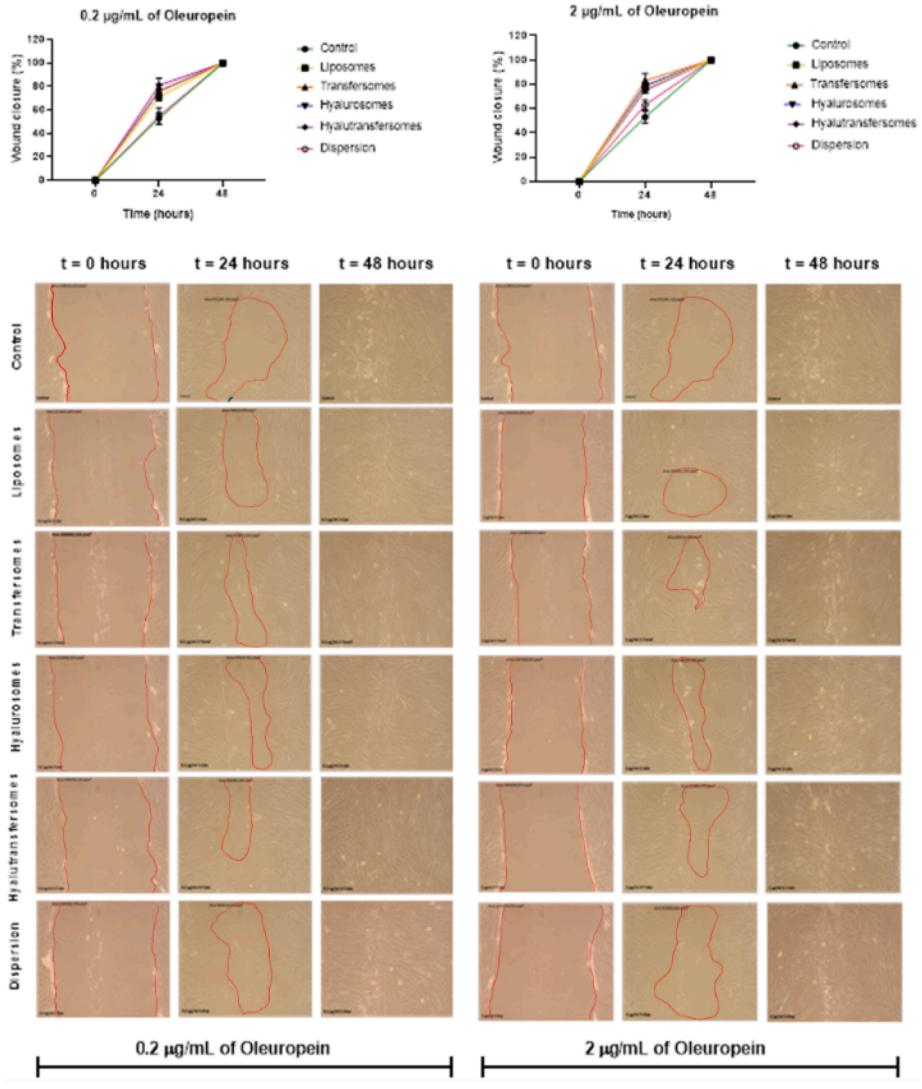

Fig. 7. Upper panel: percentage of closure of the wounded area, performed on a monolayer of normal human dermal fibroblasts, treated with oleuropein and lentisk oil in dispersion or loaded in liposomes, transfersomes, hyalurosomes and hyalutransfersomes at two different dilutions (corresponding to 0.2 and 2 µg/ml of oleuropein). Lower panel: representative images of a scratched area treated for 48 h with oleuropein and lentisk oil in dispersion or loaded in liposomes, transfersomes, hyalurosomes and hyalutransfersomes at two different dilutions (corresponding to 0.2 and 2 µg/mL of oleuropein). Mean values (bars) ± standard deviations are reported, n = 4. Symbol **** indicates values significantly different from that of cells untreated (negative control) or treated with the dispersion, $p < 0.0001$.

MMP-2 (Figure S1), cytokines IL-6 and IL-8 released were measured (Fig. 8).

The treatment of cells stimulated with lipopolysaccharide led to an increased production of inflammatory markers (MMP-1, MMP-2, IL-6 and IL-8). The treatment of the stimulated and scratched cells with oleuropein and lentisk oil in dispersion or loaded in the vesicles counteracted the overproduction of MMP-1 and IL-6, while their effectiveness was lower for MMP-2 (see Supplementary Material, Figure S2) and IL-8, as the production of these markers was not efficiently inhibited in comparison with the positive control cells. Specifically, only oleuropein

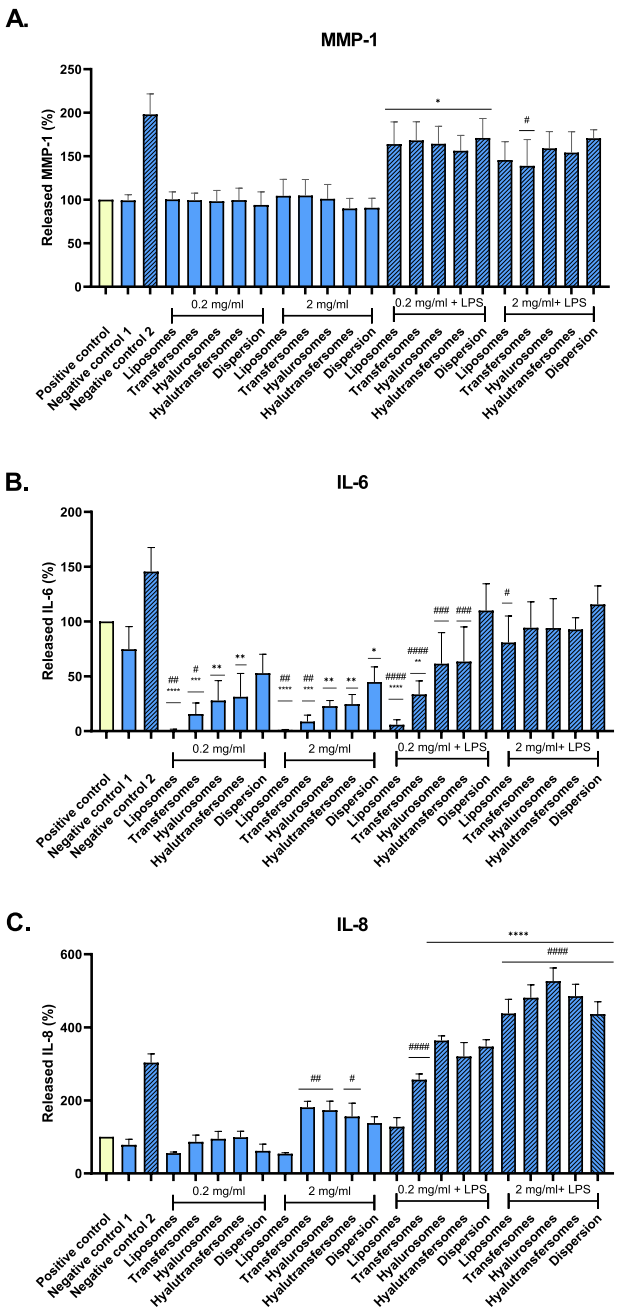

Fig. 8. Percentage of MMP-1 (A), IL-6 (B) and IL–8 (C) released from primary normal human dermal fibroblasts pre-treated with lipopolysaccharide and treated with oleuropein and lentisk oil in dispersion or loaded in liposomes, transfersomes, hyalurosomes and hyalutransfersomes at two different dilutions (corresponding to 0.2 and 2 μg/mL of oleuropein). Mean values ± standard deviations are reported, n = 3. Symbol * indicates values statistical different from positive control (untreated, unscratched, unstimulated cells): * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Symbol $^{\#}$ indicates values statistical different from negative control 1 (untreated, scratched, unstimulated cells) and negative control 2 (untreated, scratched, stimulated cells): $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$; $^{\#\#\#\#}$ $p < 0.0001$.

and lentisk oil loaded liposomes down-regulated the production of IL-8 in scratched primary normal human dermal fibroblasts, while, when the phytochemicals were loaded in other vesicles (transfersomes, hyalurosomes and hyalutransfersomes) an up-regulation of this chemokine was founded.

4. Discussion

The skin is the outermost organ of our organism, which is continuously subjected to chemical and physical insults that may modify its homeostasis. The general audience is increasingly interested to protective and beneficial products for skin treatment, especially those containing natural ingredients, however, the majority of these formulations are not effective due to the low absorption and bioavailability of natural molecules [31–33]. To this purpose, several innovative formulations of plant-derived bioactive molecules have been tested to improve its efficacy. Given that, this study focused on i) co-loading of oleuropein and lentisk oil in phospholipid vesicles, thus allowing to associate the beneficial and complementary properties of these phytochemicals in a unique formulation, which has optimal carrier ability in the skin and ii) evaluation of their effect on the protection of skin against oxidative stress caused by lipopolysaccharide. The obtained results of the current study are summarized in Fig. 9.

Indeed, liposomes have been largely used for skin delivery of bioactive molecules, however, their ability to cross the skin was not confirmed and the delivered payload often remains confined in the upper layers [34]. For this reason, the liposome formulation was improved adding a surfactant in transfersomes, or sodium hyaluronate in hyalurosomes or even both in hyalutransfersomes, since these enriched and more performant vesicles have proven to be excellent skin carriers [35]. Empty vesicles were smaller than the corresponding drug-loaded ones, and highly negatively charged, but slightly polydispersed, probably because the loaded phytochemicals are mostly lipophilic and intercalate inside the bilayer, contributing to the rearrangement of the final assembling [3637]. The presence of the phytochemicals also affected the homogeneity of the dispersions, while the surface charge was not affected by them confirming their intercalation inside the vesicles and not at the vesicle surface [38,39]. Despite the presence of sodium hyaluronate, which immobilises the vesicles and usually ensure a good stability, the prepared vesicle dispersions had a shelf-life of maximum 6 months, after this time the vesicles broke, aggregated, and become unmeasurable. The instability can be related to the high concentration of lipophilic phytochemicals intercalated in the bilayer, which modify its assembly and make it less stable [40]. Additionally, the small size of the vesicles corresponded to a high specific surface area that, increased the interactions among particles facilitating the occurrence of instability phenomena such as aggregation and fusion [41]. However, it is important that the vesicles remain small during the storage and maintain unchanged their physico-chemical characteristics, avoiding the release of payloads. To prevent vesicle instability, a cryoprotectant was added to the dispersions, which were freeze-dried, stored and redispersed before the use. To find the most appropriate cryoprotectant, different sugars were tested and mannitol was selected, according to previous study [42–44]. During the freeze-drying process, the sugar replaced the water in the internal spaces among the bilayers bridging phospholipids by hydrogen bonds, and avoiding the collapse and break of vesicles [42]. Moreover, in absence of water, sugars formed glassy matrixes, highly viscous, which limited the mobility of the vesicles, their fusion and the break of bilayers caused by ice formation [45,46]. The freeze-dried dispersions with and without mannitol were stored and rehydrated by gentle shaking and the size of the vesicles did not changed after drying and rehydration, irrespective to the presence of mannitol, according to the results of Susa et al. which, after freeze-drying, obtained vesicles still comparable to the undried ones [45]. However, the mannitol exserted a key role during the storage of freezer-dried dispersion, since, after the rehydration at 6 and 18 months, the size of vesicles without mannitol strongly increased, while that of vesicles prepared with mannitol remained constant during the all period (18 months). Luckily, the addition of mannitol during the freeze-drying process, did not interfere with the release of phytochemicals after the rehydration. Indeed, the release rate of the phytochemicals, obtained with the vesicles prepared with the sugar was perfectly comparable with

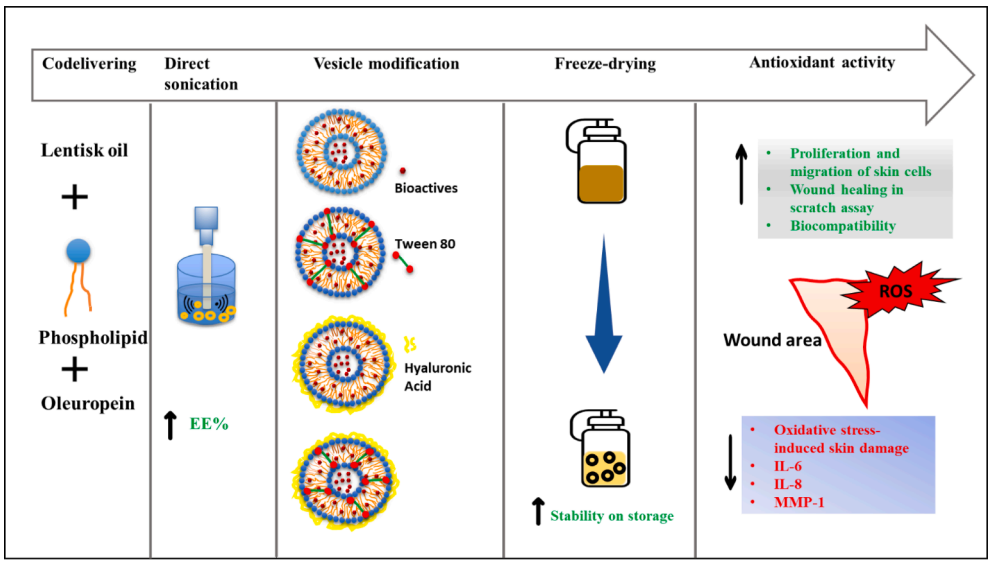

Fig. 9. Representative cartoon of the performed studies.

that obtained with the corresponding vesicles prepared without mannitol and in both cases the control of the release was more effective in comparison with that obtained with the dispersion, used as reference. Additionally, the vesicles containing sodium hyaluronate controlled the release in a better extent than the other vesicles, probably thanks to the immobilizing structure created by the polymer [35,47]. Biological assessments confirmed the effectiveness of oleuropein and lentisk oil in association, especially when loaded in phospholipid vesicles specifically tailored to improve their anti-inflammatory activity at skin level. Indeed, the phytochemicals loaded in vesicles permitted the closure of the scratched area already at 24 h, while their dispersion allowed the wound closure only at 48 h, probably favouring the proliferation and migration of fibroblasts in the lesions [48]. To better understand the healing process and the cytokines involved, the scratched area was also treated with lipopolysaccharide simulating an infected wound and the expression of the proinflammatory markers by cells. MMP-1, MMP-2, IL-6 and IL-8 levels were monitored, as they stimulate the infiltration and activation of neutrophils and other phagocytic cells into the wound, thus inducing inflammation and free radical generation, along with activation of matrix metalloproteases. Especially, metalloproteases are mainly involved in the turnover and remodelling of the dermis by means of degradation of collagen and other proteins of extracellular matrix [49]. In normal skin, the amount of metalloproteases is very low as they are kept in their inactive form thanks to their bound with endogenous inhibitors. The stimulation or prolongation of the inflammatory process led to the production of high amount of them, especially metalloprotease-1, which is involved in the collagen type1 breakdown [50], while metalloprotease-2 is mainly a co-adjuvant of metalloprotease-1 activity as it completed the degradation of fibrillar collagen and cleave the collagen type IV [51]. Oleuropein and lentisk oil loaded vesicles inhibited the production of metalloproteases-1, probably preventing collagen degradation and permitting a faster wound healing. At the same time, the production of IL-6 was effectively inhibited by the tested phytochemical loaded vesicles, especially using the lowest concentration, while the inhibition of IL-8 was evident only using liposomes and transfersomes at the lowest concentration. Despite the ineffective inhibition of IL-8, the obtained results are encouraging as IL-6 is mostly involved in the control of IL-8 production [52], thus the inhibition of IL-6 production may in turn reduce or control the liberation of IL-8 [53]. Moreover, the low effectiveness of the formulations against IL-8 may be also due to their chemical properties, indeed it has been demonstrated that among non-steroidal anti-inflammatory drugs, only some of them, are capable of effectively reduce the plasma levels of IL-8 [53].

Overall results demonstrated that the loading of oleuropein and lentisk oil in phospholipid vesicles, in particular liposomes and transfersomes, permits to control the release of payloads in the skin and promoted their efficacy in inflammation processes inhibiting the production of the mediators of the inflammation. Moreover, these formulations fully respond to the need of the modern society of using natural and safe products.

5. Conclusions

Oleuropein and lentisk oil were efficiently co-loaded in phospholipid vesicles modified with tween 80 (transfersomes) or sodium hyaluronate (hyalurosomes) or even with their combination (hyalutransfersomes). The addition of mannitol in their dispersion facilitated the rehydration after freezer-drying up to 18 months. Phytochemicals loaded vesicles were biocompatible and promoted the proliferation of human dermal fibroblasts in the scratched area, down-regulating the production of metalloprotease-1 and IL-6, which are involved in the onset of the inflammatory process. Overall results suggested that these formulations are suitable for the prevention or treatment of skin diseases, especially those connected with inflammatory processes such as psoriasis, acne or even skin aging.

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

The authors are unable or have chosen not to specify which data has been used.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2023.02.018.

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