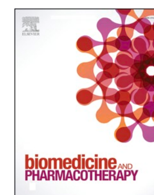
Biomedicine & Pharmacotherapy 144 (2021) 112351

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha



Formulation of liposomes loading lentisk oil to ameliorate topical delivery, attenuate oxidative stress damage and improve cell migration in scratch assay

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ARTICLE INFO

Keywords:
Pistacia lentiscus
Liposomes
Keratinocytes & fibroblasts
Wound healing
Scratch assay
Skin permeation studies, confocal microscopy

ABSTRACT

Pistacia lentiscus L. is a sclerophyllous shrub capable of growing under harsh climatic conditions especially in the Mediterranean Basin. Different products can be obtained from this plant, such as essential oil, mastic gum or even fixed oil. The last is well known for its flavor which is mainly exploited in the food industry. Additionally, it has been traditionally used in the treatment of skin diseases, but, at the moment, any suitable formulation for skin delivery has been formulated and its biological effects was not deeply confirmed. Given that, in the present study, the lentisk oil has been formulated in liposomes at different concentrations (10, 20, 30 mg/ml) and their physicochemical, technological and main biological properties have been evaluated. Vesicles were prepared by using natural soy lecithin and a green and organic solvent free method, thus obtaining spherical, small (~ 118 nm), homogeneously dispersed (0.27) and highly negatively charged (~ −62 mV) vesicles. The used amount of oil loaded in liposomes (10, 20, 30 mg/ml) modulated the penetration ability of vesicles in the skin, favoring the deposition of the payload in the deeper strata. The loading in the vesicles potentiated the ability of oil to counteract the damaging effects caused by hydrogen peroxide in keratinocytes and fibroblasts and facilitate their migration in a cell monolayer lesion. Overall findings suggested that the incorporation of lentisk oil in liposomes made from soy lecithin can be an alternative and natural approach to exploit it in pharmaceutical ad cosmetical applications and manufacturing natural products suitable for the treatment of skin lesions.

1. Introduction

Pistacia lentiscus L. commonly known as lentisk or mastic tree in Greece, is a sclerophyllous Mediterranean shrub belonging to the Anacardiaceae family [1]. The fruits, galls, resin and leaves of *P. lentiscus* have been traditionally used in folk medicine to treat a wide range of diseases in the whole Mediterranean Basin, where it grows wild with a large geographical and bio-climatical distribution range [2]. The main traditional products obtained from this plant are the edible oil from the berries and the mastic gum, a resin secreted by incising the tree bark [3]. The fixed oil has been used mainly as food dressing, flavouring, colorant and antioxidant agent, especially in Tunisia and Greece, but also as ethnomedical remedy for the treatment of gastrointestinal upsets, gastric ulcers or skin illness. In Sardinia, this oil was mainly used as flavour in cuisine, but also as ointment to treat wounds and as foot deodorant [4]. In the Middle East, lentisk oil was particularly appreciated for its antiseptic properties, and was used to treat wounds and gastric ulcers. Since ancient times, many beneficial properties have been ascribed to this oil, Greeks and Latins used mastic oil as diuretic, anti-inflammatory, antiseptic and healing agent to treat gastric ulcers, local skin diseases and coughs and colds [5,6]. These traditional uses and its nutritional properties have been confirmed by both in vitro and

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https://doi.org/10.1016/j.biopha.2021.112351
Received 30 August 2021; Received in revised form 17 October 2021; Accepted 19 October 2021
Available online 28 October 2021
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in vivo studies [7–9]. In addition, the chemical composition of the lentisk fixed oil has been investigated disclosing a high content of bioactives such as fatty acids, especially oleic, palmitic and linoleic, and phenolic compounds, such as tocopherols, carotenoids and anthocyanins [10,11]. The unsaponifiable fraction of the fixed oil is responsible of its beneficial effect in the healing of the injured skin [3]. The antioxidant compounds promote the repair of skin damages and inhibit lipid oxidation and depletion of antioxidant defence enzymes [12].

A modern strategy, to improve the efficacy at skin level of such lipid phytocomplexes, is their incorporation into nanocarriers. However, the *P. lentiscus* fixed oil was never loaded into nanocarriers while the essential oil obtained from this plant was encapsulated in polymeric nanoparticles specifically designed for skin application [13].

For the first time in this study, the *P. lentiscus* fixed oil has been loaded in liposomes, which have been previously used to load several essential oils but only few fixed oils whose incorporation allowed to improve their beneficial activities (e.g. *Nigella sativa* seed oil and neem oil) [14,15]. Liposomes were made with natural occurring soy lecithin and the lentisk oil was loaded at increasing concentrations (10, 20, 30 mg/ml). The main physicochemical (mean diameter, polydispersity index, zeta potential) and technological (entrapment efficiency, storage stability and skin penetration ability) properties of vesicles were evaluated and a preliminary evaluation of their biological abilities, such as biocompatibility, protection against oxidative damages and in vitro promotion of migration of keratinocytes and fibroblasts in a cell monolayer lesion, has been carried out as well.

2. Materials and methods

2.1. Materials

Soy lecithin was purchased from Galeno (Potenza, Italy). The *P. lentiscus* oil was a kind gift of a local enterprise, SSA Mediflora (Pula, CA, Italy). All the chemical products and solvents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Cell medium, foetal bovine serum, penicillin, streptomycin and all the other reagents and plastic for cell culture were purchased from Life Technologies Europe (Monza, Italy).

2.2. Sample preparation

Soy lecithin (60 mg/ml) and lentisk oil (10 or 20 or 30 mg/ml) were weighed in a glass vial and left hydrating overnight with water (2 ml) to promote the swelling of the phospholipid. The obtained dispersions were sonicated (5 s on and 2 s off, 20 cycles; 13 µ of probe amplitude) with a high intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, United Kingdom), to obtain 10lentisk-, 20lentisk- and 30lentisk-liposomes (Table 1).

The free bioactives contained in the oil and not entrapped inside the vesicle bilayer were separated dialysing the samples (1 ml) against water (2.5 litres). Dialysis tubing (Spectra/Por® membranes, 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands) were filled with the vesicle dispersions and transferred in a bath of distilled water (2,5 litres) maintained under stirring and at room temperature for 4 h. The water was replaced every hour. The used water (10 litres total) was able to theoretically remove all the bioactives contained in 1 ml of liposome dispersions.

Table 1
Amount of each component used to prepare empty and lentisk oil loaded liposomes.

	Lentisk oil (mg/ml)	Soy lecithin (mg/ml)	Water (ml)
Empty liposomes	–	60	1
10lentisk-liposomes	10	60	1
20lentisk -liposomes	20	60	1
30lentisk -liposomes	30	60	1

2.3. Vesicle characterization

Cryogenic electron transmission microscopy (cryo-TEM) analyses were performed by using a Tecnai F20 TEM (FEI Company). Briefly, a thin aqueous film was formed on a glow-discharged holey carbon grid and vitrified by plunging into ethane, using a Vitrobot (FEI Company, Eindhoven, The Netherlands), which was then observed in a low dose mode, at 200 kV and at a temperature around ~−172 °C [16].

The average diameter, polydispersity index and zeta potential were determined by means of Photon Correlation Spectroscopy method by using a Zetasizer Ultra (Malvern Panalytical Ltd, Worcestershire, UK). Before the analysis, samples were diluted (1:100) with water to be optically clear and avoid the attenuation of the laser beam by the particles along with the reduction of the scattered light that can be detected [19].

Entrapment efficiency was calculated as the percentage of the amount of bioactives initially used versus that found in dialyzed samples. The quantification of lentisk oil was performed measuring fatty acid methyl ester (FAME) by gas chromatography coupled to flame ionization detector after derivatization with sodium methoxide.

2.4. Stability studies

The vesicle stability was assessed by monitoring the vesicle average size and zeta potential over 90 days of storage at room temperature (25 ± 1 °C).

2.5. In vitro skin delivery studies

Experiments were performed under non-occlusive conditions by using Franz cells (diffusion area 0.785 cm^2) and new-born pig skin. One-day-old pigs were provided by a local slaughterhouse. The skin, stored at −80 °C, was cut in specimens (n = 6 for each formulation), which were pre-hydrated in saline at 25 °C for 12 h. When properly hydrated, they were sandwiched between donor and receptor compartments of the Franz diffusion cells. The receptor was filled with 5.5 ml of saline, continuously stirred and thermostated at 37 ± 1 °C. Vesicles were labelled with 1,2-dioleolyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl, 0.035 mg/ml; phospho-rhodamine) and applied (100 µl) on the skin surface. After 4 and 8 h of treatment, the skin specimens were gently washed with distilled water, the diffusion area punched out and rapidly frozen at −80 °C. Sections of skin (7 µm thickness) were cut with a cryostat (Leica CM1950, Barcelona, Spain) orthogonally (in the *z* axis) to the surface and examined under a FluoView FV1000 inverted confocal microscope (Olympus, Barcelona, Spain) equipped with an Ultraviolet-Visible light laser. Images with a field size of 1024 × 1024 µm were generated by using an UPlanSApo 20 × objective NA 0.75. Phospho-rhodamine was excited at 559 nm and detected at 578 nm.

2.6. Biocompatibility and protection provided by liposomes against cell oxidative stress

Human keratinocytes (HaCaT) and primary mouse embryonic fibroblasts (3T3) (ATCC collection, Manassas, VA, USA) were grown as monolayer in 75 cm^2 flasks, incubated in 100% humidity and 5% CO_2 at 37 °C, by using DMEM with high glucose (4500 mg/l glucose), supplemented with foetal bovine serum (10%), penicillin and streptomycin (1%), as culture medium.

The biocompatibility of samples was evaluated seeding keratinocytes and fibroblasts (7,5 $\times 10^3$ cells/well) into 96-well plates and incubating them for 24 h. The lentisk oil in dispersion or loaded into liposomes at different dilutions (1:500, 1:1.000, 1:10.000, 1:100.000, corresponding

to 60, 30, 3, 0.3 μg/ml of oil using 30lentisk-liposomes and dispersion; 40, 20, 2, 0.2 μg/ml of oil using 20lentisk-liposomes and 20, 10, 1, 0.1 μg/ml of oil using 10lentisk-liposomes) was added. Cells were incubated for 48 h and after their viability was measured using the MTT [3(4,5-dimethylthiazolyl-2)−2, 5-diphenyltetrazolium bromide] colorimetric assay. Cells were washed 3 times with fresh medium and 100 μl of MTT reagent (0.5 mg/ml in PBS) was added in each well. After 2–3 h, the formed formazan crystals were dissolved in dimethyl sulfoxide and their concentration was spectrophotometrically quantified at 570 nm with a microplate reader (Synergy 4, Reader BioTek Instruments, AHSI S.P.A, Bernareggio, Italy).

The ability of formulations to avoid damages caused by oxidative stress was evaluated stressing keratinocytes and fibroblasts (7,5 $\times 10^3$ cells/well) with hydrogen peroxide (30% diluted 1:30.000 *v/v* with PBS) for 4 h and simultaneously treating them with the lentisk oil in dispersion or loaded in liposomes, diluted (1:10000) with medium to reach 3 μg/ml of oil using 30lentisk-liposomes and dispersion, 2 μg/ml of oil using 20lentisk-liposomes and 1 μg/ml of oil using 10lentisk-liposomes. At the end of each experiment, the survival of cells was measured by using the MTT test. Viability was calculated as percent of viability of cells incubated with samples versus the viability of untreated control cells (100% viability).

2.7. In vitro scratch assay

The ability of the lentisk oil in dispersion or loaded into liposomes to stimulate both cell proliferation and migration was evaluated by measuring the speed rate of the wound healing as a function of the time of exposition to the samples (scratch assay). Cells were cultured in 6-well plates until the complete confluence was reached. Then, a linear scratch was generated by using a sterile plastic pipette tip. The scattered fragments of cells were removed by gently washing with fresh medium. The cells were treated with lentisk oil in dispersion or loaded in liposomes properly diluted (1:10000) with medium (3 μg/ml of oil using 30lentisk-liposomes and dispersion, 2 μg/ml of oil using 20lentisk-liposomes and 1 μg/ml of oil using 10lentisk-liposomes) and incubated for 48 h. Untreated cells and cells treated with the aqueous dispersion of the essential oil were used as controls. The changes of the area of the lesion were monitored by using an optical microscope (10 × objective) and the related images were captured at initial time zero to measure the wounded area immediately after scratching (a_0) and at 24, and 48 h (a_Δ) to measure the areas during the treatment. The captured images were quantified by Java's image J software (http://rsb.info.nih.gov) by measuring the area of the wound [18]. The migration of cells toward the wounds was expressed as percentage of wound closure: $WC\% = [(a_0 - a_\Delta / a_0] \times 100\%$.

2.8. Statistical analysis of data

Results are expressed as the mean ± standard deviation. Analysis of variance (ANOVA) was used for multiple comparisons of means, and the Tukey's test and Student's *t*-test were performed to substantiate differences between groups using XL Statistics for Windows. The differences were considered statistically significant for $p < 0.05$.

3. Results

3.1. Vesicle characterization

The lentisk oil was loaded in liposomes and the maximum amount to be loaded was found by a pre-formulation study, in which increasing amount of oil, from 10 mg/ml to 50 mg/ml, were used. 40 and 50 mg/ml of lentisk oil led the formation of large vesicles, highly polydispersed and instable, while those loading 10, 20 and 30 mg/ml of oil were small in size, homogeneously dispersed and highly negatively charged. Given that, 10, 20 and 30 mg/ml of lentisk oil were selected as suitable concentrations to be incorporated in liposomes. Cryo-TEM images disclosed the formation of small vesicles, homogeneously dispersed and with a spherical and uniform shape (Fig. 1).

Photon correlation spectroscopy analyses confirmed the findings of cryo-TEM analyses, as the formation of small and homogeneous systems was detected (Table 2).

Empty liposomes, without lentisk oil, were small (~122 nm), slightly polydispersed (0.26) and negatively charged (~−60 mV). The loading of 10 mg/ml of lentisk oil allowed a significant decrease of the mean diameter, which was ~104 nm, thus significantly smaller than that of the other formulations ($p < 0.05$, versus the values of other vesicles), while the polydispersity index and the surface charge remained almost unchanged. Differently, the loading of 20 and 30 mg/ml of the oil led the formation of slightly bigger vesicles, with diameter similar to that of the empty ones (~121 nm, $p > 0.05$ among the mean diameter of empty, 20lentisk- and 30lentisk-liposomes). Even the homogeneity of the vesicles was affected by the amount of lentisk oil incorporated, but with a reverse trend as the lower value of polydispersity index has been found for 30lentisk-liposomes. The zeta potential remained strongly negative, irrespective of the used amount of oil and was mainly due to the negative group of phospholipids at the pH of dispersions, ~5.5 [19]. All tested liposomes incorporated high amount of lentisk oil, which increased as the amount of lentisk oil used for their preparation also increased.

The mean diameter of empty liposomes stored at room temperature for 90 days, slightly increased up to ~200 nm and the zeta potential became more negative (Fig. 2). The mean diameter of 10lentisk- and 20lentisk-liposomes increased only at 90 days up to ~150 nm while any important variation was detected for the other parameters. Liposomes loading the higher amount of lentisk oil, remained almost constant, disclosing a better stability on storage of these vesicles (Fig. 2).

3.2. In vitro skin delivery

The ability of liposomes to promote the delivery of lentisk oil to the skin was evaluated in vitro by using new-born pig skin and vesicles labelled with a phospholipid linked to the fluorescent rhodamine (phospho-rhodamine), which allowed the visualization of the vesicle distribution in the different strata of the skin (Fig. 3).

The vesicles pathway was strongly affected by the amount of lentisk oil incorporated. Indeed, empty vesicles were confined on the skin surface, and any significant passage of vesicles up to the deeper skin layers has been detected. Similarly, using the lower amount of oil the fluorescence provided by vesicles was mainly confined on the skin surface and a lower fluorescence was detectable in the deeper strata, irrespective of the duration of the treatment (4 or 8 h). The use of higher amount of oil (20 and 30 mg/ml) led to a dipper distribution of the vesicles, especially after 8 h of treatment. At 4 h, the red fluorescence was mainly evident on the skin surface (especially for 30lentisk-liposomes) and in the deeper strata was less intense. At 8 h of treatment the fluorescence was mainly evident and well distributed in the deeper strata, especially in the dermis confirming a strong penetration of lipid components. Probably the free lentisk oil could act as penetration enhancer by promoting the fluidification of lipid lamellar matrix imbibing the corneocytes and favouring the passage of the intact vesicles.

3.3. Biocompatibility and protection provided by liposomes against oxidative stress in both keratinocytes and fibroblasts

The most representative cells of the skin, keratinocytes and fibroblasts, were used to evaluate the cytotoxicity of samples (Fig. 4). Using the lentisk oil dispersion at the lower dilutions, (corresponding to 60–30 μg/ml of oil) the cell viability was lower (~77%, $p < 0.05$ versus the viability obtained incubating the cells with all other samples and dilutions), while at the lower concentrations (3 and 0,3 μg/ml) was ~93%. The same viability value ($p > 0.05$) was obtained loading the oil

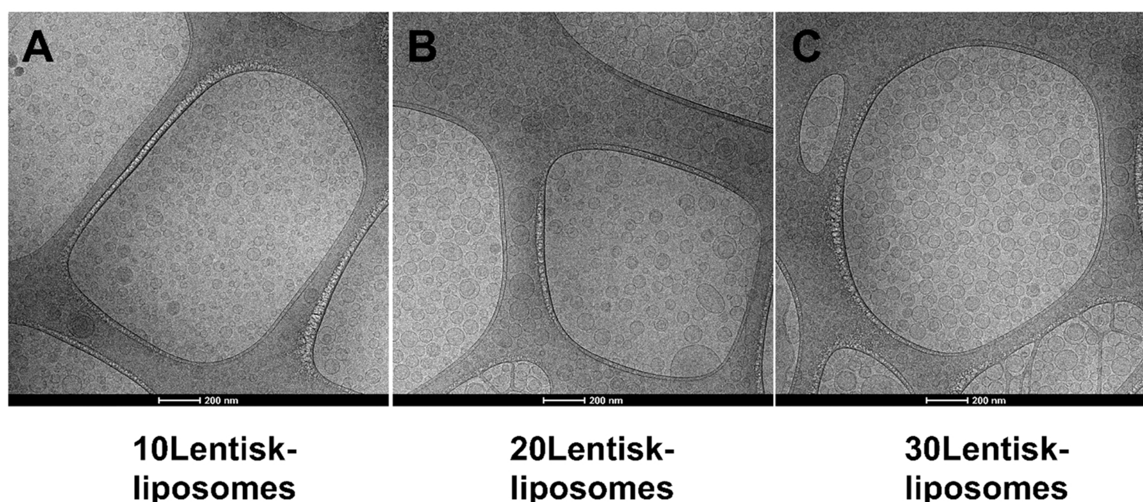

Fig. 1. Representative Cryo-TEM images of 10lentisk-liposomes (A), 20lentisk-liposomes (B) and 30lentisk-liposomes (C).

Table 2
Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency of empty vesicles and 10lentisk-liposomes, 20lentisk-liposomes and 30lentisk-liposomes. Samples were diluted with water (1:100) before the analysis. Mean values ± standard deviations are reported (n = 6). Symbol * indicates values statistically different from the others.

	Mean diameter (nm)	Polydispersity index (PI)	Zeta Potential (mV)	Entrapment efficiency (%)
Empty liposomes	121 ± 3	0.26	−60 ± 7	–
10lentisk-liposomes	*104 ± 8	0.27	−63 ± 5	72 ± 7
20lentisk -liposomes	116 ± 15	0.25	−67 ± 5	82 ± 9
30lentisk -liposomes	126 ± 13	0.23	−67 ± 3	85 ± 5

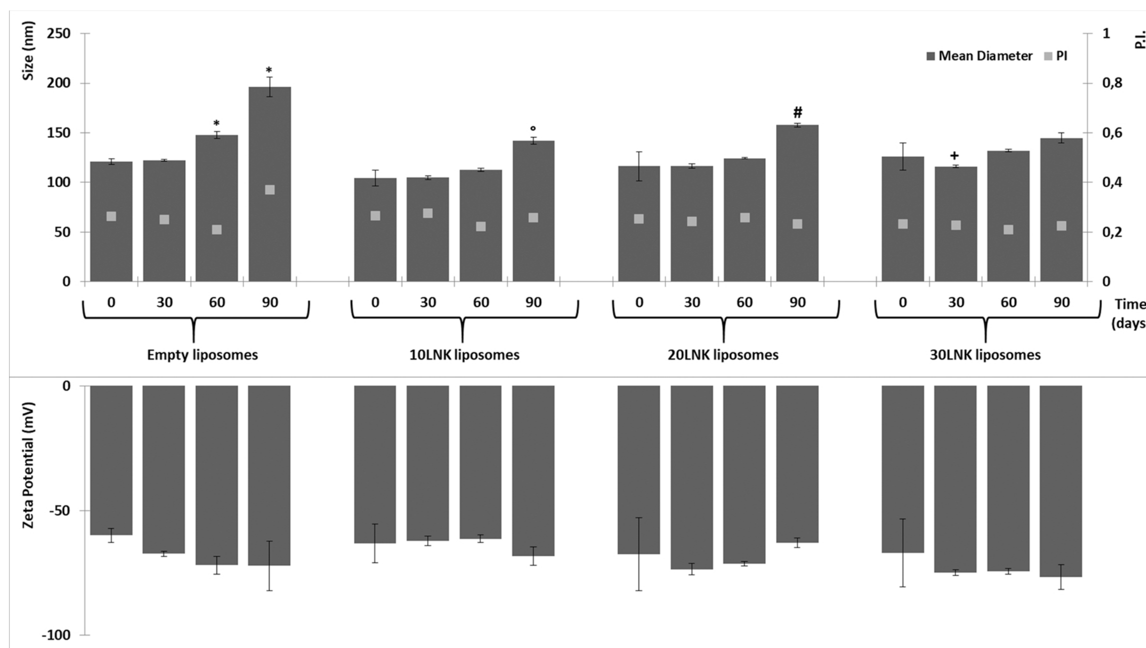

Fig. 2. Mean diameter, polydispersity index and zeta potential of empty liposomes, 10lentisk-liposomes, 20lentisk-liposomes and 30lentisk-liposomes measured by Photon Correlation Spectroscopy, during 90 days of storage at 25 °C. The mean values ± standard deviations (error bars) are reported (n = 6). Each symbol (*, °, #, +), indicates a value statistically different from the others.

in liposomes at lower dilutions and higher concentrations (60–30 or 40–20 or 20–10 µg/ml of oil). Using the three lentisk oil formulations at lower concentrations (3–0.3 or 2–0.2 or 1–0,1 µg/ml of oil) the cell viability was improved up to ~112% ($p > 0.05$ among the groups), disclosing a proliferative effect of lentisk-liposomes at these dilutions.

The incubation of fibroblasts with lentisk oil, in dispersion or loaded in 10lentisk-liposomes, increased the cell viability up to ~112%, irrespective to the used dilution ($p > 0.05$). The incubation with 20lentisk- and 30lentisk-liposomes at lower dilutions (60–40 or 40–20 µg/ml of oil) provided the same cell viability (~112%, $p > 0.05$ versus the

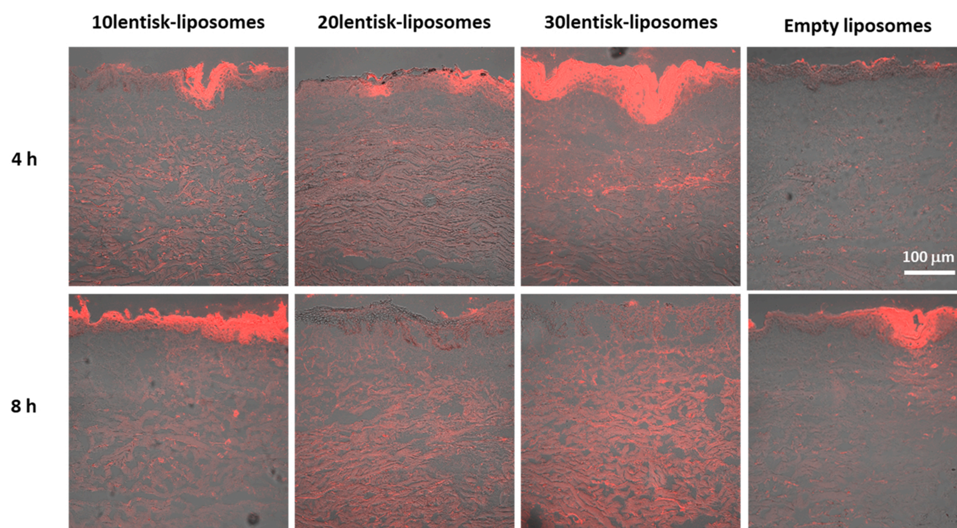

Fig. 3. Representative confocal laser scanning microscopy images of phospho-rhodamine (red) distribution in a skin section (z axis) after 4 and 8 h of treatment with empty liposomes, 10lentisk-liposomes, 20lentisk-liposomes and 30lentisk-liposomes.

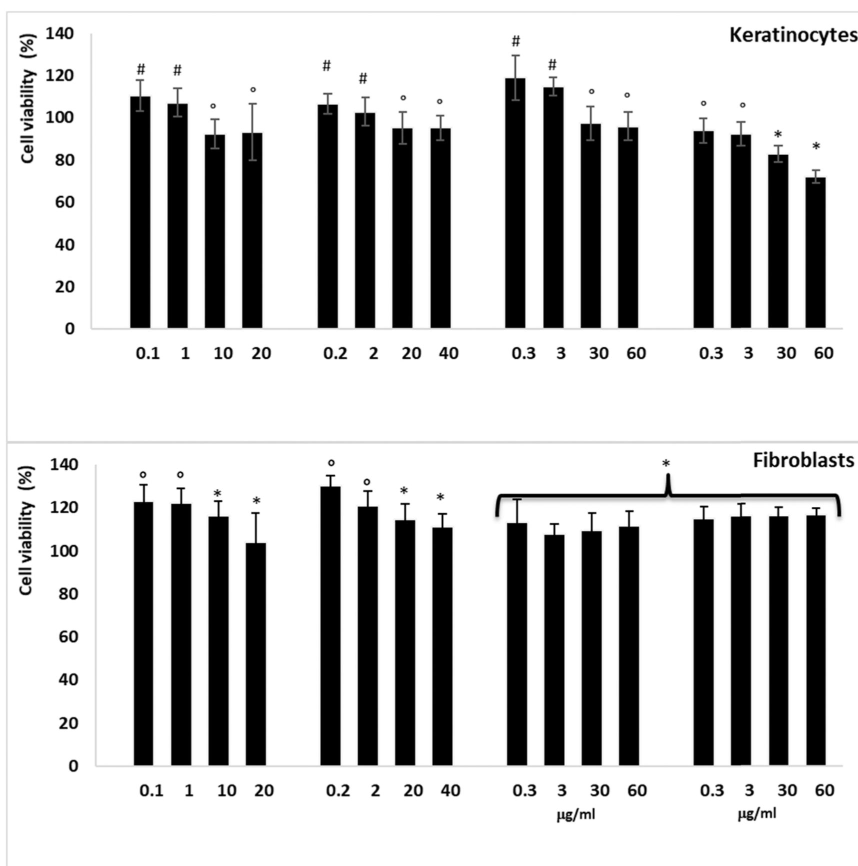

Fig. 4. Cell viability of human keratinocytes and fibroblasts treated for 48 h with lentisk oil in dispersion or loaded in vesicles diluted 500, 1000, 10,000 and 100,000 folds. Data are reported as mean values ± standard deviations (n = 9) of cell viability expressed as the percentage of untreated cells (100% of viability). Each symbol (#, *, °) indicates the same value.

viability of samples incubated with the dispersion) while the lower concentrations (4–0.4 or 2–0.2 μg/ml of oil) provided the highest values of viability, ~123%, ($p < 0.05$ versus the viability of samples incubated with samples and dilutions).

Considering that lentisk-liposomes were not toxic at the tested dilutions and the dilution 1/100,000, corresponding to concentrations 3, 2 or 1 μg/ml of oil provided proliferation of keratinocites and fibroblasts, this dilution was selected to evaluate the bioactivity of formulations in further studies.

The ability of formulations to counteract the toxic effect of hydrogen peroxide in cells were evaluated as well (Fig. 5). The treatment of cells with hydrogen peroxide led to a significant reduction of keratinocytes

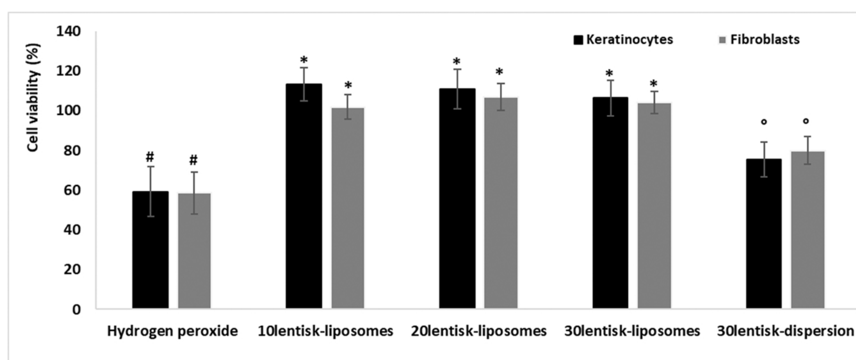

Fig. 5. Protective effect of lentisk oil in dispersion (3 μg/ml) or loaded in 10lentisk-liposomes (1 μg/ml), 20lentisk-liposomes (2 μg/ml) and 30lentisk-liposomes (3 μg/ml), against hydrogen peroxide-induced oxidative stress in keratinocytes and fibroblasts. Data are reported as mean values ± standard deviation of cell viability expressed as the percentage of untreated cells (100% viability). Each symbol (*, °,#) indicates the same value.

and fibroblasts viability, which reached ~58% ($p < 0.05$ versus other values). The treatment of stressed cells with lentisk oil in dispersion led to a significant increase of cell viability up to ~77% ($p < 0.05$ versus other values), while the loading in vesicles ensure a protection of both keratinocytes and fibroblasts irrespective of the used formulation, as the cell viability was ~106% ($P < 0.05$ versus the values provided by the oil dispersion). Results confirmed the capability of the tested nanovesicles of inhibiting the toxic effect provided by the treatment with hydrogen peroxide.

3.4. *In vitro scratch assay*

In vitro scratch assay was carried out on a cell monolayer of keratinocytes and fibroblasts to evaluate the capability of oil in dispersion or loaded in liposomes of promoting the wound closure by favouring the cell migration. As previously reported, the samples were diluted to reach the concentration, which provided higher cell proliferation (3 μg/ml of oil using 30lentisk-liposomes and dispersion, 2 μg/ml of oil using 20lentisk-liposomes and 1 μg/ml of oil using 10lentisk-liposomes). The thickness of lesions was measured at different time points to evaluate the ability of formulations to favour not only the cell proliferation but also their migrations versus the scratched region free of cell (Figs. 6 and 7). At 24 h, the percent closure of untreated cells was ~20% and reached ~40% at 48 h. When the cells were treated with oil in dispersion or loaded into 10lentisk-liposomes, the behaviour was slightly better: ~20% at 24 h and ~60% at 48 h. A significant reduction

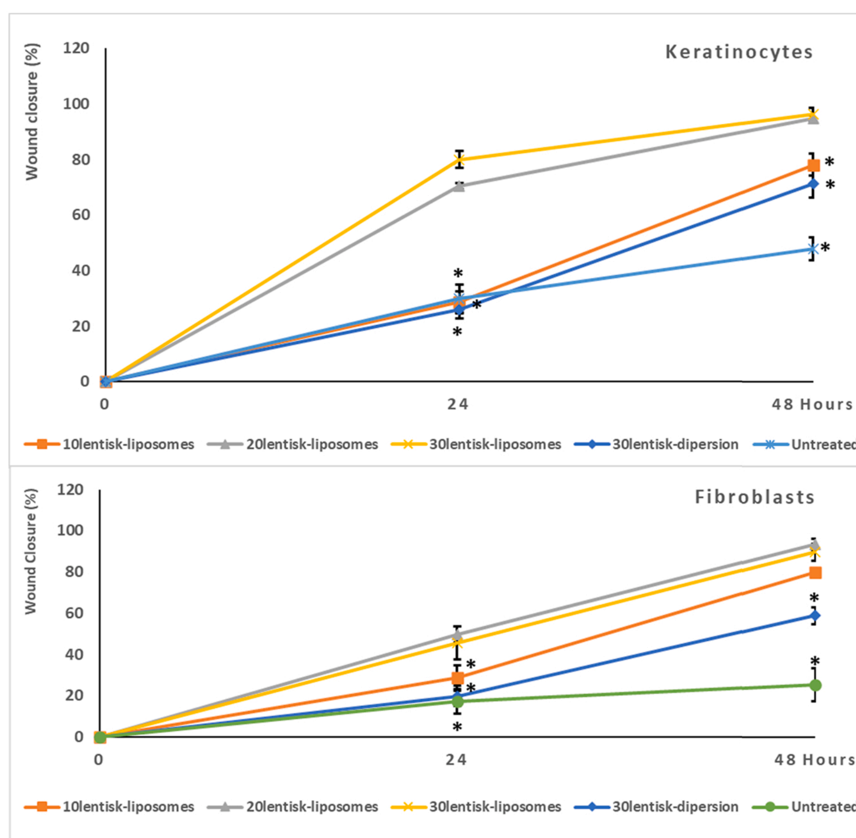

Fig. 6. Percentage of closure of a wound performed in a monolayer of keratinocytes and fibroblasts untreated or treated with lentisk oil in dispersion (3 μg/ml) or loaded in 10lentisk-liposomes (1 μg/ml), 20lentisk-liposomes (2 μg/ml) and 30lentisk-liposomes (3 μg/ml), as a function of the time. Mean values ± standard deviations (error bars) are reported (n = 6). Symbol * indicates values statistically different from 20lentisk and 30lentisk-liposomes.

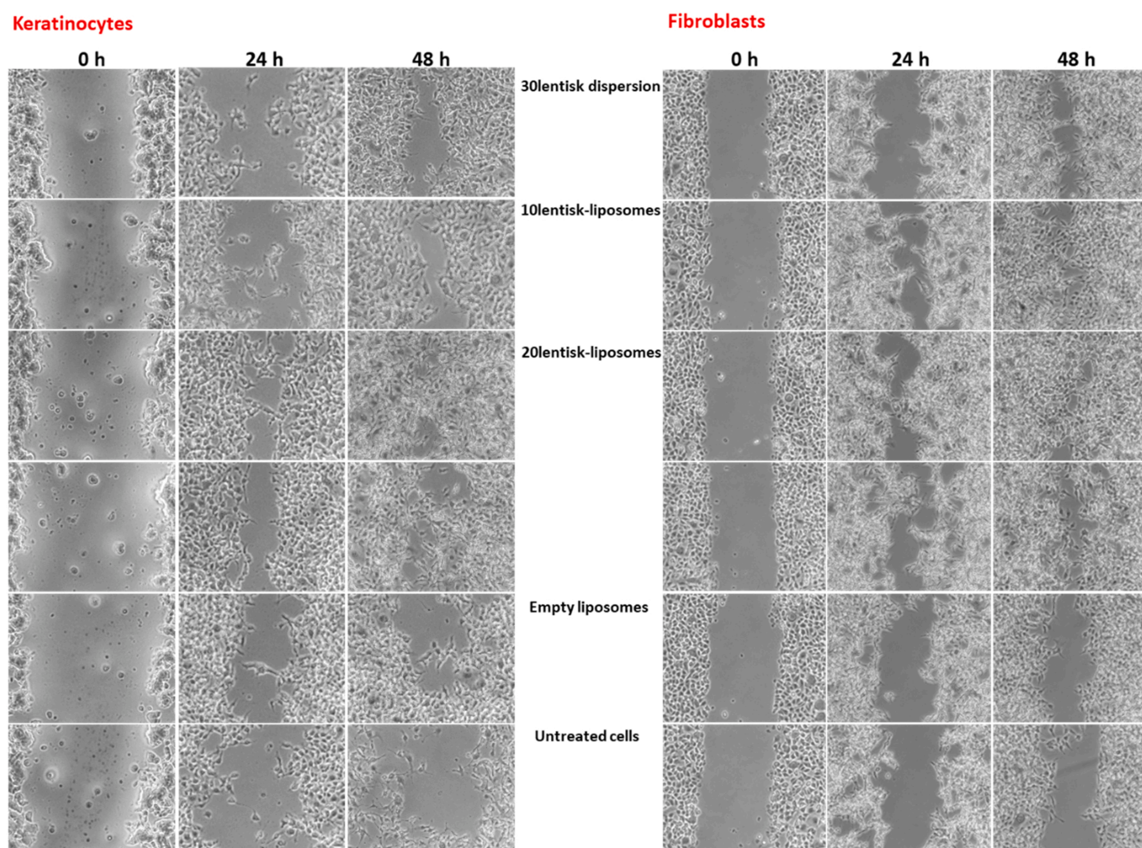

Fig. 7. Representative images of wound closure in a monolayer of keratinocytes and fibroblasts untreated or treated with lentisk oil in dispersion (3 μg/ml) or loaded in 10lentisk-liposomes (1 μg/ml), 20lentisk-liposomes (2 μg/ml) and 30lentisk-liposomes (3 μg/ml) at 0, 24 and 48 h.

($p < 0.05$) of the wound thickness was reached by using liposomes loading 20 and 30 mg/ml of lentisk oil (20- and 30lentisk-liposomes), being the closure ~80% at 24 h and almost complete (~100% closure), at 48 h, disclosing an optimal potential of these formulations in accelerating the healing of a wound performed in a cell monolayer.

4. Discussion

Pistacia lentiscus is a resilient plant capable of growing under hard climatic conditions characterized by nutrient and water scarcity and long-term exposure to extensive solar radiation and high temperatures [20]. To resist to these conditions, it produces several secondary metabolites, which can positively affect their curative and beneficial effects [21]. Its fixed oil obtained from the berries is rich in unsaturated fatty acids (~70%), especially oleic acid, which is dominant, linoleic and palmitic acids, while only ~25% of saturated fatty acids are present [22]. Moreover, several phenolic compounds are contained in the oil and they are responsible for oil stability and antioxidant properties [23]. Thanks to this content, lentisk oil can protect the skin cells from oxidative injury inhibiting the lipid oxidation and subsequent malondialdehyde formation, inducted by treatment with hydrogen peroxide [12]. The lipid peroxidation product (malondialdehyde) if accumulated in tissue, readily interacts with functional groups of proteins, lipoproteins, DNA, and RNA involving different pathological states [24]. It was disclosed that lentisk oil has a positive impact on skin reparation owing to its antioxidant properties and ability to promote cell proliferation, collagen synthesis and dermal reconstruction [25]. Additionally, it can repair the lipid barrier function of skin, similarly to other natural oils, since its moisturizing properties [25]. It was also proven that the fixed oil of lentisk promotes proliferation of fibroblasts and new synthesis of collagen [26]. Indeed, the collagen density in tissue of rats treated with this oil was significantly higher than that found with other treatments. The in vivo anti-inflammatory activity of oil was confirmed and related with its ability to inhibit or reduce the production of inflammatory mediators involved in the acute inflammatory response [27]. Lentisk oil seems to act as wound healing agent on one hand by the reduced production of the inflammatory mediators, on the other hand thank to the antioxidant activity and stimulation of the production of antioxidant enzymes [27,28]. These previous studies confirmed the wound healing properties of lentisk oil, but its suitable formulation in highly-effective carries tailored for the skin delivery was not previously developed. In the light of this, this study was mainly aimed at loading the oil in stable and effective liposomes and evaluating the main benefits of its incorporation into them. Liposomes were prepared by using lecithin from soy (60 mg/ml), which is a natural occurring mixture of phospholipids, being phosphatidylcholine the main component associated with phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol and small amounts of other triglycerides, fatty acids, and carbohydrates [29]. Soy lecithin is usually separated from the crude vegetable oil source using non-toxic solvent and chromatographic procedures with low consumption of equipment, materials and energy, these positive factors are reflected in its low commercial price [22]. The use of lecithin to incorporate lentisk oil permitted to manufacture natural nanotechnological liposomes with a simple and easy scalable preparation method and only using natural plant-derived components [31]. The resulting vesicles were stable for 3 months of storage at room temperature, especially when the highest amount of oil (30lentisk-liposomes) was used, probably because the oil intercalated in the bilayer giving a high fluidity and deformability of it. The amount of oil incorporated was also capable of positively affecting the penetration of vesicles in the deeper skin strata, indeed liposomes loading the higher amount of lentisk oil (30 mg/ml) accumulated especially in the dermis, which is the

stratum mainly involved in the regeneration of the skin during the wound healing process. In the present study, in addition to the formulation suitability for skin application and its natural and scalable production, the improvement of biological properties provided by the loading was also dilucidated. Actually, the lentisk oil in dispersion or loaded in liposomes was not toxic also if used at high concentrations. The oil dispersion slightly stimulated the proliferation of fibroblasts and not at all that of keratinocytes while its loading in liposomes at selected dilution (1/10,000) improved the proliferation of both keratinocytes and fibroblasts. Cell proliferation is not always associated to cell migration, being the latter a key parameter in the promotion of lesion closure. Given that, the ability of formulations to affect lesion closure was evaluated by the scratch assay. Results underlined that lentisk oil in dispersion provided only a low migration of keratinocytes and fibroblasts in the lesion zone, even if, in biocompatibility test, it favored proliferation of fibroblasts and not that of keratinocytes probably because the two effects are mediated by different biological mechanisms [32]. According to biocompatibility test, at the used dilution, the 20lentisk and 30lentisk-liposomes improved the proliferation of keratinocytes in a better extent than the oil dispersion and this positive effect was associated to a better cell migration in scratch assay. The improved rate of closure provided by lentisk-liposomes in fibroblast lesions can be also related to a collective migration driven by improved collagen production [33]. Indeed, 3T3 fibroblasts can synthetize collagen [34]. Finally, lentisk oil in dispersion partially counteracted the toxic effect of hydrogen peroxide in cells while its loading in vesicles ensured a better protection guarantying the restoring of the healthy condition probably because the vesicles facilitate the internalization of the polyphenols contained in the oil, which, inside the cells are capable of scavenging the free radicals in a better extent than the dispersion [25]. Then, lentisk-liposomes can positively affect the local treatment of skin lesions, due to a synergic effect on reduction of oxidative stress, promotion of proliferation and migration of keratinocytes and fibroblasts and restoring effect of skin lipid barrier functions mostly expressed as emollient action [25,35]. This effect is improved by the incorporation of oil in liposomes, because the phospholipids, as the main components of cell membranes, are potent regulators of epithelial barrier function [36]. Overall findings suggested the high potential of lentisk oil loaded liposomes in the treatment of skin lesions mainly associated to oxidative stress.

5. Conclusions

The fixed oil obtained from the berries of *P. lentiscus* has been incorporated into totally green and natural liposomes only composed of lecithin from soy. The combination of these two plant-derived products led the formation of a natural nanotechnological formulation, ideal for the treatment of skin wounds. Overall results suggested that the loading of lentisk oil into liposomes (especially 30 mg/ml) is a promising strategy to prepare natural and biocompatible nanoformulations, being both lentisk oil and soy phosphatidylcholine obtained from plants, which naturally assemble to form nanovesicles. In addition, this combination of natural occurring components guarantees the stability of the system in dispersion and promote the accumulation of the bioactives in the skin especially in the dermis, and their ability to counteract damages induces by oxidative processes, thus promoting their beneficial effect on lesion regeneration and healing.

CRediT authorship contribution statement

Mohamad Allaw: Investigation, Formal analysis, Data curation, Writing – original draft preparation. **Maria Manconi**: Supervision, Data curation, Writing – original draft preparation. **Pierluigi Caboni**: Investigation, Writing – review & editing. **Gianluigi Bacchetta**: Supervision, Methodology, Validation, Writing – review & editing. **Elvira Escribano-Ferrer**: Methodology, Investigation. **Josè Esteban Peris**: Methodology, Investigation, Data curation. **Amparo Nacher**: Methodology, Validation, Writing – review & editing. **Octavio Diez-Sales**: Methodology, Validation, Writing – review & editing. **Maria Letizia Manca**: Supervision, Methodology, Validation, Writing – review & editing.

Declaration of Competing Interest

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

Data Availability

The authors declare that this manuscript do not includes a statement on how/if data supporting the research is available.

Acknowledgements

The authors thank MEDLENTISK project “Partnership for the exchange of best practices on lentisk tree fixed oil, an emblematic non-timber forest product in the Mediterranean” co-funded by the Erasmus+Programme of the European Union for providing analytical equipment and materials.

References

[1] V.K. Pachi, E.V. Mikropoulou, P. Gkiouvetidis, K. Siafakas, A. Argyropoulou, A. Angelis, S. Mitakou, M. Halabalaki, Traditional uses, phytochemistry and pharmacology of Chios mastic gum (Pistacia lentiscus var. Chia, Anacardiaceae): a review, J. Ethnopharmacol. 254 (2020), 112485.

[2] T. Yi, J. Wen, A. Golan-Goldhirsh, D.E. Parfitt, Phylogenetics and reticulate evolution in Pistacia (Anacardiaceae), Am. J. Bot. 95 (2008) 241–251.

[3] H. Trabelsi, O.A. Cherif, F. Sakouhi, P. Villeneuve, J. Renaud, N. Barouh, S. Boukhchina, P. Mayer, Total lipid content, fatty acids and 4-desmethylsterols accumulation in developing fruit of Pistacia lentiscus L. growing wild in Tunisia, Food Chem. 131 (2012) 434–440.

[4] E. Milia, S.M. Bullitta, G. Mastandrea, B. Szotáková, A. Schoubben, L. Langhansová, M. Quartu, A. Bortone, S. Eick, Leaves and fruits preparations of Pistacia lentiscus L.: a review on the ethnopharmacological uses and implications in inflammation and infection, Antibiotics 10 (2021) 425. **10** 425.

[5] F. Siano, A. Cutignano, S. Moccia, G.L. Russo, M.G. Volpe, G. Picariello, Phytochemical characterization and effects on cell proliferation of lentisk (Pistacia lentiscus) berry oil: a revalued source of phenolics, Plant Foods Hum. Nutr. 4 (2020) 487–494. *2020 75*.

[6] Anon EBSCOhost | 143625046 | Wound healing effect of Pistacia lentiscus L. seed oil: confirmation of its uses in Mediterranean traditional medicine.

[7] G. Orrù, C. Demontis, A. Mameli, E. Tuveri, P. Coni, G. Pichiri, F. Coghe, A. Rosa, P. Rossi, G. D'hallewin, The selective interaction of Pistacia lentiscus oil vs. human streptococci, an old functional food revisited with new tools, Front. Microbiol. 8 (2017) 2067.

[8] I.M. Boutemine, M. Amri, Z.C. Amir, C. Fitting, S. Mecherara-Idjeri, K. Layaida, N. Sennoun, S. Berkane, J.M. Cavaillon, C. Touil-Boukoffa, Gastro-protective, therapeutic and anti-inflammatory activities of Pistacia lentiscus L. fatty oil against ethanol-induced gastric ulcers in rats, J. Ethnopharmacol. 224 (2018) 273–282.

[9] S.Ben Khedir, S. Bardaa, N. Chabchoub, D. Moalla, Z. Sahnoun, T. Rebai, The healing effect of Pistacia lentiscus fruit oil on laser burn, Pharm. Biol. 55 (2017) 1407–1414.

[10] F. Mezni, M.L. Khouja, S. Gregoire, L. Martine, A. Khaldi, O. Berdeaux, Effect of growing area on tocopherols, carotenoids and fatty acid composition of Pistacia lentiscus edible oil, Nat. Prod. Res. 28 (2014) 1225–1230.

[11] L. Longo, A. Scardino, G. Vasapollo, Identification and quantification of anthocyanins in the berries of Pistacia lentiscus L., Phillyrea latifolia L. and Rubia peregrina L, Innov. Food Sci. Emerg. Technol. 8 (2007) 360–364.

[12] S. ben Khedir, D. Moalla, N. Jardak, M. Mzid, Z. Sahnoun, T. Rebai, Pistacia lentiscus fruit oil reduces oxidative stress in human skin explants caused by hydrogen peroxide, Biotech. Histochem. 91 (2016) 480–491.

[13] I. Vrouvaki, E. Koutra, M. Kornaros, K. Avgoustakis, F.N. Lamari, S. Hatziantoniou, Polymeric nanoparticles of Pistacia lentiscus var. Chia essential oil for cutaneous applications, Pharmaceutics 12 (2020).

[14] Z.T. Rushmi, N. Akter, R.J. Mow, M. Afroz, M. Kazi, M. de Matas, M. Rahman, M. H. Shariare, The impact of formulation attributes and process parameters on black seed oil loaded liposomes and their performance in animal models of analgesia, Saudi Pharm. J. 25 (2017) 404–412.

[15] M.L. Manca, M. Manconi, M.C. Meloni, F. Marongiu, M. Allaw, I. Usach, J.E. Peris, E. Escribano-Ferrer, C.I.G. Tuberoso, G. Gutierrez, M. Matos, M. Ghavam, Nanotechnology for natural medicine: formulation of neem oil loaded

phospholipid vesicles modified with argan oil as a strategy to protect the skin from oxidative stress and promote wound healing, Antioxidants 10 (2021) 670.
[16] J. Vitonyte, M.L. Manca, C. Caddeo, D. Valenti, J.E. Peris, I. Usach, A. Nacher, M. Matos, G. Gutiérrez, G. Orrù, X. Fernàndez-Busquets, A.M. Fadda, M. Manconi, Bifunctional viscous nanovesicles co-loaded with resveratrol and gallic acid for skin protection against microbial and oxidative injuries, Eur. J. Pharm. Biopharm. 114 (2017) 278–287.
[18] M. Allaw, M. Manconi, M. Aroffu, F. Marongiu, M. Porceddu, G. Bacchetta, I. Usach, R.A. Rached, H.N. Rajha, R.G. Maroun, J.L. Pedraz, T.B. Lopez-Mendez, A. M. Fadda, M.L. Manca, Extraction, characterization and incorporation of hypericum scruglii extract in ad hoc formulated phospholipid vesicles designed for the treatment of skin diseases connected with oxidative stress, Pharmaceutics 12 (2020) 1–20.
[19] M.Z. Fiume, Final report on the safety assessment of Lecithin and Hydrogenated Lecithin, Int. J. Toxicol. 20 (2001) 21–45.
[20] N.S. Margaris, Adaptive strategies in plants dominating mediterranean-type ecosystems. *Ecosystems of the world ll* Mediterr. -Type shrublands, 1981, pp. 309–315.
[21] Y. Li, D. Kong, Y. Fu, M.R. Sussman, H. Wu, The effect of developmental and environmental factors on secondary metabolites in medicinal plants, Plant Physiol. Biochem. 148 (2020) 80–89.
[22] D. Wissal, J. Nahida, C. Emna, B. Maroua, F. Saloua, O. Semia, M. Wissem, Chemical composition of Lentisk (Pistacia lentiscus L.) seed oil, Afr. J. Agric. Res. 8 (2013) 1395–1400.
[23] F. Siano, A. Cutignano, S. Moccia, G.L. Russo, M.G. Volpe, G. Picariello, Phytochemical characterization and effects on cell proliferation of Lentisk (Pistacia lentiscus) berry oil: a revalued source of phenolics, Plant Foods Hum. Nutr. 75 (2020) 1–8.
[24] A. Ayala, M.F. Muñoz, S. Argüelles, Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal, Oxid. Med. Cell. Longev. 2014 (2014), 360438.
[25] N. Poljšak, S. Kreft, N. Kočevar Glavač, Vegetable butters and oils in skin wound healing: Scientific evidence for new opportunities in dermatology, Phytother. Res. 34 (2020) 254–269.
[26] S. ben Khedir, S. Bardaa, N. Chabchoub, D. Moalla, Z. Sahnoun, T. Rebai, The healing effect of Pistacia lentiscus fruit oil on laser burn, Pharm. Biol. 55 (2017) 1407–1414.
[27] S. ben Khedir, M. Mzid, S. Bardaa, D. Moalla, Z. Sahnoun, T. Rebai, In vivo evaluation of the anti-inflammatory effect of Pistacia lentiscus fruit oil and its effects on oxidative stress, Evid. -Based Complement. Altern. Med. 2016 (2016), 6108203.
[28] H. Jemai, M. Bouaziz, I. Fki, A. el Feki, S.S.- Chemico-biological, Hypolipidimic and antioxidant activities of oleuropein and its hydrolysis derivative-rich extracts from chemlali olive leaves, Chem. Biol. Interact. 176 (2008).
[29] A. Joshi, S.G. Paratkar, B.N. Thorat, Modification of lecithin by physical, chemical and enzymatic methods, Eur. J. Lipid Sci. Technol. 108 (2006) 363–373.
[31] J.S. Rhee, M.G. Shin, Analysis of phosphatidylcholine in soy lecithins by HPLC, J. Am. Oil Chemists' Soc. 59 (1982) 98–99.
[32] H Z, D. HF, D M, Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) peceptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways, J. Biol. Chem. 276 (2001) 26969–26979.
[33] S.Y. Wang, H. Kim, G. Kwak, H.Y. Yoon, S.D. Jo, J.E. Lee, D. Cho, I.C. Kwon, S. H. Kim, Development of biocompatible HA hydrogels embedded with a new synthetic peptide promoting cellular migration for advanced wound care management, Adv. Sci. 5 (2018), 1800852.
[34] B. Goldberg, Collagen synthesis as a marker for cell type in mouse 3T3 lines, Cell 11 (1977) 169–172.
[35] M.C. Abdeldjelil, A. Bensegueni, A. Messaï, A. Agabou, H. Benazzouz, Med. Use Pist. lentiscus fixed oil Constant Prov., North-East Algeria vol 4 2014.
[36] W. Gehring, J. Wenz, M. Gloor, Influence of topically applied ceramide/phospholipid mixture on the barrier function of intact skin, atopic skin and experimentally induced barrier damage, Int. J. Cosmet. Sci. 19 (1997) 143–156.