Università degli Studi di Cagliari

PHD DEGREE IN
Life, Environmental and Drug Sciences

Cycle XXXIII

Protection of skin and treatment of lesions using phospholipid vesicles tailored for the topical delivery of phytochemicals from Sardinian flora

CHIM/09

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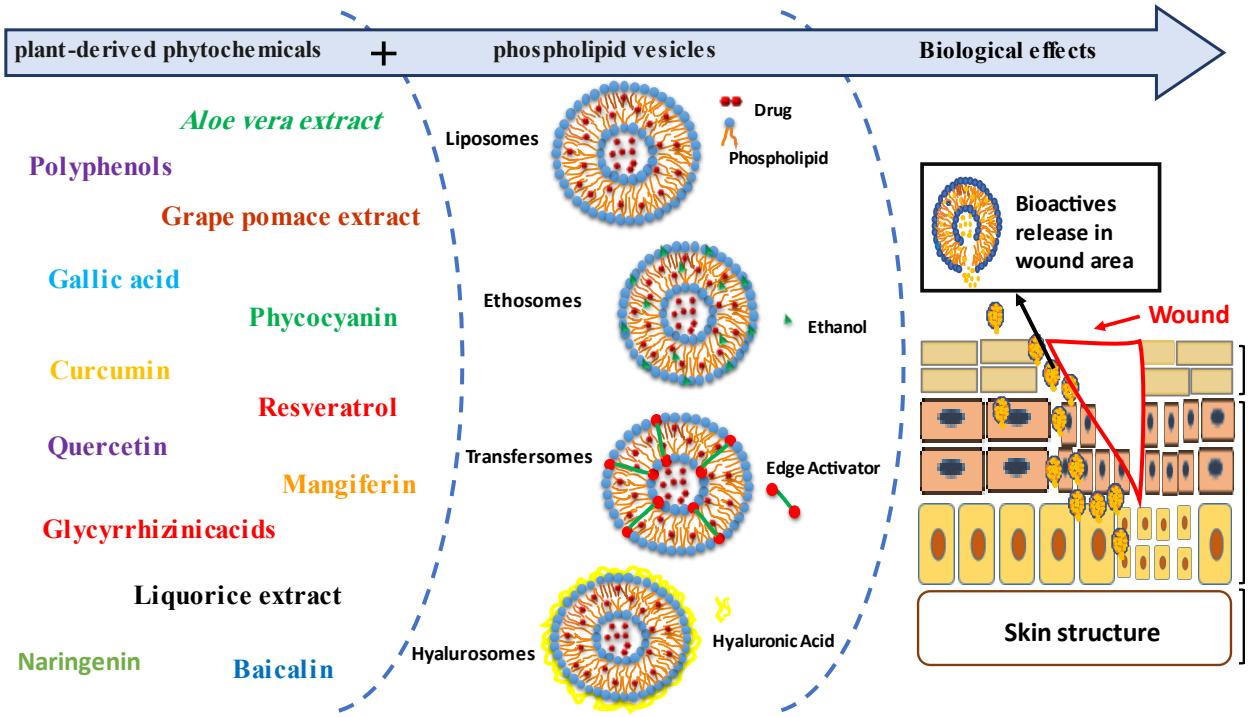
Final exam. Academic Year 2019 – 2020
Thesis defence: January 2022 Session

Introduction



From plants to phospholipid vesicle products: a comprehensive review on the incorporation of plant-derived phytochemicals into phospholipid vesicles designed for skin applications with special focus on scalability and in vitro and in vivo efficacy

Abstract

Natural bioactives, especially those derived from plants, have gained a great popularity throughout the scientific community and among the final users, as they are considered an ideal alternative to synthetic drugs to prevent chronic diseases and treat minor affections especially at skin level. To substantiate their activities, different plant-derived molecules and extracts have been tested, mainly in *in vitro* studies. In addition, in the last decade, several studies were centred on their incorporation in innovative and effective nanocarriers aiming at improving their beneficial properties upon application on the skin. Among the proposed nanocarriers, phospholipid vesicles appeared to be the most effective thanks to their ability to interact with the skin components and reach the deeper strata, their high biocompatibility and feasibility.

This review aims at providing a comprehensive analysis of the studies performed on phospholipid vesicles as topical carriers for plant-derived bioactive molecules. The different formulations used have been reported and discussed along with the different techniques applied for their preparation, with special attention to low dissipative, environmentally friendly and scalable methods. Moreover, the *in vitro* and *in vivo* performances of obtained formulations have been analysed. Interesting opportunities and challenges for the integrated utilisation of these natural formulations are proposed as well.

From plants to phospholipid vesicles: a comprehensive review on the incorporation of phytochemicals into phospholipid vesicles designed for skin applications with special focus on scalability and *in vitro* and *in vivo* efficacy

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Abstract

Natural bioactives, especially those derived from plants, have gained a great popularity throughout the scientific community and among the final users, as they are considered an ideal alternative to synthetic drugs to prevent chronic diseases and treat minor affections, especially at skin level. To substantiate their activities, different plant-derived molecules and extracts have been tested, mainly *in vitro*. In addition, in the last decades, several studies have been centred on their incorporation into innovative and effective nanocarriers aiming at improving their beneficial properties upon application on the skin. Among the proposed nanocarriers, phospholipid vesicles appeared to be the most effective thanks to their ability to interact with the skin components and reach the deeper strata, along with their high biocompatibility and feasibility.

This review aims at providing a comprehensive analysis of the studies performed on phospholipid vesicles as topical carriers for plant-derived bioactive molecules. The different formulations tested have been reported and discussed along with the different techniques used for their preparation, with special attention to low dissipative, environmentally friendly and scalable methods. Moreover, the *in vitro* and *in vivo* performances of obtained formulations have been analysed. Interesting opportunities and challenges for the integrated utilisation of these natural formulations are proposed as well.

Key Words: Phospholipid vesicles; Skin structure; Preparation techniques; Liposome-like vesicles; *In vitro* studies; *In vivo* studies.

Introduction

Plants have been, for years, the basis of traditional medicine in several countries and continue to be considered an important source of bioactive molecules, which are widely used to prepare home-made remedies based on their local availability [1]. Indeed, plants are composed by several active ingredients, having a unique chemical diversity, biological activities and drug-like properties [2]. Their beneficial effects are often similar to those obtained by using synthetic drugs, but with reduced side effects, that significantly improved the compliance and satisfactoriness of the patients.

At the beginning of 1930s, the use of natural remedies has been partially abandoned because of the increased production of synthetic drugs, which for a while have replaced the natural molecules, and even now are considered the first choice for a wide variety of diseases [3]. A different trend on natural therapies and treatments of some diseases has been detected in the past few decades, as a consequence of the globalization and the increased interest in knowing how to have healthy lifestyles and avoid concerns, especially those linked to environmental pollution and excessive intake of junk food, drugs, alcohol and smoke. In the light of this, the scientific community has started new studies on the effects of herbal products all over the world, to substantiate their uses in commercial formulations. A large variety of new products, as pharmaceuticals, dietary supplements, functional foods and skin ointments devoted to enhance beauty, avoid aging, treat major or minor illnesses, based on natural plant-derived ingredients, have been developed to specifically meet the demand of modern society [4,5]. Recent results provided evidence that the best approach to effectively potentiate the beneficial properties of plant-derived bioactive molecules, is based on their combination with nanocarriers usually used in pharmaceutical nanotechnologies to improve the efficacy of a wide range of synthetic drugs [6,7]. These advanced systems can deliver both chemicals and phytochemicals, protect them from physical and chemical degradation and enhance their solubility, bioavailability and pharmacological activity [7]. Among nanotechnological carriers, those used the most for the delivery of natural compounds are phospholipid vesicles, as they are composed of safe and biocompatible ingredients, such as phospholipids, capable of self-assembling to form closed vesicles. These vesicles can encapsulate hydrophilic molecules in the water core, incorporate lipophilic molecules within the bilayer and interact with biological membrane, cells and tissues, thus improving the effectiveness of chemically-different payloads. The most common phospholipid vesicles are called liposomes and are only composed of water and phospholipids, which make them highly similar to biological membranes, thus fully meeting the demand of the modern final users [3]. In the last decades, basic liposomes have been modified by the addition of specific additives, which permit to design vesicles specifically tailored for topical application, with improved affinity to the inter-corneocyte matrix, and capable of promoting the deposition of payloads in the deeper strata [8] [9,10]. These advanced vesicles facilitate the topical administration not only of synthetic drugs but also of phytochemicals and phytocomplexes. Topical dosage forms address important advantages such as compliance of patients as it is non-invasive, can be self-administered without the need of specialized personnel, and usually side effects are effectively reduced [11]. Moreover, their usage is a key strategy to protect and care the skin and its functions, by means of defence from the external insults, thus ensuring the maintenance of healthy body. Indeed, the skin is the organ subjected the most to daily aggressions by chemical products, pollution, stress, irradiation from infrared and ultraviolet sources, mechanical insults and abrasions,

which in turn threatens its equilibrium, functions and beauty [12]. All these external factors may cause important modifications on this tissue, often recognized as premature aging, actinic damage, inflammation, burns, oedema, and long-term illness. Moreover, the variation of skin homeostasis may be reflected also in the entire human body causing serious and, uneasy to treat, diseases.

Taking into account the essential importance of skin in healthy body and the promising effectiveness of phospholipid vesicles as carriers of natural molecules, the aim of this review is to critically examine the most significative studies based on the use of phospholipid vesicles specifically tailored for the cutaneous delivery of plant-derived bioactive molecules. The first part of the review provides a brief description of the skin structure and the most used vesicles along with a critical analysis of their composition and the preparation methods. Particular attention has been devoted to the scalability and economical suitability of the formulated and prepared products. The second part of the review is mainly focused on the effectiveness of phospholipid vesicles in promoting the cutaneous delivery of plant-derived bioactive molecules and to potentiate their biological activity, evaluating the studies performed *in vitro*, by using mainly cells, and *in vivo*, by using specific animal models. Finally, challenges and future perspectives are presented for the actual utilisation of some phyto-phospholipid vesicles to manufacture environmentally friendly with high added value commercial products.

1. Skin structure and functions

Human skin is organized as a complex layered structure consisting of the outermost epidermis, the underlying dermis and the subcutaneous hypodermis. Hair follicles, sweat glands, sebaceous glands, nerves, lymphatics, and blood vessels are located within the skin, especially in the dermis [13,14]. The outermost layer of the epidermis is called stratum corneum and is the main barrier of our body capable of preventing unwanted materials from entering, and control the loss of water by the sweating, which in turn regulate the body temperature and remove undesired compounds from the circulation [15]. The stratum corneum contained a low amount of water (~25%) and its surface is completely coated by a blend of hygroscopic molecules (the natural moisturizing factor, NMF) and lipids. It has a bricks and mortar structure, where the cells are the bricks embedded in a lipid matrix (mortar). Corneocytes, the main cells of stratum corneum, are unviable keratinocytes, which during the upward differentiation process lose both nucleus and water and form a cornified envelop. These structural transformations are responsible for the high mechanical resistance and the effective barrier function of this stratum [16,17]. Corneocytes are organized in different layers (18–20) and are cohesively held together by cell-junctions so called corneo-desmosomes. The extracellular matrix, which embedded corneocytes is mainly composes of ceramides (40–50%), cholesterol (25%), and non-essential fatty acids (10-15%) and is organized into a peculiar multilamellar structure, which is the main responsible for the regulation of the permeability of the cutaneous barrier [18]. Cholesterol contained in the

extracellular matrix modulates the molecular organization within the lamellar bilayers, while enzymes and antimicrobial peptides improve the barrier function [19,20]. Behind the stratum corneum, there is the viable epidermis, a stratified epithelium consisting of keratinocytes, which are constantly renewed thanks to the proliferation of stem cells at the stratum basal [21,22]. During their maturation, keratinocytes synthesize and express various structural proteins and lipids. Their basal proliferation, metabolic differentiation and final desquamation in the outermost layer is a balanced process of the skin in healthy conditions, and a complete renewal approximately takes place every 28 days [23]. On the contrary, when an imbalance between proliferation and desquamation occurs, some skin disorders can be detected. In particular, when the rate of the desquamation process is decreased, epidermal cell retention (ichthyoses) can be observed, while when the proliferation is increased the formation of parakeratotic scales (psoriasis) can be evidenced [24]. In addition, the epidermis provides epithelial defence thanks to its composition: antimicrobial peptides; pro-inflammatory cytokines and interferons; and adaptive immunity by antigen presenting cells, such as epidermal Langerhans and dendritic cells, mediating a T cell response.

The underlying layer of the human skin is the dermis, which consists of connective tissue formed by intertwined bundles of collagen and elastin immersed in a fundamental substance formed by hyaluronic acid and proteoglycans. The presence of elastin and collagen ensures flexibility, strength and protection, of the deeper anatomical structures and their network support the cells, among which the most representative are fibroblasts [25]. This layer also contains blood and lymph vessels and several appendages, hair follicles, sebaceous glands and sweat glands, which are important target sites for drug and bioactive's delivery.

The deepest layer of the skin is the hypodermis, formed by fat cells entrapped into the fibre network. It provides the main mechanical support and thermal isolation of our body, protecting it from physical shock and cold [26].

The entire skin surface is coated with a hydrolipidic film consisting of a water-soluble part from sweat and environment (lactates, sodium chloride, citrates, amino acids) and a fat-soluble part from sebaceous secretion and cell catabolism (glycerides, fatty acids, esterified waxes) [27]. The skin also possesses many enzymes capable of metabolizing topically applied compounds and involved in the keratinocyte maturation and desquamation process, formation of natural moisturizing factor (NMF) and general homeostasis.

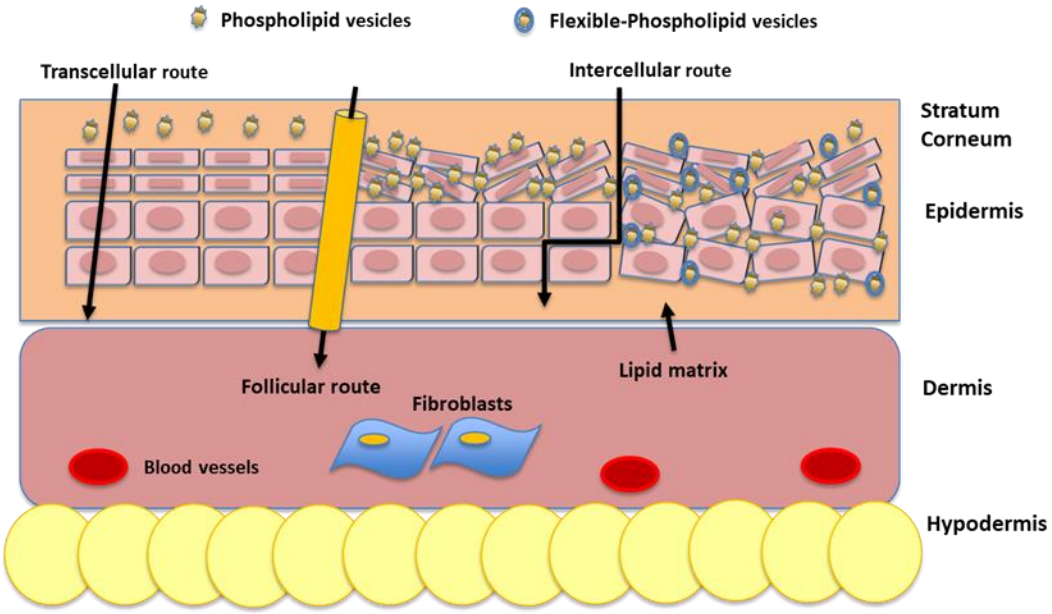

Figure 1. Representative cartoon of skin structure and the possible interaction between vesicles and skin.

Overlay, the skin represents an ideal site for the application of external molecules, with important implications and clinical challenges for their local or systemic delivery due to its easy accessibility and large surface area [28]. In particular, herbal products are usually applied on the skin to protect it from chemical and physical external insults or to promote the healing of local pathologies or lesions.

2. From liposomes to phospholipid vesicles loaded phytochemicals, specifically tailored for skin application

Phospholipid vesicles are promising carriers for the delivery of phytochemicals into and through the skin, due to their high biocompatibility and similarity with biological membranes, affinity with skin components and versatility, as they are capable of simultaneously loading hydrophilic and lipophilic molecules [29]. Phospholipids are safe components, which have been approved for pharmaceutical and cosmetic uses, being the main components of the membrane of cells and capable of providing a nourish effect on the skin [30,31]. They are special surfactants basically composed of two fatty acids chains, a glycerol unit, a phosphate and another polar group, which are organized to form a molecule with a hydrophilic head and two lipophilic tails, responsible for its amphiphilic properties. Differently from other surfactants, phospholipids spontaneously aggregated in water to form closed bilayers (vesicles) composed by one or more lipophilic lamellae, one water core and different concentric hydrophilic water compartments [32]. This organization in which aqueous compartments and hydrophobic moieties are simultaneously present in their internal structure makes them more versatile in comparison with other nanocarriers. This peculiar structure confers special biological properties to the vesicles, especially in terms of interaction with biological membranes, tissues, and cells, thus promoting their carrier ability [33]. Soy lecithin, egg lecithin, marine lecithin and milk phospholipids

are those mainly used for their preparation. Alternatively, phospholipids should be synthetic or semisynthetic, the later are generally synthesized by starting from naturally occurring molecules, among them dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine, or dimyristoylphosphatidylcholine (DMPC) are pure molecules highly used in pharmaceutical applications [34].

Liposomes have been discovered in 1965 by Bangham, but only in 1976 their potential as drug delivery systems has been exploited thanks to the brilliant intuition of the scientist Gregoriadis [35]. Ever since, many studies have been performed to underlying their effectiveness as cutaneous delivery systems, but several results highlighted that basic liposomes were not capable of successfully crossing the main barrier of the skin (stratum corneum) remaining confined at the skin surface [36]. During the time, alternative vesicles containing new additives, specifically chosen to improve their performances at skin level, have been developed. Ethanol (from 10% to 45%) has been one of the first additive used by Touitou and collaborators in 1997 to modify phospholipid vesicles thus obtaining ethosomes [37–40]. They are soft vesicles, which facilitate the delivery of payloads into and through the skin [41,42]. Subsequently, ultra-flexible liposomes so called Transfersomes® have been formulated by Cevc and co-workers [43] by adding an edge activator, which is generally a single chain surfactant (sodium cholate, sodium deoxycholate, Span 60, Span 65, Span 80, Tween 20, Tween 60, Tween 80 and dipotassium glycyrrhizinate). Transfersomes promote the passage of the payloads into and through the skin thanks to their high deformability. They can penetrate intact up to the viable epidermis, by carrying high amounts of payload with an effectiveness comparable to that of a subcutaneous injection [44–47]. Alternatively, liposomes have been modified by the addition of water co-solvents different from ethanol, such as glycerol to prepare glycerosomes [48]. Glycerol is a harmless, non-toxic, and non-irritating additive widely used in pharmaceutical and cosmetic products, which can improve the fluidity of the bilayer along with the delivery performances of vesicles [49,50]. Other vesicles tailored for skin application are the so-called penetration enhancer containing vesicles (PEVs), firstly introduced by Manconi and co-workers in 2009 [51]. These vesicles were prepared by adding a hydrophilic skin penetration enhancer containing a glycol group (propylene glycol, diethylene glycol monoethyl ether (Transcutol®), labrasol) or a lipophilic one (cineol) [42,52,53]. Propylene glycol has been widely used, as it improves the entrapment efficiency of loaded molecules and their deposition in the deeper strata of the skin [54–56]. More recently, phospholipids have been combined with sodium hyaluronate to prepare hyalurosomes, that are defined immobilized vesicles especially effective for the treatment of skin lesions [57,58]. Hyaluronan, as a main component of the connective tissue, acts as stimulator of the proliferation of cells, especially keratinocytes and fibroblasts improving the efficacy of formulations in the treatment of skin lesions. All these vesicles

have been mainly used for the skin delivery of synthetic drugs but, more recently, this technology has been applied to ameliorate the efficacy and beneficial effect of phytochemicals as well. Phyto-phospholipid vesicles were obtained by the combination of plant-derived bioactives with natural phospholipids, especially soy phosphatidylcholine, which also derived from plants.

3. Phytochemical loaded phospholipid vesicles: preparation methods

The intense development and commercialization of phospholipid vesicles at the beginning of the XXI century, has improved the need of finding new preparation methods simple and easily reproducible at industrial level. According to Bangham, liposomes have been firstly prepared by direct dispersion of phospholipids in water [59]. Alternatively, in 1976 Gregoriadis proposed the thin-film hydration method, which has been the method used the most at laboratory level, even if it involves different steps, the use of organic solvents and special equipment [60]. More recently, alternative preparation methods such as the reverse-phase evaporation, injection, electroformation, microfluidic, freeze drying of double emulsions, membrane extrusion, direct hydration, sonication, detergent depletion and heating, have been proposed and tested [61,62]. Most of these methods remained useful only at laboratory level because are very complex, time consuming and require hard and energetically dissipative conditions and again specific equipment. In addition, most of them involve the use of organic solvents, which must be carefully removed. The development of easy and scalable methods, involving the use of few steps, chemicals and times, should be an important strategy to improve the spreading of phospholipid vesicles at industrial level. Indeed, the high cost of both manufacturing and materials, limit their application in food and cosmetic industries [63]. Additionally, when natural bioactive molecules are used, the green preparation became of great importance for the marketability of the final products. Unfortunately, at the moment, the main research studies reported in literature, concerning the formulation of phospholipid vesicles loading natural chemicals or phytocomplexes, still involved complex and unscalable preparation methods and the use of several chemicals. One of the natural molecules loaded the most in phospholipid vesicles is curcumin, as it has been loaded in different phospholipid vesicles by using several preparation methods. Chen and co-workers prepared curcumin loaded vesicles by using the conventional thin film hydration method, which involves the dissolution of curcumin and phospholipid in chloroform, the drying at 50 °C under vacuum by using a rotary evaporator, the hydration of the obtained film with a phosphate buffer saline solution at pH 6.5, under stirring, for 30 min at 60 °C, and finally, the sonication (3 min, 80 W) of the dispersion by using a probe sonicator to obtain small vesicles and homogeneous dispersions [64]. Jin et al. obtained curcumin loaded liposomes using milk fat globule membrane phospholipids or soybean lecithin and the previously-reported preparation method [65]. As well as, Pathan et al. developed and prepared curcumin loaded ethosomes tailored for transdermal application, by means of the lipid film hydration

method, by using ethanol and water as hydrating medium [66]. Cheng et al loaded curcumin in special liposomes, so called rhamnolipids modified liposomes, which were obtained by means of ethanol-injection method [67]. Phospholipids (10 mg/ml) were mixed with different amounts of rhamnolipids dissolved in absolute ethanol, the resulting solution was then dropped into a phosphate buffer saline solution (pH 7, 5 mM) and stirred for 30 min. The ethanol was eliminated at 45 °C under reduced pressure thus obtaining the vesicle dispersion. Li et al, used a different and more complex preparation method, as curcumin (50 mg/ml) was dissolved in dimethyl sulfoxide and the phospholipid (20 mg/ml) was dissolved in tert-butanol, then the obtained solutions were mixed together and sterilized through a 0.22-μm filter. Aliquots of this solution were frozen in a dry ice acetone bath and lyophilized to remove the solvents and stored at −20 °C until their hydration and use.

As such, the lipid film formation and subsequent hydration is the most common and used method, at laboratory level, to load not only curcumin but all the phytochemicals and phytocomplexes derived from plants, into phospholipid vesicles. It was used by Oliveira de Moraes Nogueira and co-workers to encapsulate phycocyanin, a bili-protein obtained from spirulina, in large multilamellar liposomes [68]; by Aisha et al. to load orthosiphon stamineus ethanolic extract into liposomes [69]; by Pavaloiu et al, to load the polyphenols extracted from leaves of Lycium barbarum into liposomes. Often the thin film hydration method is even combined with sonication and extrusion, thus adding more steps during the preparation [70].

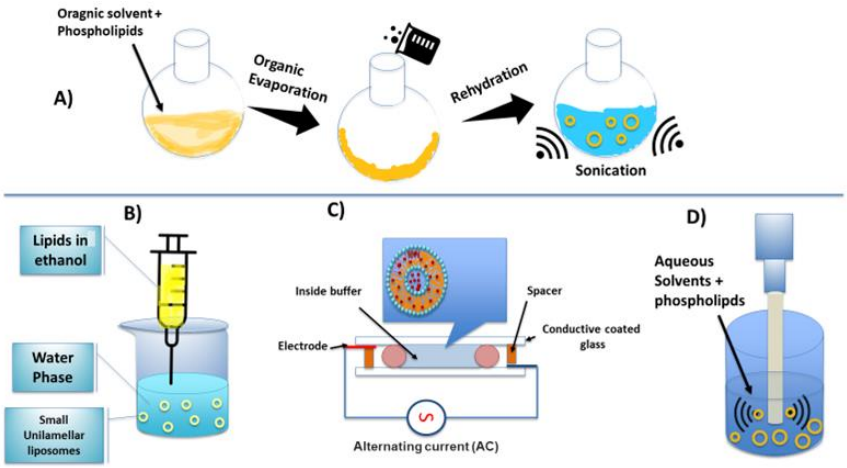

Figure 2. Representative cartoon of the principal methods used for the preparation of phospholipid vesicles, A) Thin-film hydration method, B) Ethanol injection, C) Electroformation, D) direct hydration and sonication.

Recently, Cheng and co-authors proposed an alternative, organic solvent-free encapsulation method to load curcumin into liposomes specifically designed for oral administration [71]. It is a pH-driven method, based on the self-assembling of phospholipids in water and the diffusion of lipophilic phytochemicals (such as curcumin and rutin) across liposomal membranes, driven by hydrophobic force. The method involves the deprotonation and dissolution of hydrophobic molecules under

alkaline conditions followed by neutralization, which led the encapsulation of the precipitated hydrophobic phytochemical. They prepared the same vesicles by also using the film-hydration method and they concluded that the pH-driven method is a promising technique to load lipophilic phytochemicals in liposomes, since it does not require the use of organic solvents and is easily scalable at industrial level. However, the method involves different steps and mostly the use of sodium hydroxide and hydrochloric acid.

Another scalable method, proposed to prepare liposomes without the use of toxic solvents, is the microfluidization technique, which only need a microfluidizer equipment traditionally used in the pharmaceutical industry [72]. Vesicle components are directly suspended in water in a dedicated chamber, where the high fluid pressures generate high shear forces, avoiding thermal process technique and facilitating the self-assembling of phospholipids in bilayered vesicles [62]. Gibis et al. loaded the hibiscus extract in soy lecithin liposomes, by using microfluidization technique, which involves the dissolution of phospholipids in acetate buffer or in the extractive solution [73]. The dispersion was pre-emulsified by using a high-shear disperser and subsequently passed five times through a microfluidizer at a pressure of 22,500 psi. Similarly, liposomes loaded curcumin have been prepared using this technique by Tai et al [74]. In this study, the method was modified in a low sustainable moiety because the vesicles were prepared by formation of thin lipid film and its hydration, after that, a microfluidizer was used not to form the vesicles but only to decrease their size. Dag and Oztop loaded green tea extract in soy lecithin liposomes by high pressure microfluidization or ultrasonication [75]. Vesicles prepared by microfluidization were more stable and more antioxidant then that prepared by ultrasonication. The microfluidization method alone and not combined with other techniques, was not largely used to prepare phospholipid vesicles loading phytochemicals.

Other simple, economic and scalable method is that firstly proposed by Mozafari and co-workers, so-called heating or Mozafari method [76]. It can be performed in one hour and one step, only using a single apparatus avoiding the use of potentially toxic solvents [77]. It involves heating (40–120°C) and stirring of the components, in the presence of a hydrating agent (propylene glycol, glycerol, or sorbitol), followed by their hydration with water. Polyols, especially glycerol, could be safely used as a hydrating agent due to its water solubility and high biocompatibility [78]. Mortazavi et al. reported that phospholipid vesicles prepared by the heating method did not cause toxicity to cultured cells, that appeared to be ideal to deliver natural bioactive molecules [63]. Jahanfar et al. loaded rosemary extract into liposomes by using this method, without the utilization of toxic solvents or detergents [79]. Aiming at finding a scalable preparation method, curcumin was incorporated by Zhao et al into liposomes and ethosomes designed for skin delivery, by using ethanol injection method [80]. Curcumin and phospholipid were dissolved in ethanol (3 ml) and the solution was slowly added into

distilled water (10 ml) maintained under stirring. The removal of ethanol under vacuum by using a rotary evaporator, allowed the formation of liposomes. Curcumin loaded ethosomes were prepared by using the same method but avoiding the evaporation of ethanol. The used method to obtain ethosomes was simple and it did not involve the use of toxic solvents but only ethanol.

Another method widely used to incorporate plant-derived bioactive molecules is the direct sonication or energic agitation of vesicle components dispersed in aqueous phase [81]. Indeed, as well known, phospholipids dispersed in water naturally assemble in lamellar vesicles, the lipophilic molecules, if present in dispersion, spontaneously intercalate inside the lipid bilayer while the hydrophilic ones remain solubilized in the water phase. This natural process can be accelerated providing adequate energy by sonication or strong mechanical stirring [82]. The direct agitation or sonication of vesicle components is also a simple, scalable and economic method, which avoid the use of toxic organic solvents or polyols like the method proposed by Mozafari. In this procedure, the heating and the addition of polyols are possible but not mandatory, then it seems to be more suitable for the preparation of plant-derived bioactive molecules [83]. Phycocyanin vesicles were obtained by dispersing in water all the components (bioactive and phospholipids) and sonicating the dispersion with a high intensity ultrasonic disintegrator [84]. As well, curcumin, quercetin, grape pomace extracts, mangiferin, tocopherol, neem oil have been incorporated in phospholipid vesicles, modified by using polyols (i.e. glycerol or propylene glycol) or several penetration enhancers or surfactants or even polymers [85–90]. Overall results confirmed the suitability of this method and the high biocompatibility of the obtained vesicles, thus it seem to be especially suitable to formulate phyto-nano-phospholipid vesicles [91–93].

4. Biological effects of phytochemicals loaded into phospholipid vesicles on skin

The loading of beneficial molecules into phospholipid vesicles is a modern and valuable approach to maximize the bioavailability of plant-derived bioactive molecules and their phytocomplexes in the skin. Indeed, several *in vitro* and *in vivo* studies have been performed to evaluate their efficacy. It was generally observed that the loading into liposomes or modified phospholipid vesicles improve the phytochemical deposition in the skin and thus their efficacy. The composition of the vesicles is a key parameter, which affects the structure and fluidity of the bilayer, as well as their performance as skin carriers and the final bioactive effectiveness. In Table 1 the most commonly used vesicles, along with their main composition and effectiveness have been reported.

Table 1. phytochemical used, type of phospholipid vesicles obtained, biological effects, main results and references.

Natural compounds	**Type of vesicles used**	**Biological effects**	**Results (*in vitro* and/or *in vivo*)**	**Reference**
Ascorbate	Liposomes	Antioxidant effect	-improved penetration of the payload into and through the skin and prevention of the skin damages induced by UVA and UVB irradiations	[94]
Anthocyanin	Liposomes	Antioxidant effect	-Enhanced loading efficiency, anthocyanin stability, scavenging activity and skin permeability	[95]
Rosmary extract	Ethosomes-Liposomes	Antioxidant effect	-Enhanced penetration of the bioactives contained in the phytocomplexes up to the dermis and promotion of their antioxidant effect.	[96]
Resveratrol	Transfersomes	Antioxidant effect	-Improved stability, solubility, bioavailability, safety, and *in vitro* transdermal delivery	[97]
Curcumin	Transfersomes	Antioxidant effect	-Delivery of curcumin in the deeper strata of the skin in comparison with the dispersion of curcumin used as reference.	[98]
Grape	Hyalu-transfersomes	Antioxidant effect	-Efficient promotion of the beneficial properties of the extract, by means of reduction of death of both fibroblasts and keratinocytes, induced by oxidative stress	[99]
Grape pomace	Liposomes and Glycerosomes	Antioxidant effect	-protection of the skin from the damaging effect induced by using hydrogen peroxide	[100]
Citrus limon (L.) Osbeck var. pompia Camarda	Hyalurosomes andGly-hyalurosomes	Antioxidant effect	-Promotion of the protective effect against oxidative stress induced in skin cells	[101]
Asparagus racemosus root extract	Liposomes	Anti-inflammatory effect	-Amelioration of the anti-inflammatory activity of extract, and effective inhibition of tumour necrosis factor-α	[102]
Mangiferin	Ethosomes and transethosomes	Anti-inflammatory effect	-Improved beneficial effect of mangiferin loaded in nanovesicles	[103]
Glycyrrhizin, glycyrrhizic and glycyrrhizinic acids	Transfersomes	Anti-inflammatory effect	-effective reduction of skin inflammation in human volunteers.	[104]
Baicalin	Transfersomes and gellan-transfersomes	Anti-inflammatory effect	-Effective reduction of damages and of skin inflammation induced by TPA application in an *in vivo* mice model.	[105]
Naringenin	Transfersomes	Anti-inflammatory effect	-Increased deposition of naringenin in the skin and low irritating effect of formulations.	[106]
Lycopene extract	Transfersomesand Ethosomes	Anti-inflammatory effect	-Increased permeation and retention of Lycopene extract both *In vitro* and *in vivo* Reduction of epidermal hyperplasia and inflammatory cell infiltration	[107]
Aloe vera gel	Liposomes	Wound healing activity	-Enhanced bioavailability and skin care properties of *Aloe vela* leaf gel extract	[108,109]
Fraxinus angustifolia leaves Extract	Ethosomes and transcutol ethylene	Wound healing activity	-Highest antioxidant and anti-inflammatory effects and improved *in vivo* healing of the wounded area	[89]

	glycol containing vesicles			
Hypericum scruglii extract	Glycerosomes, Gel-glycerosomes and Hyal-glycerosomes	Wound healing activity	-Promotion of cell proliferation and migration in the scratched performed in a cell monolayer	[110]
Liquorice extract	Liposomes and hyalurosomes	Wound healing activity	-Promotion of cell proliferation and migration in the scratched performed in a cell monolayer effective reduction of damages and of skin inflammation induced by TPA application in an *in vivo* mice model	[111]
Curcumin	Propilen glycol liposomes	Wound healing activity	-Effective promotion of the healing of acute and chronic wounds	[112]
Gallic acid	Liposomes	Wound healing activity	-Promotion/Improvement of bone regeneration in Wistar rats with calvarial defects	[113]
Curcumin	Hyalurosomes	Wound healing activity	-Effective promotion of the healing and closure of skin wounds.	[114]
Mangiferin	Glycethosomes	Wound healing activity	-high biocompatibility and effective reduction of damages induced by TPA application in an *in vivo* mice mode	[115]
Mangiferin	Transfersomes and glycol-transfersomes	Wound healing activity	-Promotion of mangiferin deposition in epidermis and dermis. Effective protection of fibroblastsfrom oxidative stress and stimulation of their proliferation. Effective reduction of damages induced by TPA application in an *in vivo* mice model	[90]
Moringa oleifera leaf extract	Phytosomes	Wound healing activity	-Higher stimulation of the proliferation and migration of the main representative cells of the skin.	[116,117]
Phycocyanin	Hyalurosomes and PG-hyalurosomes	Wound healing activity	-High protection and restoring ability against skin lesions.	[58]
Glycyrrhetinic acid	Hyalurosomes	Wound healing activity	-High biocompatibility and stimulation of fibroblast proliferation.	[118]
Capsaicin	Niosomes and Liposomes	Antipsoriatic applications	-Reduction of sundesired side effects and increase of therapeutic efficacy	[119]
Psoralen	Ethosomes	Antipsoriatic applications	*In vitro* studies demonstrated that the deposition of psoralen was 6.56-fold higher when ethosomes were used respect to the ethanolic solution. Similarly, *in vivo* skin microdialysis showed that the peak concentration and area under the curve of psoralen were approximately 3.37 and 2.34 times higher when it was incorporated into liposomes. Enhanced permeation and skin deposition of psoralen delivered by ethosomes can help to reduce its toxicity and improve the efficacy of long-term psoralen treatment	[120]
Psoralen	Liposomes Ethosomes	Antipsoriatic applications	-*In vitro* skin permeation studies showed that the permeability of psoralen when loaded into ethosomes was higher than that obtained by using liposomes. Indeed, transdermal flux and skin deposition of psoralen delivered by ethosomes, were 3.50 and 2.15 times	[121]

			higher than those achieved by using liposomes	
Thymoquinone	Ethosomes	Antipsoriatic applications	-High anti-psoriatic activity	[122]
Astaxanthin	Cationic liposomes	Cosmeceutical and antiaging applications	-Prevention of UV-induced skin thickening, collagen reduction and inhibition of melanin production.	[123]

4.1. Antioxidant effect

The demand for effective natural antioxidants to protect the skin from external insults, contaminants and free radicals have been increasing in the last few years and it is expected to further increase in the near future. Several creams, gels, serums or lotions are present in the market, but their efficacy is not uniquely demonstrated. To this propose, a large number of studies have been performed by using different phospholipid vesicles to load several phytochemicals and to evaluate their capability of protecting the skin from aging and damages caused by oxidative stress. Serrano et al. prepared ascorbate loaded liposomes, which were capable of improving the penetration of this payload into and through the skin [94]. The application of the vesicle dispersion in the skin effectively prevented the damages induced by UVA and UVB irradiations, thus confirming the antioxidant and anti-inflammatory properties of ascorbate. Anthocyanin, another natural antioxidant, was loaded by Lee et al. in liposomes, which exhibited markedly enhanced loading efficiency, anthocyanin stability, scavenging activity and skin permeability [95]. Alternatively to liposomes, Yucel and co-workers evaluated the antioxidant activity along with the inhibitory effects against collagenase and elastase enzymes of rosemary extract loaded into ethosomes, which were compared with liposomes. When applied on the skin, ethosomes were more effective than liposomes in improving the passage of the components contained in the phytocomplex up to the dermis thus promoting their antiaging effect [96]. Other phospholipid vesicles widely used for the skin delivery of natural molecules are transfersomes or deformable vesicles, which can squeeze through this barrier reaching the deeper skin strata [124]. Wu et al. demonstrated that these ultradeformable vesicles improve the stability, solubility, bioavailability, safety, and *in vitro* transdermal delivery of resveratrol [97]. Ternullo et al. [98] prepared deformable vesicles by using sodium deoxycholate as edge activator to deliver curcumin at skin level. The presence of this edge activator strongly ameliorated the vesicle performances. Indeed, in ex vivo experiments performed by using human skin, these carriers delivered curcumin in the deeper strata of the skin in comparison with the non-liposomal curcumin used as reference. They also improved the ability of the payload to counteract the production of nitric oxide induced in cells and to inhibit microbial activity, avoiding their replication and growth. Transfersomes have also been combined with hyaluronan to obtain hybrid vesicles so called hyalo-transfersomes, which were used to load an extract obtained from grape pomace [99]. In comparison

with the extract in dispersion, these vesicles effectively promoted the beneficial properties of the extract, reducing the death of fibroblasts and keratinocytes, induced by oxidative stress, and improved the healing of a wound performed in a cell monolayer. Another antioxidant extract obtained from grape pomace, as the most abundant by-product produced during the wine-making chain, was also loaded in liposomes and glycerosomes modified with a commercial mixture of surfactants of natural origin (Montanov 68) [100]. Vesicular formulations protected the skin from the damaging effect of hydrogen peroxide restoring the healthy conditions. It is important to note that in the last studies, the phytocomplexes were prepared by using an agri-food by-product, thus obtaining not only formulations by using only natural components but also green and environmentally-friendly systems, based on the transformation of by-product in a resource, according to the circular economy, and thanks to the optimal performances of phospholipid vesicles.

Differently to transferosomes, Manconi et al. used hyalurosomes and hyalurosomes modified with glycerol (gly-hyalurosomes) to improve the skin delivery of an extract obtained from a cultivar of citrus, called *pompia* [101]. Results confirmed that the phytocomplex in dispersion was highly antioxidant but its loading into vesicles promoted the protective effect against oxidative stress induced in skin cells. The combination of the different molecules contained in the phytocomplex with the carrier ability of liposomes, the hydrating properties of glycerol and the mechanical resistance and regenerative activity of sodium hyaluronate, led the formation of attractive and effective natural formulation suitable for skin protection against oxidative injuries.

4.2. Antiinflammatory effect

Several studies evaluated the anti-inflammatory activity of phytochemicals loaded into phospholipid vesicles. Plangsombat et al. prepared liposomes entrapping Asparagus racemosus root extract and evaluated their anti-inflammatory activity in the monocytic leukemia cell line [102]. Liposomes were prepared by using different methods: thin-film hydration, reverse-phase evaporation and polyol dilution methods. The incorporation of the extract into the vesicles ameliorated its anti-inflammatory activity, evaluated as percentage of inhibition of tumour necrosis factor-α (~52% at a concentration of 1μg/ml of extract). Results confirmed the potential of extract loaded liposomes to be used as topical effective formulation to prevent inflammatory responses. In another study, ethosomes and transethosomes were designed by Sguizzato and colleagues, as topical delivery systems for mangiferin, a natural glucosyl xanthone with antioxidant and anti-inflammatory activity [103]. The antioxidant and anti-inflammatory effect were evaluated in human keratinocytes exposed to cigarette smoke as an oxidative and inflammatory challenger, by measuring their ability to induce an antioxidant response and anti-inflammatory status (IL-6 and NF-kB). *In vitro* data demonstrated the effectiveness of mangiferin loaded into nanovesicles in protecting cells from damages. Similarly,

liposomes were used to load hydroxy-citrate, the most abundant organic acid component of the fruit rind of *Garcinia cambogia* [125]. It is a natural citrate insoluble in water and unable to permeate through the skin barrier. Its delivery in liposomes increased by approximately 4 times the intracellular accumulation of hydroxy-citrate in macrophages and reduced by 10 times the amount of hydroxy-citrate required to effectively counteract nitric oxide, reactive oxygen species, and prostaglandin E_2 production as confirmed by *in vitro* studies. Glycyrrhizin, glycyrrhizic and glycyrrhizinic acids were loaded too into transfersomes to be delivered in the skin since their anti-inflammatory and antiallergic effects [104]. The *in vivo* anti-inflammatory study on human healthy volunteers, demonstrated that the incorporation of bioactives in transfersomes significantly reduced the skin inflammation suggesting their possible use as topical anti-inflammatory systems. Transfersomes were also modified by adding some water co-solvents to improve their loading and delivery ability. Manconi et al. combined transfersomes with a gellan nanohydrogel to incorporate baicalin in high amount and obtain the so called self-assembling core-shell gellan-transfersomes [105]. Their optimal performances were confirmed *in vitro* by the high deposition of payload in the skin (~11% in the whole skin) and especially in the deeper tissue (~8% in the dermis) and *in vivo* by the reduced inflammation in mice. The topical application of these formulations on the damaged skin provided its complete restoration mainly by inhibiting all the studied inflammatory markers. Tsai et al. prepared naringenin loaded transfersomes by using Tween 80 as edge activator [106]. The stability of formulations and their skin irritation and deposition were also evaluated for the assessment of the clinical utility. Saturated aqueous solution and Tween 80 solution of naringenin were both used as controls. Transfersomes permitted to increase the deposition of naringenin in the skin about 7.3-11.8-folds respect to the saturated solution and 1.2~1.9-fold respect to the Tween 80 solution. Results of skin irritation test confirmed the low irritating effect of formulations, suggesting its potential therapeutic application. Differently, Ascenso et al. incorporated lycopene extract into transfersomes prepared with phosphatidylcholine and tween 80 and ethosomes prepared with phosphatidylcholine and ethanol [107]. *In vitro* and *in vivo* experiments underlined that the skin permeation and retention was higher when lycopene loaded ethosomes were used. In order to test the penetration behaviour in inflamed skin, the vesicles were applied on an anthralin-induced ear oedema model. The extract loaded in both transferosomes and ethosomes reduced the levels of epidermal hyperplasia and inflammatory cell infiltration in comparison with the plain lycopene extract, confirming the optimal performances of phospholipid vesicles in the delivery of pure phytochemicals and also phytocomplexes. These results are important because the extraction of the total phytocomplex from plants is easier and low dissipative in comparison with the procedures involved in the purification and production of pure

single molecules. In addition, phytocomplexes are usually more effective than the single molecules due to the synergic activity of their different components.

4.3. Wound and burn healing activity

Several extracts or pure phytochemicals have been loaded into phospholipid vesicles and their effect of wound healing has been evaluated by using either *in vitro* or *in vivo* models. *Aloe vera* was traditionally used in the treatment of skin lesions because its gel extract obtained from the leaves exserts a direct effect on the healing process, accelerating the rate of lesion contraction and collagen synthesis, probably due to its high content in mannose-6-phosphate [108]. Indeed, this sugar can increase macrophage activity and therefore stimulate fibroblast metabolism and collagen synthesis. The gel was loaded in high concentration in soy lecithin liposomes, which increased type I collagen synthesis, in normal human neonatal skin fibroblasts, in higher extent than the unloaded extract. Accordingly, the bioavailability and skin care properties of *Aloe vera* leaf gel extract was significantly enhanced by its incorporation in liposomes, suggesting their potential as an effective skin care formulation [109]. Similarly, the polyphenolic phytocomplex extracted from Fraxinus angustifolia leaves was incorporated by Moulaoui et al., into different nanovesicles, namely ethosomes and transcutol or ethylene glycol containing vesicles. They played a key role in improving the efficacy of the extract in wound healing process. Indeed, the highest antioxidant and anti-inflammatory effects were provided by the phytocomplex incorporated in ethylene glycol containing vesicles, which favored *in vivo* the healing of the wounded area in a greater extent than the extract in dispersion [89]. Another extract delivered in phospholipid vesicles was that obtained from *Hypericum scruglii,* an endangered endemic plant of Sardinia (Italy) [110]. It was loaded in glycerosomes or glycerosomes modified with a maltodextrin (glucidex) and a polymer (gelatin or hyaluronan). The basic liposomes were also prepared and used as reference. The formulation efficacy was proved in *in vitro* tests, which underlined the ability of vesicle to promote cell proliferation and migration in the scratched cell monolayer. Liposomes and hyalurosomes were also formulated by Castangia et al. [111] to load the phytocomplex obtained from the liquorice. Extract loaded hyalurosomes addressed *in vitro* an effective cell proliferation and migration, thus speeding-up the healing of a wound performed in a cell monolayer. In addition, the gelling properties of the polymer, led the formation of a viscous system, which can be easily spread in the skin avoiding loss of the formulation and promoting the re-epitelization and the remission of the damages induced by using 12-O-tetradecanoylphorbol-13-acetate (TPA) in mice [126]. Not only polymers but also co-solvents, seem to be good candidate as additives in the formulation of vesicles, capable of promoting the effectiveness of natural substances. General results confirmed the suitability of phospholipid vesicles as skin carriers of phytocomplexes, being the lasts more effective than the single natural molecules since the synergic ability of their

components to promote healing and tissue regeneration through multiple and connected mechanisms [127]. Pure phytochemicals have been delivered in phospholipid vesicles as well, to accelerate the wound healing. Kianvash et al. formulated propylene glycol liposomes to deliver curmumin and the efficacy was evaluated *in vivo,* by using a model of burn/wounds in rats [112]. Curcumin loaded propylene glycol liposomes were small in size, homogeneously dispersed and capable of effectively promoting the healing of acute and chronic wounds. Altan et al. incorporated gallic acid in liposomes and compared its effect with that of free gallic acid, in powder [113]. As expected, the incorporation in liposomes protected gallic acid from degradation thus prolonging its effect. The comparison of incorporated and not incorporated gallic acid confirmed that vesicles significantly improved bone regeneration in Wistar rats with calvarial defects. The healing process was associated with a decreased inflammation and nuclear factor κ-B and an increased osteoprotegerin and bone morphogenetic protein-2 expression. El-refaie et al [114] demonstrated that curcumin loaded hyalurosomes dispersed in a conventional gel was more effective in promoting the healing and closure of wounds than the free curcumin dispersed in the same gel. Indeed, after 11 days of treatment the skin of mice appeared again normal and without scar, as also confirmed by histological evaluation. The combination of gel and hyalurosomes has been considered an innovative approach to improve the penetration of curcumin in the deeper strata of the skin, especially dermis, and protecting it against degradation. Pleguezuelos-Villa et al. [115] developed new vesicles in which a mixture of co-solvents (mainly glycerol and ethanol) was used as hydrating medium. Mangiferin was loaded in high amount and the resulting vesicles were highly biocompatible and promoted the healing of the wound induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in a mouse model, in a better extent than the mangiferin in dispersion. The same mangiferin was incorporated by Allaw-Pleguezuelos et al. in transfersomes and glycol-transfersomes, containing glycerol and/or propylene glycol, and mucin. Formulations were effective against oxidative stress and promoted the healing of skin wounds both *in vitro* and *in vivo* [90]. In particular, glycol-transfersomes promoted the deposition of mangiferin in epidermis and dermis, effectively protected fibroblasts from oxidative stress and stimulated their proliferation, while their wound healing and anti-inflammatory efficacy were confirmed *in vivo*.

Another approach especially used for enabling the natural compounds to cross the skin barrier, is their incorporation into phytosomes, which peculiarity is that bioactive and phospholipid interact with each other to form a stable complex. These systems are capable of stabilizing both bioactive and final formulation thus promoting the interaction with the skin [116]. Given that, Zhou et al. [117] loaded the phytocomplex obtained from Moringa oleifera leaf into phytosomes aiming at promoting the protective effect of the bioactives when applied in the skin. *In vitro* results have confirmed the better performances of phytosomes, which stimulated in a better extent than the dispersion, the proliferation

and migration of the main representative cells of the skin. These systems have been proposed to be formulated into a functional wound dressing. Castangia et al. used different vesicles to incapsulate phycocyanin (a natural plant-derived protein): hyalurosomes, or hyalurosomes modified with polyethylene glycol. Phycocyanin loaded in hyalurosomes and especially that modified with polyethylene glycol, protected and restored the skin lesions better than the phycocyanin solution, and seemed to be ideal for the formulation of advanced cosmetic products. The presence of the polymer confers viscosity to the dispersion favouring its application on the skin and improving their carrier ability [58]. Based on this results, Abruzzo et al. [118] prepared hyalurosomes to load glycyrrhetinic acid, which were stable on storage, highly biocompatible and also capable of stimulating fibroblast proliferation. Furthermore, *in vitro* release studies, performed by using different dressings impregnated with the vesicle dispersions, demonstrated that a high amount of glycyrrhetinic acid reach the wound site, 60 minutes after application.

4.5. Antipsoriatic applications

Psoriasis, an incurable autoimmune skin disease, is one of the most common immune-mediated disorders. Due to its high diffusion and difficulty to be treated, in the last decades, natural products have gained attention because of their high efficiency and low toxicity. Flavonoids, such as luteolin, quercetin, naringin, phenyl-propanoids like psoralene, curcumin, gallic acid and resveratrol, alkaloids such as capsaicin are some of the natural molecules that have been incorporated into phospholipid vesicles and tested for the treatment of psoriasis. Gupta et al delivered capsaicin in liposomes, niosomes and gel-emulsions to improve its therapeutic effect. The loading in these systems significantly reduced its secondary and undesired effects and increased the therapeutic efficacy thanks to a better skin permeation through the hyperproliferative region. Moreover, the enhanced accumulation of capsaicin within the skin might prolong its effect acting as a depot from which the payload is released in a controlled manner [119]. Zhang et al aiming at improving skin deposition and permeation of psoralen isolated from *Fructus psoraleae*, incorporated this molecule into ethosomes. *In vitro* studies demonstrated that the deposition of psoralen was 6.56-fold higher when ethosomes were used in comparison with the ethanolic solution. Similarly, *in vivo* skin microdialysis showed that the concentration and the area under the curve of psoralen were approximately 3.37 and 2.34 times higher when it was incorporated into liposomes. Enhanced permeation and skin deposition of psoralen delivered by ethosomes can help to reduce its toxicity and improve its efficacy especially when long-term psoralen treatment re required [120]. Again, Zhang et al [121] developed a novel psoralen transdermal delivery system employing ethosomes and comparing them with liposomes used as reference. *In vitro* skin permeation studies showed that the permeability of psoralen, when loaded

into ethosomes, was higher than that obtained by using liposomes. Indeed, transdermal flux and skin deposition of psoralen delivered by ethosomes, were 3.50 and 2.15 times higher than those achieved by using liposomes. Ethosomes and liposomes were highly biocompatible and did not show any adverse effect following daily application *in vivo* to rat for 7 days. In another study performed by Negi et al., thymoquinone, a lipid soluble benzoquinone known as the major active ingredient of volatile oil of Nigella sativa with high anti-psoriatic activity, was incorporated into ethosomes and their anti-psoriatic potential was tested by employing mouse-tail model [128]. The anti-psoriatic drug activity was observed to be substantially higher in the case of thymoquinone loaded ethosomes in gel in comparison with plain thymoquinone contained in Nigella Sativa extract. The promising outcomes of the current studies confirmed the superiority of thymoquinone loaded into ethosomes as vesicular systems for the management of psoriasis in topical applications.

4.4. Cosmeceutical and antiaging applications

The application of liposomes as carriers for the delivery of bioactive molecules to the skin was firstly introduced by Mezei et al. in 1980 [129], which loaded triamcinolone into phospholipid vesicles obtaining an increased bioavailability in epidermis and dermis, where the activity of the drug was required. As reported before, due to the controversial results on the efficacy of liposomes, in the past decades, several studies have been focused on the design of modified lamellar vesicles capable of overcoming the problems associated with skin delivery, thus leading to the development of new classes of lamellar vesicles. Given the needs of modern society, these new vesicles have also been used as carriers for natural bioactives especially in cosmetic and cosmeceutical preparations. These vesicles can exert hydrating and emollient effects thanks to their ability to intercalate into the stratum corneum facilitating the incorporation of water or can act as true carriers improving the distribution of the bioactives in the skin layers, especially the deeper ones. Natural antioxidant molecules (e.g., curcumin, quercetin, resveratrol) or plant extracts (phytocomplexes) have shown beneficial and protective effects since ancient times and are being exploited more and more for cosmeceutical uses. As known, these compounds are low bioavailable, especially in the skin, and their incorporation into nanocarriers seems to be the only convenient approach capable of improving their local efficacy. The use of phospholipid vesicles in cosmeceuticals is not widespread yet, mainly due to the little knowledge on their preparation at industrial level, however few studies can be found in literature, which have proven the efficacy of these systems. *In vitro* and *in vivo* studies have been carried out to demonstrate the improved cosmeceutical effect of different bioactives when incorporated into vesicles, by reducing pigmentation disorders, skin aging and solar exposure effects. The incorporation of the bioactives into vesicles favour the penetration through the stratum corneum thus promoting

their activities at the damaged site [130]. Astaxanthin was loaded in cationic liposomes and applied to the skin before UV exposure. This treatment prevented the UV-induced skin thickening, collagen reduction induced by UV exposure and inhibited melanin production [131].

Conclusion

Considering all the studies and results reported in this review, phospholipid vesicles seem to be optimal carriers for the delivery of plant-derived bioactive molecules at skin level, as by using them is possible to manufacture environmentally friendly and effectively marketable products capable of protecting, maintaining in health conditions and caring the skin. Indeed, new preparation methods like the microfluidification, the heating procedure or the direct dispersion followed by sonication or stirring, have been advanced. These methods are free of toxic solvents, not energetically dissipative and easily scalable, thus ideal to be used to produce not expensive commercial products, highly biocompatible. Using soy lecithin as phospholipid to load phytocomplexes is possible to prepare completely natural and green formulations with effective beneficial properties. Moreover, the performed studies suggest that the phytocomplexes are usually more efficacy than the single bioactive molecules due the synergic activities of the different chemicals and their effectiveness is usually improved by their loading into phospholipid vesicles. Actually, using phospholipid vesicles is possible to manufacture totally green formulations, which fully meet the awareness of modern consumers. Encapsulation of phytocomplexes into phospholipid vesicles with increased skin penetration ability is receiving considerable attention in the recent years. A wide range of phytochemicals can be incorporated either as pure bioactives or as phytocomplexes, into phospholipid vesicles to improve their delivery into and through the skin for different applications.

In particular, phytocomplexes obtained from autochthonous species or agri-food by-products or even waste may represent a clever alternative to synthetic active molecules, which in turn may obtain a highest distribution on the market thanks to their main claims related to improved biodiversity and exploitation of circular economy.

However, to ensure the success of this natural-based phospholipid systems both at clinical and commercial level, many problems/challenges still need to be addressed. Firstly, the safety of the different formulations used need to be further explored. For example, studies focused on toxicology of phospholipid vesicles during both short and long term application on the skin, have to be deeply evaluated, especially those containing hight amount of ethanol or surfactants. From a commercial point of view, specific evaluation based on the formulation and design of the systems, along with the preparation methods and the scalability of the entire process are still required.

Aknowledgements: This research was supported by the Italian Ministry of Education, University and Scientific Research (MIUR), Grant No. PON A.A. 2017/2018, Molecole bioattive naturali veicolate in vescicole lamellari per la cura delle lesioni cutanee (cod = DOT1304004).

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Aim of the thesis

To exploit phytochemicals obtained from the Sardinian flora to prepare natural and environmentally-friendly formulations based on phospholipid vesicles specifically tailored for the treatment of skin damages and lesions.

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

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Abstract

An extract of *Hypericum scruglii*, an endangered endemic plant of Sardinia (Italy), was prepared and characterized. It was loaded in special phospholipid vesicles, glycerosomes, which were modified by adding maltodextrin (glucidex) and a polymer (gelatin or hyaluronan). The corresponding liposomes were also prepared and used as reference. The vesicles disclosed suitable physicochemical features for skin delivery. Indeed, their mean diameter ranged from 120 to 160 nm, they were homogeneously dispersed (polydispersity index ≤ 0.30), and their zeta potential was highly negative (~−45 mV). The vesicle dispersions maintained unchanged characteristics during 60 days of storage, were highly biocompatible, and were able to protect keratinocytes against damages due to oxidative stress induced by treating them with hydrogen peroxide. Vesicles were also capable of promoting cell proliferation and migration in vitro by means of a scratch wound assay. The results confirmed the fruitful delivery of the extract of *H. scruglii* in glycerosomes modified with glucidex and gelatin and their promising ability for skin protection and treatment.

1. Introduction

The skin represents the main barrier of our body and it is specifically designed to perform different functions such as protection from various external insults, maintenance of the internal homeostasis, and sensory perception (Gefen, 2019). Indeed, it protects the body from various daily challenges, avoiding excessive loss of water and providing a defense against mechanical insults, passage of xenobiotics, and absorption of chemicals and physical pollutants (Fore, 2006). As the outermost barrier, skin is constantly exposed to a prooxidative environment (i.e., solar UVA and UVB radiation, air pollution), which can generate a large amount of reactive oxygen species (Birch-machin and Swalwell, 2010; Birch-Machin et al., 2013; Packer and Valacchi, 2002). The overproduction of these species can overwhelm the elaborated defense system of the human body, causing premature skin aging due to the induction of DNA and mitochondrial damage, lipid peroxidation, and activation of inflammatory signaling pathways. In addition, oxidative stress might negatively affect the natural healing process of skin lesions, increasing the risk of developing complications including infections, sepsis, or chronic wounds (Church et al., 2006; Godbout and Glaser, 2006).

The daily use of natural antioxidants applied topically represents a valuable strategy aimed at protecting the skin from external insults and promoting the regeneration and healing of damaged tissues (Briganti and Picardo, 2003; Tabassum and Hamdani, 2014). Due to increased interest in natural medicine, a large number of plant-derived antioxidants have been tested as potential bioactives for skin protection and repair. In vitro results obtained until now are encouraging, even if their effectiveness in vivo still has to be confirmed (Chua et al., 2012; De Luca et al., 2016; Halliwell, 2008; Nicolaou, 2013). The use of innovative skin delivery systems, such as penetration enhancers or nanocarriers, represents a modern and valuable approach to maximize the local bioavailability of natural antioxidants (Cristiano et al., 2020; Sala et al., 2018; Zhang et al., 2013). Recent studies underlined the key role played by ad hoc formulated nanocarriers in improving the effectiveness of natural antioxidants in in vivo models of skin diseases (Coradini et al., 2014; Pivetta et al., 2018; Scalia et al., 2013).

Considering these challenges, in the present study, the extract of *Hypericum scruglii* Bacch., Brullo, and Salmeri (Hypericaceae) was loaded in ad hoc formulated phospholipid vesicles. *H. scruglii* is an endangered endemic plant species of the Sardinia Island (Bacchetta et al., 2010) and it is considered as a plant of high pharmaceutical and conservation value (Porceddu et al., 2020; Sanna et al., 2018). In addition, these species are known for their ability to produce a wide spectrum of secondary metabolites, such as naphthodianthrones (hypericin and pseudohypericin), phloroglucinols (hyperforin and adhyperforin), phenolic acids, flavonoids (hyperoside, rutin, or quercitrin), xanthones, and terpenes (Mandrone et al., 2017). In popular medicine, the oleolite of *Hypericum*, also

called “St. John’s Wort Oil” and obtained by maceration of *H. perforatum* L. flowers in a vegetable oil, is well-known for its beneficial properties in skin diseases. It is traditionally used to treat burns, wounds, bedsores, and myalgias due to the high content of valuable components with antibacterial, antiviral, anti-inflammatory, astringent, and restoring properties (Sanna et al., 2018; Stojanovic et al., 2013). Plant species belonging to the genus *Hypericum* has been used since ancient times for the treatment of different diseases and, recently, scientific researchers focused their attention on the phytochemicals contained in these plants, confirming the high content in polyphenols (Saddiqe et al., 2010; Zhang et al., 2020, 2019). In addition, the extract of *H. scruglii* disclosed excellent antioxidant activity and inhibitory effects on elastase. The latter is a protease of the chymotrypsin family, responsible for the rupture of elastin as well as collagen and fibronectin (Chiocchio et al., 2018).

The phytochemical composition and biological properties of *H. scruglii* have not been previously studied, but Mandrone et al. (Mandrone et al., 2017) have recently identified in this plant the presence of shikimic and chlorogenic acids, two derivatives of phloroglucinols, quercitrin, hyperoside, and hypericin, confirming their chemotaxonomic meaning. They also described the ability of these metabolites to exert antioxidant activity and inhibition of α-glucosidase.

In the present study, a hydro-alcoholic extract of the aerial parts of *H. scruglii* was obtained by a maceration process and its main components have been identified. The dried extract was loaded in ad hoc formulated glycerosomes aiming at improving its effectiveness. Glycerosomes were further modified by adding a natural dextrin (glucidex) and a natural polymer (gelatin or hyaluronan). Corresponding liposomes were prepared as well and used as reference. Vesicles were fully characterized, and their biocompatibility was evaluated along with their ability to protect keratinocytes from oxidative stress and promote the closure of skin lesions. Finally, the uptake of vesicles by cells was assessed by using a confocal microscope.

2. Materials and Methods

2.1. Materials

Plants of *H. scruglii* were collected from the natural population of Funtanamela (Laconi, Italy) in July 2019. To ensure correct identification of the plant samples, the collected specimens were compared with the herbarium material harvested by Bacchetta at al. (Bacchetta et al., 2010) in the same locality and preserved in the Herbarium CAG (University of Cagliari, Italy).

Lipids S75 (S75), a mixture of phospholipids (~70% soy phosphatidylcholine, 9% phosphatidylethanolamine, and 3% lysophosphatidylcholine), triglycerides, and fatty acids were purchased from AVG S.r.l. (Garbagnate Milanese, Milan, Italy), a local supplier for Lipoid GmbH (Ludwigshafen, Germany). Ethanol, glycerol, gelatin, 1,2-dioleolyl-snglycero-3-

phosphoethanolamine-N-lissamine-sulfo-rhodamineB, 5(6)-carboxyfluorescein, Hoechst 33342, and all other reagents and solvents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Analytical standards of fatty acids were purchased from Supelco Analytical (Bellefonte, PA, USA). Standard cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin 3-O-glucoside, delfinidin-3-O-glucoside, catechins, epicatechins, and gallic acid were purchased from Extrasynthese (Lyon, France). 2,2-diphenyl-1-pikryl-hydrazyl (DPPH) and Trolox were purchased from Sigma-Aldrich (St-Quentin Fallavier, France). Folin–Ciocalteu and sodium carbonate were purchased from Sigma-Aldrich (Darmstadt, Germany). All reagents and plastics for cell cultures were purchased from Life Technologies Europe (Monza, Italy).

2.2. Extraction of Phytocomplexes

The aerial parts of the plant were cleaned, washed, and left to dry in the dark for 30 days at room temperature. The dried aerial parts (100 g) were grinded to favor the extraction of the active components and then, dispersed in a mixture (1 L) of water and ethanol (30:70 *v*/*v*) and left under constant stirring for 48 h at room temperature (25 °C). The obtained dispersion was centrifuged twice (30 min, 8000 rpm). The extract was separated from the coarse part (precipitate); the ethanol was removed by evaporation at 45°C and low pressure using a rotary evaporator. The remaining water was eliminated by a freeze-drying process. The extract obtained was finally stored under vacuum and protected from light.

2.3. Determination of the Total Phenolic Content

The quantification of total polyphenols was established by means of the Folin–Ciocalteu method (Libbey and Walradt, 1968). A total of 200 µL of the extract was added to 1 mL of a diluted Folin–Ciocalteu solution and 800 µL of sodium carbonate. Samples were heated at 60 °C for 10 min and then, cooled in the refrigerator for another 10 min. The optical density was finally measured at a wavelength of 750 nm. A calibration curve was also built by using gallic acid as reference. Results were expressed as milligrams of gallic acid equivalent per gram of dry matter (mg GAE/g DM).

2.4. Estimation of the Antiradical Properties of the Extracts

The antiradical capacity of the extract was assessed by means of a DPPH (2,2-diphenyl-1-pikryl-hydrazyl) colorimetric method. A total of 50 µl of the extract was added to 1.45 mL of the DPPH (0.06 mM) solution and after incubation (30 min), the optical density was measured at 515 nm (Kallithraka et al., 2005). The calibration curve was obtained by using Trolox (positive control) and results were expressed as micrograms of Trolox equivalent per milliliter (µg TE/mL).

2.5. Polyphenol Characterization and Quantification by High Performance Liquid Chromatography (HPLC)

HPLC analyses were carried out to identify and quantify the polyphenols contained in the extracts, using an HPLC–DAD (diode array detection) (Waters Alliance, Milford, MA, USA), a quaternary Waters e2695 pump, a UV–vis photodiode array spectrophotometer (Waters 2998), a control system, and Empower 3 data collection software. HPLC studies were performed by using a Discovery HS C18, 5 µm, 250 × 4.6 mm, column with a HS C18, Supelguard Discovery, 20 × 4 mm, 5 µm, precolumn maintained at 30 °C. Two mobile phases were used during the analyses: mobile phase A, containing formic acid 0.2% (*v*/*v*) in water; mobile phase B, composed of a mixture of methanol (69%), water (29%), and formic acid (0.2%) (*v/v/v*). The HPLC gradient scheme was set as follows: 0% at 3 min, 10% at 10 min, 40% at 60 min, 60% at 80 min, 80% at 105 min, 100% at 120 min and again, 0% at 140 min, followed by 20 min of stabilization at 0%. Detection was assessed by changing the wavelengths from 278 to 364 nm, aiming at detecting all the bioactives of interest. A standard curve was determined for each compound (quercetin and chlorogenic acid) in a concentration range from 1 to 0.0625 mg/mL. The five standard solutions were made by progressive dilutions with a factor of 2. The linearity evaluation showed high correlation coefficients R^2 (> 99%). The detection of peaks was based on the standard's retention time and comparison of the spectra. The limit of detection (LOD) and the limit of quantitation (LOQ) were evaluated by using the signal to noise ratio of chromatograms for blank samples: S/N = 3 and S/N = 10, respectively. The LOD of quercetin was 0.00013 mg/mL and the LOQ was 0.0005 mg/mL at 364 nm; likewise, for chlorogenic acid, the LOD was 0.00024 mg/mL and the LOQ was 0.0008 mg/mL at 324.8 nm (Rajha et al., 2019a, 2019b; Taamalli et al., 2012).

2.6. Vesicle Preparation

Phospholipid S75 (240 mg) and *H. scruglii* extract (20 mg) were weighed in a glass vial and hydrated with 2 mL of water to obtain liposomes (used as reference) or 2 ml of a mixture of glycerol and water (25:75 *v*/*v*) to obtain glycerosomes. Glycerosomes were further modified by adding, to the mixture of phospholipid and extract, a commercial dextrin (glucidex) to obtain gluglycerosomes or the combination of glucidex and one polymer (gelatin or hyaluronan) to obtain gel-gluglycerosomes and hyal-gluglycerosomes, respectively.

Table 1. Composition of *Hypericum scruglii* extract-loaded vesicles.

	S75 (mg/mL)	Extract (mg/mL)	Glucidex (mg/mL)	Gelatin (mg/mL)	Hyaluronan (mg/mL)	Glycerol (mL)	Water (mL)

Liposomes	120	10	0	0	0	-	1
Glycerosomes	120	10	0	0	0	0.25	0.75
Gluglycerosomes	120	10	25	0	0	0.25	0.75
Gel-gluglycerosomes	120	10	25	1	0	0.25	0.75
Hyal-gluglycerosomes	120	10	25	0	1	0.25	0.75

Dispersions were sonicated (40 cycles, 5 sec on and 2 sec off) with a Soniprep 150 ultrasonic disintegrator (MSE Crowley, London, UK) to obtain small vesicles homogeneously dispersed (Castangia et al., 2013). The direct sonication of dispersion is a rapid and green method as the use of organic solvents and dissipative processes is completely avoided. The composition of the formulations is reported in Table 1. Empty vesicles (i.e., without extract) were also prepared and characterized.

Vesicle dispersions were separated from the non-entrapped bioactives contained in the extract by dialysis against water (for liposomes) or the appropriate water and glycerol mixture (for glycerosomes, gluglycerosomes, gel-gluglycerosomes, and hyal-gluglycerosomes). Dispersions (1 mL) were loaded into dialysis tubes, Spectra/Por® membranes (12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands), transferred in the dialyzing medium (3 L), and dialyzed for 2 h under continuous stirring at 25 °C. The medium was refreshed after 1 h. The entrapment efficiency was calculated as the percentage of the antioxidant activity of dispersion after dialysis versus that found before dialysis (Castangia et al., 2015). The antioxidant activity of vesicle dispersions was calculated by measuring their ability to scavenge the DPPH radical. 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 (AA%) was calculated as follows:

$$AA\% = [(ABS_{DPPH} - ABS_{sample})/ABS_{DPPH}] \times 100$$

2.7. Evaluation of Physico-chemical Properties and Stability on Storage of Vesicles

The cryogenic transmission electron microscopy (cryo-TEM) method has been used to evaluate both the formation and morphology of vesicles. Specific grids covered with a holey carbon film were used to prepare the samples, which were immediately moved into an automatic plunge freezing apparatus (Vitrobot, FEI, Eindhoven, The Netherlands) aimed at controlling humidity and temperature. The formed film was then vitrified (Vitrobot, FEI Company, Eindhoven, The Netherlands) and observed by using a Tecnai F20 microscope (FEI Company) at −173 °C and 200 kV. Images were acquired by using a CCD Eagle camera (FEI Company).

Dynamic Light Scattering technique has been used to measure the average diameter and polydispersity index of vesicles by using a Zetasizer Ultra (Malvern Panalytical, Worcestershire, UK). The same Zetasizer Ultra was used to measure the zeta potential of samples, by means of the M3-PALS method. Water or the same mixture of water and glycerol used to prepare the vesicles have been used to dilute the sample (1:100), which was then analyzed at 25 °C.

A stability study was performed by measuring the size and size distribution of the vesicles stored at room temperature (25 °C) for 60 days.

2.8. Release Studies

The amount of extract released from the vesicles was measured by using a dissolution tester equipped with 6 stations (DT 720 Series—Erweka, distributed by EMME 3 SRL, Milan) according to USP requirements. Vesicle dispersions were transferred into polycarbonate dialysis tubes (Spectra/Por membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., NJ, USA), put in the baskets of the dissolution tester containing the release media (1 liter), and left under constant stirring at 37 °C for 24 h. The amount of the extract in dispersion was evaluated at 2, 4, 6, 8, and 24 h by measuring the antioxidant activity using the DPPH colorimetric test.

2.9. Cell Viability and Protection Against Oxidative Stress

Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with fetal bovine serum (10%), penicillin (100 U/mL), and streptomycin (100 µg/mL) has been used as a medium for the growth of immortalized human keratinocytes (HaCaT). Cells were maintained at 37 °C, 100% humidity, and 5% CO_2 in 75 cm^2 flasks. For the experiment, 7.5×10^3 cells were seeded in each well of a 96-well plate and after 24 h, were treated with *H. scruglii* extract in aqueous dispersion (prepared by dispersing in water the same amount of extract used in the vesicles and sonicating it to obtain a more homogeneous dispersion) or loaded in vesicles, at different concentrations of the extract (20, 2, 0.2, and 0.02 g/mL). After 48 h, 100 µl of MTT 3-(4 5-dimethylthiazol yl-2)-2 5-diphenyltetrazolium bromide) reagent (0.5 mg/mL final concentration) was added to each well and plates were incubated for 3 h; then, formazan crystals produced by alive cells were dissolved by using dimethyl sulfoxide (100 µL), and the absorbance measured at 570 nm by using a microplate reader (Synergy 4 Reader, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). Experiments were performed three times, each time in triplicate. The cell viability of treated cells is shown as a percentage of untreated control cells (100% viability).

The protection of cells against the oxidative stress induced in keratinocytes with hydrogen peroxide has been evaluated by seeding 7.5×10^3 cells/well into 96-well plates and once they reached semi-confluence, treating them with hydrogen peroxide in PBS (1:40,000) and simultaneously with *H.*

scruglii extract in aqueous dispersion (prepared as reported above) or loaded in vesicles (2 and 0.2 µg/mL of *H. scruglii* extract). Cells were incubated for 4 h and two different controls were used: untreated cells (negative control) and cells treated with hydrogen peroxide only (positive control). After 4 h of incubation, cells were washed with fresh medium, and the MTT assay was used to measure the viability, as reported above. Results are reported as the percentage of untreated cells (100% viability).

2.10. Ability of H. Scruglii Extract Loaded into Vesicles to Promote Cell Proliferation and Migration: Scratch Assay

Keratinocytes were grown until complete confluence was reached. A linear scratch was generated in the cell monolayer by using a sterile pipette tip and the scattered fragments were accurately removed. The wounded area was then treated with *H. scruglii* extract in dispersion or loaded into vesicles (0.2 µg/mL of *H. scruglii* extract) and monitored by using an optical microscope (10× objective), at scheduled time intervals (0, 12, 24, 36, and 48 h). The observation result allowed the evaluation of the ability of the different formulations to stimulate proliferation and migration of keratinocytes.

Images at time zero (t = 0 h) were captured to record the initial area of the wounds, and the recovery of the wounded monolayers due to cell migration and proliferation was evaluated at 12, 24, 36, and 48 h (t = Δ h). The captured images were quantified by Java's image J software (1.8.0_172, http://rsb.info.nih.gov) by measuring the area of the wound (Y.K.Yue, 2010). The migration of cells toward the wounds was expressed as percentage of wound closure: wound closure (%) = $[(a_{0h} - a_{\Delta h}/a_{0h}] \times 100\%$

where a_{0h} is the wounded area immediately after scratching, and $a_{\Delta h}$ is the wounded area measured at 12, 24, 36, and 48 h after scratching.

2.11. Uptake of Fluorescent Vesicles by Keratinocytes

Fluorescent vesicles, labelled with a lipophilic fluorescent marker (1,2-dioleolyl-snglycero-3-phosphoethanolamine-N-lissamine-sulfo-rhodamineB; Rho-PE, 0.025 mg/mL; red) and loaded with a hydrophilic fluorescent marker (5(6)-carboxyfluorescein; CF, 0.025 mg/mL; green), were prepared to evaluate their internalization by keratinocytes (Manca et al., 2019b). Poly-L-Lysine-coated 8-well µ-slides (Ibidi GmbH, Martinsried, Munich, Germany) were used to culture the keratinocytes, which were then treated with the vesicular formulations for 2, 4, and 24 h. At each time point, alive cells were stained with the markers for the cell nucleus (Hoechst 33342, Trihydrochloride, Trihydrate −10 mg/mL solution in water; blue) and observed by using a confocal inverted microscope FluoView FV1000 (Olympus, Barcelona, Spain) equipped with a UV–visible light laser and a 60× objective

UPLSAPO. Fluorescence excitation and emission wavelengths for RhoPE, CF, and Hoechst were 559/578, 470/535, and 360/460 nm, respectively.

2.12. Statistical Analysis of Data

The results were expressed as mean value ± standard deviation. Statistically significant differences among samples were determined by using variance analysis. The post hoc Tukey–Kramer *t*-test was used to substantiate a significant difference between the means of two specific groups. The statistical analysis was performed by using the Excel software package (Microsoft Corp, Redmond,WA, USA) equipped with a tool for statistical analysis. The minimum level of significance chosen was $p < 0.05$.

3. Results

3.1. Extract Characterization

The extraction method is schematically represented in Figure 1A, along with the assayed total phenolic content and the radical scavenging capacity of *H. scruglii* extract. The extract contained 0.13 ± 0.0018 mg GAE/g DM and exhibited an antiradical capacity of 150.4 ± 9.9 µg TE/mL.

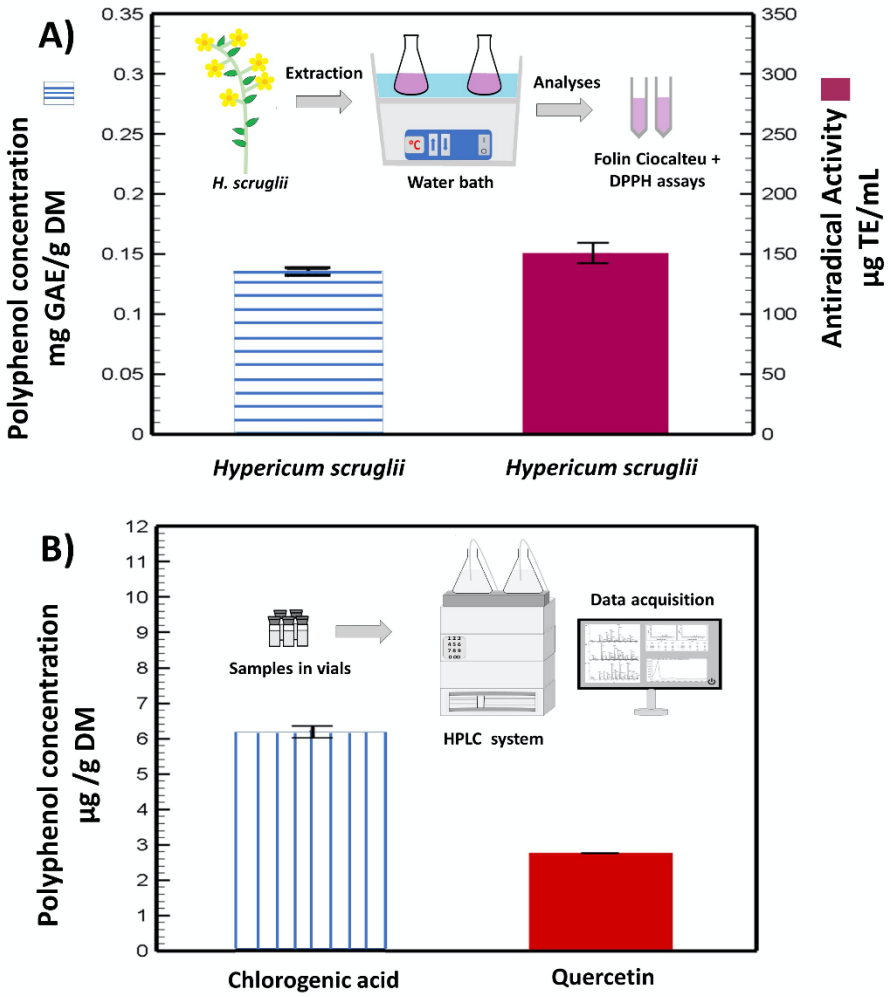

Figure 1. Polyphenol concentration and antiradical activity of the *H. scruglii* extract (inset shows the schematic representation of the process) (**A**), and chlorogenic and quercetin concentrations identified by HPLC (inset shows the schematic representation of the HPLC method) (**B**).

Two main molecules were identified and quantified in the *H. scruglii* extract by HPLC analyses: quercetin (flavonoid) and chlorogenic acid (phenolic acid). The amount of chlorogenic acid (~6 µg/g DM) was almost double that of quercetin (~3 µg/g DM) (Figure 1B).

3.2. Vesicle Characterization

Glycerosomes were used as skin delivery systems for the extract obtained from the areal parts of *H. scruglii*. The performances of glycerosomes were further improved by adding a natural dextrin (glucidex) to prepare gluglycerosomes and one polymer (gelatin or hyaluronan), thus obtaining gel-gluglycerosomes and hyal-gluglycerosomes.

Cryo-TEM analyses disclosed that *H. scruglii* extract-loaded liposomes, used as reference, were spherical and unilamellar (Figure 2A). Glycerosomes (Figure 2B,C) and gluglycerosomes (Figure 2D) assembled in spherical and oligolamellar vesicles, which appeared close-packed. The addition of gelatin did not significantly modify both morphology and structure of the glycerosomes (Figure 2E), while the addition of hyaluronan (hyal-gluglycerosomes) allowed the formation of aggregated vesicles with an irregular shape (Figure 2F). The results underlined that the glycerol facilitated the formation of concentric bilayer probably because it decreased the repulsion between the bilayer surfaces. Dextrin and gelatin did not affect the assembly probably because of their possible location in the water phase, within the lipid bilayers, and in the intervesicle's medium (M. L. Manca et al., 2015b). Hyaluronan seems to facilitate the aggregation of vesicles.

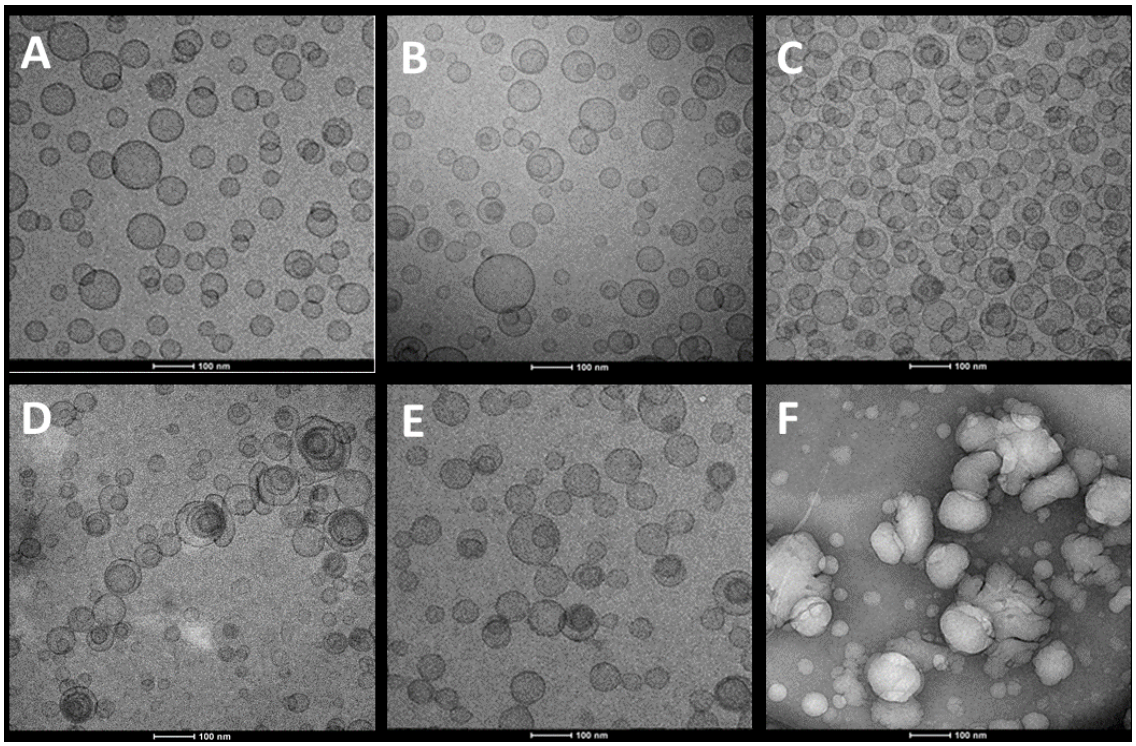

Figure 2. Representative cryo-TEM images of liposomes (**A**), glycerosomes (**B**,**C**), gluglycerosomes (**D**), gel-gluglycerosomes (**E**), and hyal-gluglycerosomes (**F**), loading *H. scruglii* extract.

The mean diameter of the vesicles was measured by means of dynamic laser light scattering technique, which provided a global estimation of the vesicle size calculated from the scattering intensity of each particle fraction (Table 2). Extract-loaded liposomes and empty vesicles were prepared and characterized as well. The mean diameter of empty vesicles was ~116 nm without statistical differences among species ($p > 0.05$). The loading of the extract did not significantly affect the size of liposomes and glycerosomes, which have a mean diameter ~120 nm. On the contrary, the

loading of the extract caused an increase in the size of gluglycerosomes with or without gelatin or hyaluronan, which disclosed the same mean diameter, ~158 nm (p > 0.05 among the three formulations of extract-loaded gluglycerosomes), according to the results reported by Akgün et al. (Akgün et al., 2019) for chitosan-coated liposomes. Indeed, for these formulations, the empty vesicles were smaller than the corresponding extract-loaded vesicles, indicating a contribution of the extract in both assembly and curvature radius of the bilayer. As previously reported, dextrin may stabilize the system thanks to their localization in both inter-lamellar and inter-vesicle medium (Catalán-Latorre, et al. 2018). In particular, its distribution in the aqueous compartment of the vesicles combined with the active component of the extract may cause a reduced curvature radius of vesicles, thus leading to the formation of bigger systems (Manconi et al., 2010). All the dispersions of *H. scruglii* extract-loaded vesicles were more homogeneously dispersed (polydispersity index ≤ 0.30) in comparison with empty vesicles (polydispersity index ~0.34), probably because the components of the extract may affect the vesicle assembly leading to the formation of vesicles more similar in size, as reported in previous studies where polyphenols influenced phospholipid assembly and reduced aggregation phenomena (Fang, 2010; Omar M Atrooz, 2014). The zeta potential was highly negative for all the extract-loaded vesicles (~−48 mV, p > 0.05 among all) irrespective of their composition. The results are in agreement with other studies, which confirmed the negative charge of liposomes made with the zwitterionic phosphatidylcholine (Akgün et al., 2019; Krämer et al., 1997). The amount of extract incorporated into the vesicles was very high (entrapment efficiency ~89%) without significant differences among the different samples. As expected, the antioxidant activity (AA, Table 2) of vesicles was very high and the composition of vesicles did not modify the antioxidant power of the extract, as already reported (M. L. Manca et al., 2015b; M.L. Manca et al., 2015c).

Table 2. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP), and entrapment efficiency (EE) of *H. scruglii* extract-loaded vesicles. Mean values ± standard deviations are reported (n = 6).

	DM (nm)		PI		ZP (mV)		EE (%)	AA (%)
	Empty	**Extract**	**Empty**	**Extract**	**Empty**	**Extract**	**Extract**	**Extract**
Liposomes	111 ± 13	122 ± 23	0.36	0.24	−39 ± 5	−55 ± 6	88 ± 9	64 ± 4
Glycerosomes	108 ± 19	117 ± 15	0.32	0.30	−41 ± 6	−53 ± 5	85 ± 7	79 ± 7
Gluglycerosomes	121 ± 21	154 ± 22	0.35	0.30	−37 ± 3	−41 ± 4	88 ± 11	84 ± 6
Gel-gluglycerosomes	114 ± 16	163 ± 33	0.34	0.25	−43 ± 7	−39 ± 6	97 ± 4	83 ± 7
Hyal-gluglycerosomes	127 ± 24	154 ± 25	0.35	0.29	−39 ± 4	−50 ± 5	88 ± 13	81 ± 9

Stability studies performed for 60 days at room temperature underlined the positive effect of the combination of dextrin and polymers on this property. Indeed, any significant variation of size and polydispersity index has been detected for these formulations, while an increase in vesicle size was detected for glycerosomes and liposomes, used as reference (Figure 3).

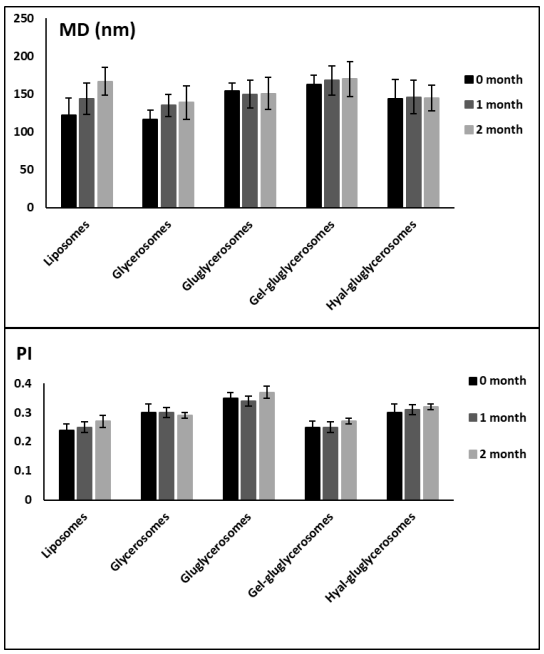

Figure 3. Mean diameter and polydispersity index (PI) of vesicles containing *H. scruglii* extract over 60 days of storage at room temperature (25 °C). Mean values ± standard deviations are reported (n = 6).

3.3. Release Studies

The amount of extract released by the vesicles during 24 h was similar for all the used vesicles. A burst release was observed at 2 h, reaching 20% of the initial amount; after, it became almost constant up to 24 h, reaching ~35%, Figure 4. Any significant differences between the formulations tested have been detected, suggesting that the bioactives contained in the extract are effectively incorporated in the vesicles. The slow release of the bioactives can be exploited at the skin level to reach an improvement in their effect as the formulation can stay at the application site as a depot, slowly releasing the actives in the damaged area and favoring the interaction with cells (Lewis et al., 2006; Murthy et al., 2004).

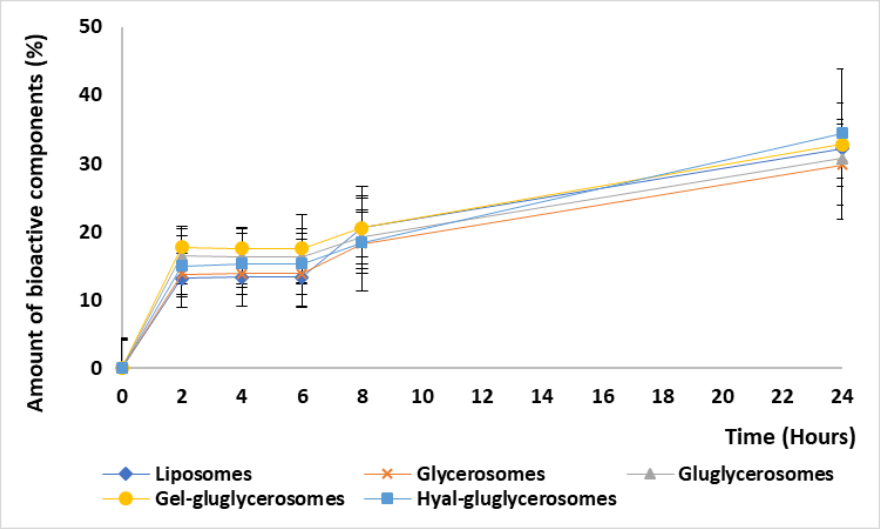

Figure 4. Amount of bioactive components (%) released from liposomes, glycerosomes, gluglycerosomes, gel-gluglycerosomes, and hyal-gluglycerosomes during 24 h of experiment. Mean values (error bars) ± standard deviations are reported (n = 3).

3.4. Biocompatibility and Protective Effect against Oxidative Stress of H. Scruglii Extract-Loaded Vesicles

The biocompatibility of the *H. scruglii* extract in aqueous dispersion or loaded in vesicles was evaluated by using keratinocytes, which are the outermost cells of the skin. No toxic effect was observed in cells treated with either vesicles or dispersion of the extract (Figure 5). Furthermore, cell proliferation was detected especially using extract-loaded vesicles, which improved the cell viability up to ≥ 150%, without significant differences among samples and concentrations ($p > 0.05$). The proliferative effect was less evident when cells were treated with the extract in dispersion as the viability was ~120% irrespective of the concentration used. The higher viability provided by treating the cells with the extract-loaded vesicles can be related to their ability to interact with cells favoring the internalization of the active components of the extract (Allen and Moase, 1996; Chithrani et al., 2010).

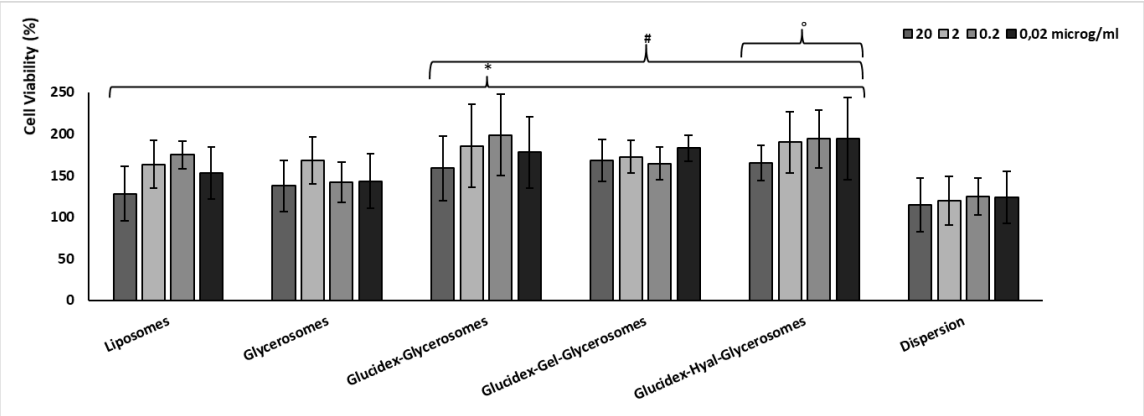

Figure 5. Viability of keratinocytes incubated for 48 h with different concentrations of *H. scruglii* extract in dispersion or loaded in vesicles. Data are reported as mean values ± standard deviations of cell viability expressed as the percentage of control (untreated cells; 100% of viability). The symbol (*) indicates values statistically different from dispersion ($p < 0.05$); (#) indicates values statistically different from glycerosomes ($p < 0.05$); (°) indicates values statistically different from liposomes ($p < 0.05$).

Likewise, *H. scruglii* extract was able to protect keratinocytes against damages induced by hydrogen peroxide, which was chosen as the oxidative agent (Figure 6). Its loading into vesicles strengthened its protective effect. Indeed, cells stressed with hydrogen peroxide showed a low viability (~62%), which did not change with the simultaneous treatment with extract in dispersion, irrespective of the used concentration (72%). When the extract was loaded in vesicles, the cell viability reached ~100% ($p > 0.05$ among cells treated with different vesicles, and $p < 0.05$ versus cells stressed with hydrogen peroxide and treated with the extract in dispersion). The treatment with the extract-loaded vesicles was able to counteract the damages induced by using hydrogen peroxide, re-establishing the healthy conditions. Therefore, the improved effectiveness of *H. scruglii* extract observed when it was incorporated in the vesicles may confirm our hypothesis according to which, thanks to their ability to interact with cells, vesicles may promote the release of the bioactives inside the cytoplasmic environment, leading the neutralization of the oxidative species. No differences in antioxidant power were detected among the different formulations.

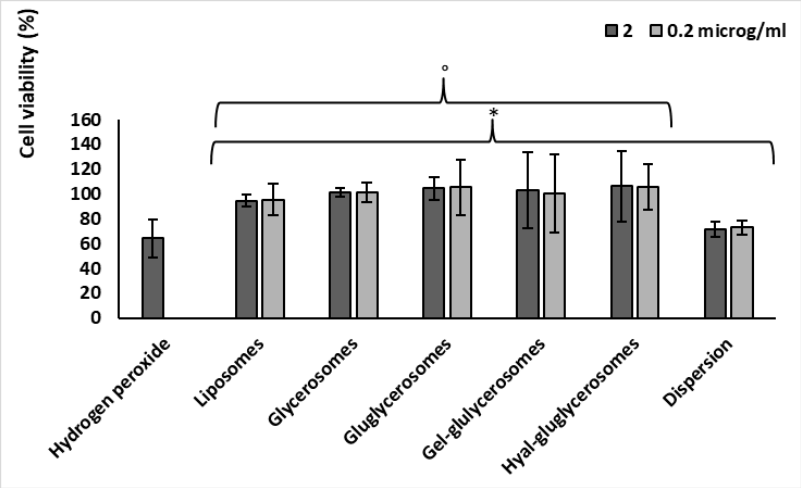

Figure 6. Viability of keratinocytes stressed with hydrogen peroxide and treated with *H. scruglii* extract in dispersion or loaded in vesicles. Data (bars) are reported as mean values ± standard deviations of cell viability expressed as the percentage of control (untreated cells; 100% of viability). The symbol (*) indicates values statistically different from that obtained with hydrogen peroxide ($p < 0.05$); (°) indicates values statistically different from that obtained with dispersion ($p < 0.05$).

3.5. Wound Healing Activity

The promotion of both proliferation and migration of keratinocytes was monitored during the treatment of the wounded area with *H. scruglii* extract in dispersion or loaded in vesicles. As shown in Figure 7, *H. scruglii* extract-loaded vesicles promoted the closure of the wound to a greater extent than the extract in dispersion, used as reference.

The scratch of untreated keratinocytes was mostly unchanged at 24 h, the proliferation and migration of cells started at 36 h, and closure of the wound was not reached at 48 h. A similar behavior was observed in cells treated with *H. scruglii* extract in dispersion, even if the proliferation and migration of cells started at 24 h. The treatment with extract-loaded vesicles stimulated to a greater extent the proliferation and migration of cells, especially when gel-gluglycerosomes were used, as they lead to the almost complete closure of the scratch already at 36 h. After 48 h, the wound was almost completely closed for cells treated with all the other vesicles, confirming one more time the key contribution of the carriers in promoting the effectiveness of the extracts. To better evaluate the differences provided by the different formulations, the wounded areas were measured and the healing areas were reported as a function of the time (Figure 7).

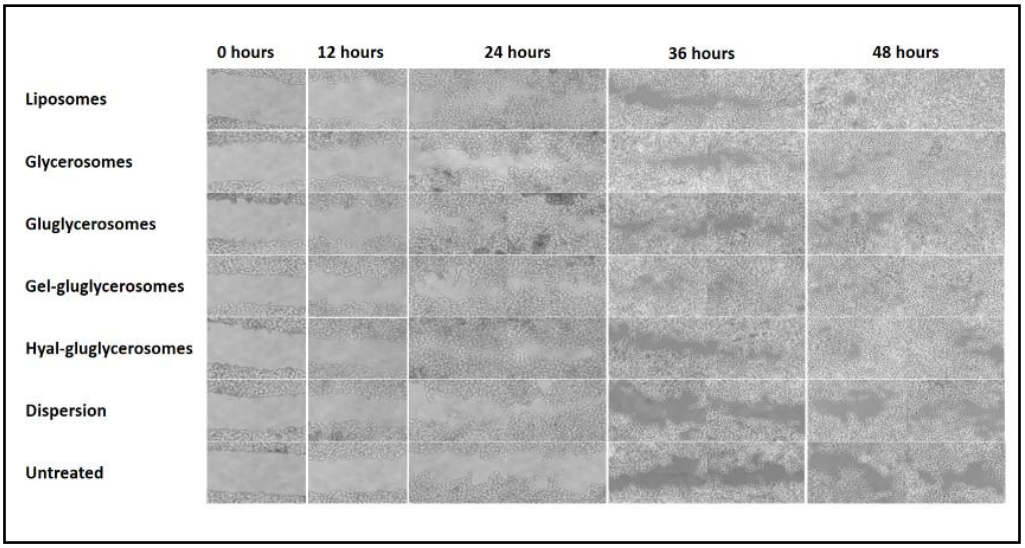

Figure 7. Representative optical microscopy images at different time points (0, 12, 24, 36, and 48 h) of the wound induced in keratinocytes and treated with *H. scruglii* extract in dispersion or loaded in vesicles.

The lesion of untreated cells was only partially closed (~50%) after 48 h, which gives an idea of the proliferation and migration speed of normal cells, mimicking the normal and healthy conditions (Figures 7 and 8). Likewise, when the cells were treated with *H. scruglii* extract in dispersion, the closure of the wound at 48h was ~50%. A faster closure of the wound was observed when the cells were treated with *H. scruglii* extract-loaded vesicles. Particularly using gel-gluglycerosomes, the % of closure reaches ~89% at 32 h and increased more at 48h to reach ~93%. Using the other phospholipid vesicles, the closure of the wound reached ~76% at 32 h and ~83% at 48 h, which, compared with the closure of the wound treated with gel-gluglycerosomes, was significantly lower (* ¤; $p < 0.05$). This study confirmed the optimal performances of all the vesicles as they promoted the delivery of *H. scruglii* extract and favored the proliferation and migration of keratinocytes. Particularly, the gel-gluglycerosoemes were found to be the most suitable formulation for the treatment of the wound induced in a monolayer of keratinocytes.

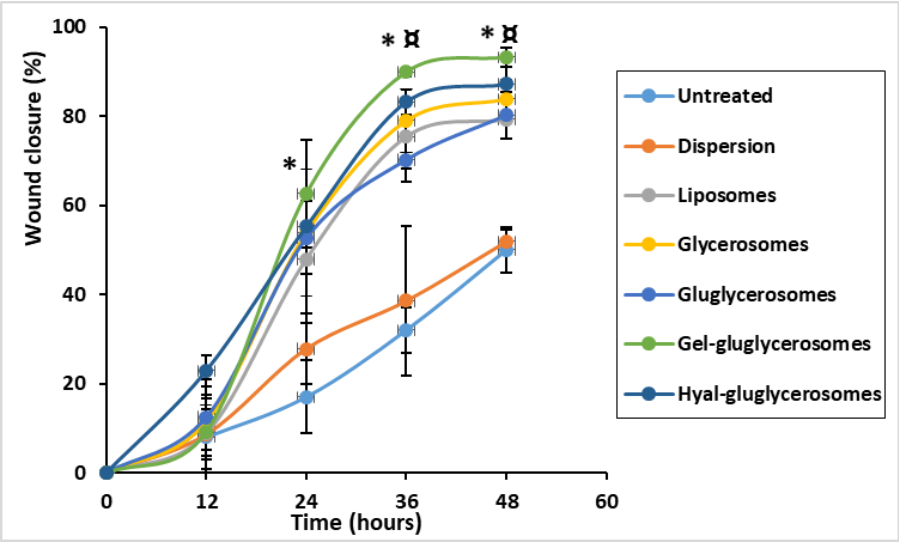

Figure 8. Percentage of wound closure. Wound healing is expressed as percentage of closure relative to the original size of wound. Mean values ± standard deviations are reported. The symbol (*) indicates values significantly different from that of untreated keratinocytes (control) and keratinocytes treated with the extract dispersion at 24, 36, and 48 h. The symbol (¤) indicates values significantly different from that of cells treated with gel-gluglycerosomes and other formulations.

3.6. Uptake of Fluorescently Labelled Vesicles by Keratinocytes

To confirm our findings, the ability of vesicles to interact with cells and promote their internalization has been evaluated. Keratinocytes were treated with fluorescent vesicles, obtained by labelling them with a lipophilic fluorescent marker (Rho-PE, 0.025 mg/mL) and loading a hydrophilic fluorescent marker (CF, 0.025 mg/mL), for 2, 4, and 24 h. Treated cells were observed by using a confocal microscope to evaluate the trend of internalization at the different time points (Figure 9).

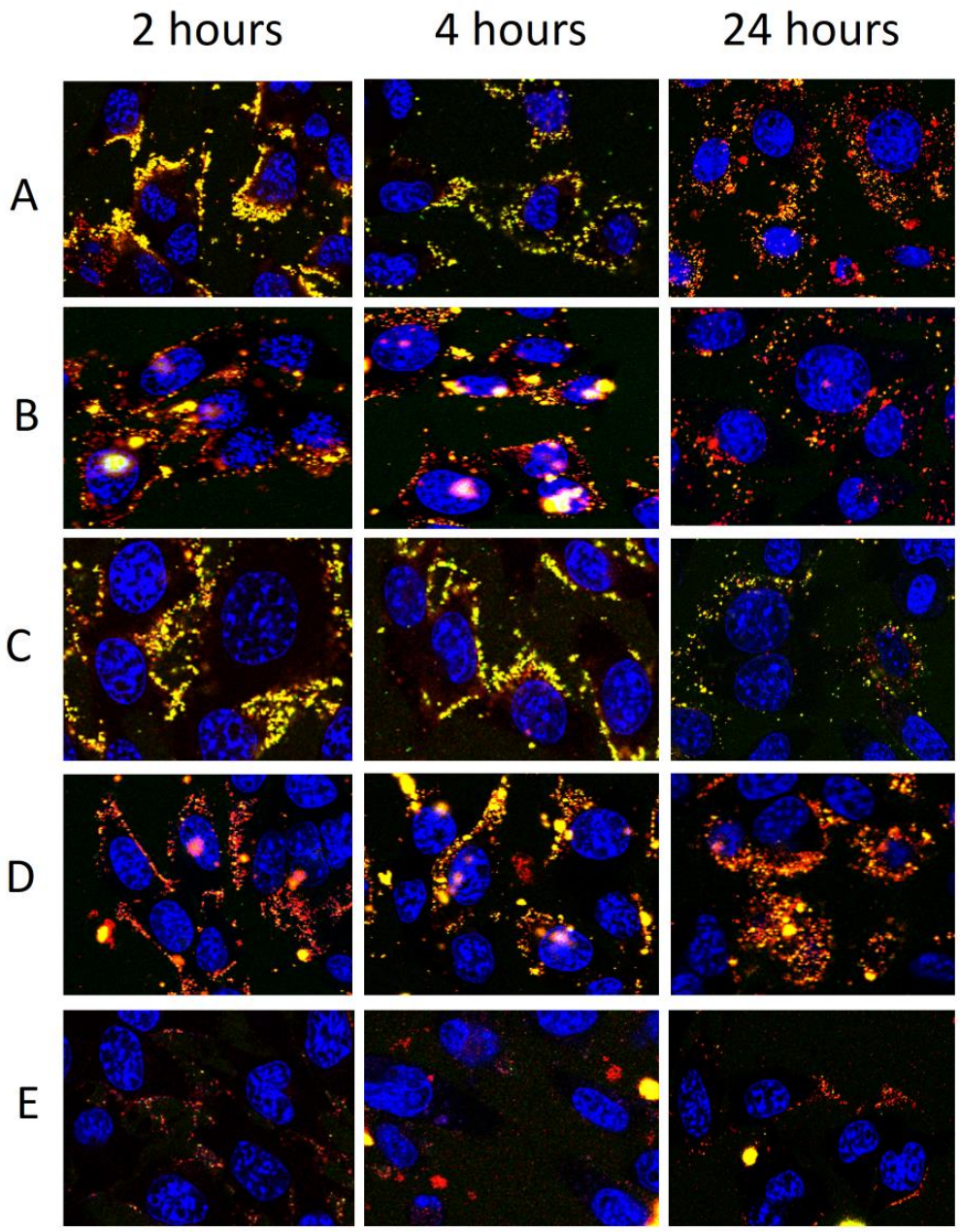

Figure 9. Confocal laser scanning microscopy images of living keratinocytes incubated for 24 h with fluorescently labelled vesicles: liposomes (**A**), glycerosomes (**B**), gluglycerosomes (**C**), gel-gluglycerosomes (**D**), and hyal-gluglycerosomes (**E**). The localization and intensity of the dyes are displayed at 2, 4, and 24 h in red for rhodamine-phosphoethanolamine, green for carboxyfluorescein, and blue for the nucleus. The orange area indicated the superposition of the markers (internalization of intact vesicles).

After 2 h of incubation, yellow fluorescence was evident around and inside the cells by using liposomes, disclosing a rapid internalization of vesicles. At 2 h of treatment with glycerosomes (with

or without glucidex and gelatin), the main fluorescence of both probes was less evident and located around the cell membrane as a small point, probably formed by aggregates of vesicles. Fluorescence was not evident at each time point inside the cells when hyal-gluglycerosomes were used, probably because of the reduced uptake due to the interaction between hyaluronan and glucidex and the quick aggregation of vesicles as disclosed in cryo-TEM images. At 4 h, using liposomes, the fluorescence of both markers was feeble, punctuated and distributed on the cell membrane denoting a rapid elimination, which was even more evident at 24 h. Using glycerosomes, gluglycerosomes, and gel-gluglycerosomes, the uptake of probes in the cytoplasm of the cells was evident at 4 and 24 h, disclosing a delayed but more effective uptake of these formulations.

4. Discussion

Different species of *Hypericum* have been widely used in the treatment of skin wounds in European phytotherapy and Turkish folk medicine (Pes et al., 2010). Mukherjee et al. (Mukherjee et al., 2000)(Mukherjee and Suresh, 2000) confirmed the effect of *H. perforatum* and *H. patulum* Thunb. extracts on in vivo lesions and Süntar et al. (Süntar et al., 2010) corroborated the healing and anti-inflammatory activities of *H. perforatum.*

In this study, the extract of *H. scruglii* was obtained from the aerial parts and its main components were identified. The composition of the extract confirmed its high antioxidant activity and antiradical activities, which were detected by in vitro tests. According to this, previous studies reported that the antioxidant capacity of *Hypericum* species is mainly related to their flavonoids and phenolic acids content (Orčić et al., 2011). Mandrone et al. (Mandrone et al., 2017), by using H-NMR, identified chlorogenic acid and a quercetin derivative in the extract obtained from *H. perforatum*. Similarly to the extract of *H. scruglii* obtained from the aerial part, the one obtained from the leaves of *H. hircinum* L. contained chlorogenic acid and quercetin (Pistelli et al., 2000). However, the extract obtained from *H. scruglii* was richer in chlorogenic acid than both *H. hircinum* and *H. perforatum* extracts. In addition, in this study, the wound healing ability of *H. scruglii* extract was confirmed and can be related to the presence of quercetin (flavonoid) and chlorogenic acid (phenolic acid). Indeed, the ABTS and DPPH assays showed that *H. scruglii* extract has higher antioxidant capacities than *H. perforatum* extracts (Mandrone et al., 2017). Their antiradical activities seem to exert enzymatic inhibition and many polyphenols, especially flavonoids (i.e., resveratrol derivatives, ellagic acid, etc.), exhibited the inhibition of tyrosinase and elastase enzymes (Cai et al., 2004; Pillaiyar et al., 2017; Wittenauer et al., 2015). A positive correlation (Pearson test) was found between the total phenolic and flavonoid content of *H. scruglii* extracts and that of tyrosinase and elastase inhibition

(Chiocchio et al., 2018). This effect is expected to increase the concentration of elastin and tyrosine, which are functional proteins and play a critical role in wound pathogenesis (Genc et al., 2020).

Accordingly, for the first time in this study, the effectiveness of the *H. scruglii* extract was demonstrated and improved by its loading in phospholipid vesicles. Indeed, in previous studies, only hypericin was loaded into the liposomes and different complexes were formulated for other aims (Derycke and De Witte, 2002; Plenagl et al., 2019), while different kinds of liposomes and phospholipid vesicles have been designed and tested, aimed at improving the efficacy of plant extracts (Manca et al., 2016d; Yang et al., 2020). The results underlined that the composition of the vesicles and the addition of specific additives can positively affect the effectiveness of the payloads, even if for each bioactive or phytocomplex, an ad hoc formulation is required (Castangia et al., 2015; Mir-palomo et al., 2016; Saravanakumar et al., 2020). Previous works strengthened the positive effect of glycerosomes on promoting the accumulation of bioactives in the different skin strata, mainly because of the moisturizing effect of glycerol, which is able to modify the ordered structure of the stratum corneum and favor the passage of bioactives (Moolakkadath et al., 2020). They have been used to deliver several natural molecules, extracts, and oils in the skin, providing an enhancement of the local bioavailability of the payload in comparison with liposomes (Manca et al., 2016a). Results confirmed that glycerosomes are versatile vesicles that can: (i) improve the delivery of several kinds of molecules (M.L. Manca et al., 2014; Manca et al., 2016a), (ii) be modified with one or more additives (Zhang and Li, 2017), and (iii) be used for different administration routes (Maria Manconi et al., 2017a).

In light of these promising findings, in the present work, the extract of *H. scruglii* has been loaded in glycerosomes modified with specific additives (maltodextrin alone or combined with gelatin or hyaluronan), which are expected to improve the stability and the viscosity of the dispersions and their ability to deliver the payloads to the skin (DiTizio et al., 2000; Dong and Rogers, 1993; Manconi et al., 2016a). Glucidex, a maltodextrin with a dextrose equivalent of 17, is a water soluble dextrin and can act as a structural component, which is dispersed in the vesicle surface and, in the water phase between the bilayers, may exert a mechanical reinforcement, improving their stability and ensuring their cryopreservation (Manconi et al., 2020; Shishova et al., 2020). In addition, this polysaccharide also seems to play a functional role in wound healing, especially in association with gelatin. Indeed, gel-gluglycerosomes provided the more rapid and effective closure of skin lesions in the scratch assay, probably because of the ability of glucidex and/or its combination with gelatin, to promote cell proliferation to a better extent than the other components of the vesicles. This result is in agreement with previous studies reporting the wound healing promotion of different polysaccharides (Hu et al., 2019; Kumar et al., 2019). The most promising efficacy of gel-gluglycerosomes can also be due to

the delayed and prolonged uptake provided by all glycerosomes in comparison with liposomes. The extract can remain inside the cells for more time, favoring their proliferation and migrations.

The addition of the dextrin, alone or in combination with the polymer (gelatin or hyaluronan), slightly affects the size of the extract-loaded vesicles, allowing a low increase in their mean diameter. The increased size can be related to its deposition on the vesicle surface (Kumar et al., 2020). However, the presence of glucidex alone or in combination with gelatin or sodium hyaluronate improved the stability of the dispersions, probably because of the formation of a more viscous and structured medium capable of avoiding aggregation and fusion, as previously reported for polymer associated vesicles (M. L. Manca et al., 2015b; Manca et al., 2020; M. Manconi et al., 2017).

In vitro results performed by using keratinocytes, as the most representative cells of the human skin, underlined the high biocompatibility of all tested vesicles. All the vesicles, irrespective of composition, were able to improve the ability of the extract to protect the keratinocytes from the damages induced by hydrogen peroxide. Indeed, the extract-loaded vesicles protect the cells to a better extent than the dispersion, used as reference.

Compared to gelatin, the combination of hyaluronan and glucidex did not give promising results in the delivery of *H. scruglii* extract, demonstrating that for each extract, it is important to find the most suitable formulation. Indeed, even if sodium hyaluronate is well-known for its capacity of stimulating cell proliferation and migration, in this case, its effect is slightly reduced in comparison with that provided by gelatin, which, in combination with glucidex and glycerosomes, ensured the optimal performance of the vesicles. Gel-gluglycerosomes seemed to be the most promising formulations considering that they were the most effective in both counteracting the oxidative stress and promoting the healing of an induced wound in cells. All these findings confirmed the key role played by the vesicles, which interact with cells favoring the release of the payload inside the cytoplasm, where they can exert their beneficial effect.

5. Conclusions

This work underlines that *H. scruglii* extract is effective in the treatment of skin lesions related to oxidative stress and its loading into specific phospholipid vesicles improved its efficacy, especially when glycerosomes were used and modified with the combination of dextrin and gelatin. These vesicles addressed great antioxidant activity along with better cell uptake and wound healing effects. The results disclosed that *H. scruglii* extract-loaded gel-gluglycerosomes are promising carriers to be used in the treatment of skin lesions connected with oxidative processes due to external insults and lack of internal homeostasis and, in addition, confirmed that for each phytocomplex, it is necessary to find the most suitable formulation, capable of effectively ensuring the highest efficacy. Overall,

the results are, however, not enough to confirm the effectiveness of *H. scruglii* extract incorporated into vesicles for the treatment of skin diseases and in vivo and clinical evaluation are needed to support in vitro results.

Author Contributions: Conceptualization, M.A. (Mohamad Allaw) and M.M.; methodology, M.A. (Matteo Aroffu), F.M. and M.P.; formal analysis, I.U., R.A.R., H.N.R. and R.G.M.; investigation, G.B., J.L.P., T.B.L.-M.; writing—original draft preparation, M.L.M., M.A. (Mohamad Allaw) and M.M.; writing—review and editing, M.M., M.L.M., A.M.F., R.G.M., G.B., J.L.P., T.B.L.-M. and M.A. (Mohamad Allaw); supervision, M.M. and M.L.M.

Funding: This research was supported by the Italian Ministry of Education, University and Scientific Research (MIUR), Grant No. PON A.A. 2017/2018, Molecole bioattive naturali veicolate in vescicole lamellari per la cura delle lesioni cutanee (cod = DOT1304004). This research was supported by FSC 2014-2020—Patto per lo Sviluppo della Regione Sardegna (RASSR14371).

Acknowledgments: This research was financially supported by Fondazione di Sardegna 2018. Authors would like to acknowledge Charbel Afif for his assistance with HPLC analyses.

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Oleuropein multicompartment nanovesicles enriched with collagen as a natural strategy for the treatment of skin wounds connected with oxidative stress

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ABSTRACT

Collagen-enriched transfersomes, glycerosomes, glytransfersomes were specifically tailored for skin delivery of oleuropein. Vesicles were prepared by direct sonication and their main physico-chemical and technological properties were measured. Biocompatibility, protective effect and promotion of the healing of a wounded cell monolayer were tested in vitro using fibroblasts. Vesicles were mainly multicompartment, small (~108 nm), slightly polydispersed (~0.27) and negatively charged (~-49 mV). Oleuropein was incorporated in high amount (~87%) and vesicles were stable during 4 months of storage. In vitro studies confirmed the low toxicity of formulations (viability ≥ 95%), their effectiveness in counteracting nitric oxide generation and damages caused by free oxygen radicals, especially when collagen-glytransfersomes were used (viability ~100%). These last vesicles also promoted the regeneration of a wounded area by promoting proliferation and migration of fibroblasts. Conclusion: Collagen-enriched vesicles seemed to be promising formulations capable of speeding-up the healing of the wounded skin.

Keywords: Phospholipid vesicles; oleuropein; collagen; oxidative damage; nitric oxide; wound healing activity.

1. INTRODUCTION

From the past few decades there has been increasing evidence that oxidative stress is strictly connected with several human chronic diseases including acute and chronic inflammation and cancer. Oxidative stress is described as an imbalanced ratio between the formation of oxidant species and the antioxidant defence capacity of the human cells (Masaki, 2010). Oxidant species are free radicals, such as reactive oxygen species (ROS) and nitrogen species (RNS) among other reactive metabolites. To manage these imbalanced conditions, antioxidants from external sources are needed to supplement and support the defence system of our body (Bjelakovic et al., 2014; Omoregie and Osagie, 2010).

The skin is the main barrier between human body and external environment as well as the first defence against damaging chemical and biological agents, and physical insults. Due to its protective role, it also is the principal target of oxidative stress, and needs a constant and effective protection from these harmful agents aiming at remaining in health status, thus safeguarding its barrier effectiveness. Maintaining the skin in healthy condition and reducing the oxidative status of skin, allows to control the lipid and DNA damages along with cell death.

Plants have been considered as a principal source of antioxidant molecules, which can counteract the damaging effects of oxidative species in human tissues. Plant-derived bioactive compounds are usually used in food and enriched-foods for the systemic prevention of oxidative stress as well as in topical formulations for skin protection (Aburjai and Natsheh, 2003; Eteraf-oskouei and Najafi, 2012; Manca et al., 2016b; Sindhi et al., 2013). In addition, plant-derived antioxidants may accelerate the healing of skin lesions, which are usually associated with high level of oxidative species (Manconi et al., 2018a).

One of the plants used the most in traditional medicine, thanks to its antioxidant effect, is the olive. Recently, several experimental, clinical, and epidemiological data confirmed and supported the traditional beliefs connected to the beneficial effect of olive derivatives. The polyphenols contained in olive leaves, olives, virgin olive oil (unrefined) and waste obtained after the olive oil production, have been tested as protective, anti-inflammatory, antimicrobial and anticancer (Bulotta et al., 2014, 2011; Iacono et al., 2010). Among the various molecules contained in the olive plants, oleuropein is the most abundant and capable of neutralizing reactive oxygen species, thus reducing their harmful effects on tissues and cells, thanks to its antioxidant activity exerted through different mechanisms (Bendini et al., 2007).

To increase stability and bioavailability of bioactive molecules applied on the skin, the use of nanocarriers seems to be the most appropriate approach as they can incorporate a wide range of molecules and facilitate their accumulation in the deeper strata of the skin. Liposomes, micelles, nanogels and polymeric nanoparticles are those used the most as topical systems, thanks to their

nanosize and delivery ability (Hira, 2018; Seong et al., 2008). Currently, special attention and efforts are devoted to the development of new, safe, green, scalable and affordable delivery systems capable of transforming traditional preparations or plant-derived bioactives into effective health-promoting products. Among the different nanocarriers, phospholipid vesicles are suitable systems especially for skin delivery, thanks to their structure, water dispersity, amphipathic lamellarity, biocompatibility, and similarity to the skin components (Castangia et al., 2015; Maghraby et al., 2008; Manconi et al., 2018c).

The structure of phospholipid vesicles as a function of its composition has been deeply investigated, as their performances can be affected by the addition of specific additives or polymers (Celia et al., 2011). Indeed, more recently, new kinds of phospholipid vesicles have been proposed, such as transfersomes and glycerosomes among others (Cevc et al., 1998; Manca et al., 2016a). The loading of plant-derived molecules such as curcumin, resveratrol, quercetin, baicalin, naringin, mangiferin, ammonium glycyrrhizate, in these ad hoc formulated phospholipid vesicles seems to address important advantages on the treatment of skin lesions and diseases (Allaw et al., 2020c; Barone et al., 2020; Manconi et al., 2018e; Pleguezuelos-Villa et al., 2020). Despite this, they were not used yet to improve the efficacy of oleuropein in the skin.

In this work for the first time, oleuropein was loaded in collagen-enriched phospholipid vesicles tailored for skin delivery. A surfactant (tween 80), a protein (collagen), considered the major structural element of connective tissues, and a biocompatible water co-solvent (glycerol) have been combined. Tween 80 was used to obtain collagen-transfersomes, glycerol to obtain collagen-glycerosomes and the combination of tween 80 and glycerol to obtain collagen-glytransfersomes. Vesicles have been prepared by direct sonication, avoiding the use of organic solvents, and were deeply characterized from a physicochemical point of view. Their biocompatibility along with their ability to reduce nitric oxide generation and damages caused by free oxygen radicals were evaluated in vitro by using fibroblasts. Moreover, their effectiveness in promoting the healing of a wound performed in a cell monolayer has been evaluated in vitro as well.

2. EXPERIMENTAL SECTION

2.1. Materials

Lipoid S75, a mixture of soy phosphatidylcholine and other lipids was purchased from AVG S.r.l. (Garbagnate Milanese, Milan, Italy), local supplier for Lipoid GmbH (Ludwigshafen, Germany). Oleuropein, collagen, tween 80, glycerol and all the other reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Cell medium, foetal bovine serum, penicillin, streptomycin and all the other reagents for cell studies were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, US).

2.2. Vesicle preparation

Phospholipid (Lipoid S75, 180 mg/ml), oleuropein (20 mg/ml), collagen (2 mg/ml) and tween 80 (7.5 mg/ml), when appropriate, were weighed in the same glass vial and hydrated: with distilled water to obtain collagen-transfersomes; with a mixture of water and glycerol (50:50), without or with tween 80, to obtain collagen-glycerosomes and collagen-glytransfersomes. Liposomes (without tween 80 and glycerol) were prepared as well and used as reference (Table 1). Dispersions were directly sonicated (25 cycles 5 sec ON and 2 sec OFF) with a Soniprep 150 sonicator (MSE Crowley, London, UK) to promote the formation of small vesicles and homogeneous systems (M Manconi et al., 2003). The non-incorporated oleuropein was removed from the dispersions (2 ml) by dialysis in water (2 l) with Spectra/Por® membranes (12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, the Netherlands). The dialysis process has been performed at room temperature for 2 h, refreshing water after 1 h to allow the complete removal of the non-incorporated payload (Maria Manconi et al., 2017b).

Table 1. Composition of vesicles.

Sample	S75 (mg/ml)	Oleuropein (mg/ml)	Tween (mg/ml)	Collagen (mg/ml)	Glycerol (ml)	Water (ml)
Liposomes	180	20	--	--	--	1
Collagen-transfersomes	180	20	7.5	2	--	1
Collagen-glycerosomes	180	20	--	2	0.5	0.5
Collagen-glytransfersomes	180	20	7.5	2	0.5	0.5

2.3. Vesicle characterization

The morphology of vesicles has been detected by Cryo-TEM analyses. Vesicle dispersions were diluted with water and then were putted on a glow-discharged holey carbon grid aiming at forming a

thin film, which was vitrified by plunging into ethane by using a Vitrobot (FEI Company, Eindhoven, the Netherlands). A Tecnai F20 TEM (FEI Company, Hillsboro, Oregon, USA) microscope was used to observe the vitreous film in a low-dose mode at 200 kV and at a temperature around -173°C.

Photon Correlation Spectroscopy method has been used to measure both average diameter and polydispersity index as a measure of the size distribution width, by using a Zetasizer Ultra-ZS (Malvern Instruments, Worcestershire, UK). The Zetasizer Ultra has been also used to measure the surface charge of vesicles (zeta potential) by means of the M3-PALS (Mixed Mode Measurement-Phase Analysis Light Scattering) technique, which measures the electrophoretic mobility of particles in dispersion (Celia et al., 2014). Before each measurements samples were diluted (1:1000 dilution) 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 (Mir-Palomo et al., 2016).

The antioxidant activity of dispersions before and after the dialysis process has been measured to calculate the entrapment efficiency (%) by means of DPPH colorimetric test. Briefly, vesicle dispersions (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 blank as previously reported (Allaw et al., 2020a). All the experiments were performed in triplicate. The antioxidant activity was calculated as follows:

$$\text{Antioxidant Activity \%} = [(ABS_{DPPH} - ABS_{sample}) / ABS_{DPPH}] \times 100.$$

The stability of the vesicles was assessed monitoring mean diameter, polydispersity index, zeta potential and entrapment efficiency over 4 months of storage at room temperature (25±2°C).

2.4. Drug release

The amount of oleuropein released from the vesicles was measured using a dissolution tester equipped with 6 stations (DT 720 Series-Erweka, distributed by EMME 3 SRL, Milan, Italy) according to USP requirements (Rockville, 2008). Oleuropein dispersed in a mixture of water and glycerol (50:50) was used as reference. Oleuropein in dispersion or loaded in the vesicles (0.5 ml) were transferred into polycarbonate dialysis tubes diluted up to 2 ml with water (Spectra/Por membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, the Netherlands). The tubes were placed in the baskets of the dissolution tester containing the release media (1 l) and the samples were left under constant stirring at 37°C for 96 h replacing the release medium with fresh one at different time points (2, 4, 6, 8, 24, 48 and 96 hours). The amount of oleuropein was measured as a function of the antioxidant activity by means of the DPPH colorimetric test. The absorbance was converted into percentage released using a standard curve and experiments were performed in triplicates to ensure accuracy.

2.5. *In vitro* evaluation of biocompatibility and protective effect of oleuropein loaded vesicles against oxidative damages in cells

Flasks (75-cm^2) have been used to allow the growth of mouse embryonic fibroblasts (3T3, ATCC collection, Manassas, Virginia, US). Cells were incubated with 100% humidity and 5% CO_2 at 37°C. Dulbecco's Modified Eagle Medium (DMEM) high glucose, was used as growth medium, which was enriched with 10% foetal bovine serum and 1% penicillin and streptomycin. 3-[4,5-dimethylthiazol-2-yl]-3,5 diphenyl tetrazolium bromide (MTT) colorimetric test has been used to measure the cell viability (Maria Letizia Manca et al., 2014b). Briefly, cells (7.5 x 10^3/well) were seeded into 96-well plates, cultured for 24 hours, and then treated for 48 hours with oleuropein in dispersion or loaded into the vesicles, properly diluted with medium to achieve the final tested concentration (20, 2, 0.2, 0.02 μg/ml). After 48 hours of treatment, the MTT solution (100 □l, 0.5 mg/ml final concentration) was added to each well, 3 hours later was removed and the formed formazan crystals were dissolved with dimethyl sulfoxide (100 □l). The absorbance of the violet solution was read at 570 nm by using a microplate reader (Multiskan EX, Thermo Fisher Scientific Inc., Waltham, MA, US). Results have been reported as percentage of live cells in comparison with untreated control cells (100% cell viability).

To evaluate the protective effect of oleuropein (in dispersion or loaded in vesicles) against oxidative damage, the cells were seeded into 96-well plates, incubated for 24 h, and then exposed simultaneously to hydrogen peroxide (1:50000 dilution) and oleuropein containing samples properly diluted to reach the desired concentration (2 μg/ml of oleuropein). After 4 h, the cells were washed with PBS, and the MTT assay was performed to evaluate the protective effect of oleuropein in dispersion or loaded into the vesicles against damages and death caused by oxidative stress. Untreated cells (100% viability) were used as a positive control, and cells exposed to hydrogen peroxide only, were used as a negative control.

2.6. Inhibition effect of formulations in nitric oxide generation

Keratinocytes (2×10^9 cells/well) were preincubated with 2 μg/ml of oleuropein (in dispersion or loaded in vesicles) for 1 hour, then, lipopolysaccharide (LPS, 1 μg/ml) was added to each well. Cells were then incubated for 20 hours at 37°C and 5% CO_2. After incubation, cell culture medium (100 μl) was withdrawn, transferred into a new 96-well plate, and mixed with Griess reagent solution (100 μl). After 5 min of incubation at 25°C in the dark, the absorbance was measured at 540 nm by using a microplate reader. The diluted sodium nitrite in the culture media ranging from 0 to 100 μM was used as a standard curve to calculate the amount of nitrite in culture medium. The amount of nitrite was calculated as the ratio between the released nitrite versus that released in control cells (treated with lipopolysaccharide only), considered as 100% of nitrite release.

2.7. Scratch wound assay

The scratch assay, which consist in performing a wound in a cell monolayer, has been carried out aiming at evaluating the ability of vesicles to stimulate the proliferation and migration of fibroblasts (Lauder et al., 1998). 6-well plates have been used to culture the cells until they reach a confluent monolayer, which was scratched along the vertical axis of each well using a sterile plastic pipette tip, as reported previously (M. L. Manca et al., 2015b). The cells were treated for 48 hours with vesicle dispersions (containing 2 μg/ml oleuropein). Untreated cells along with cells treated with oleuropein in dispersion were used as negative and positive control, respectively. A light microscope equipped with a 10× objective has been used to monitor speed of cell migration and wound closure.

Images at time zero were captured to record the initial area of the wound, and the recovery of the wounded monolayers was measured at 12, 24, 32 and 48 hours. The captured images were quantified by Java's image J software (http://rsb.info.nih.gov) measuring the area of the wound (Yue et al., 2010). The migration of cells toward the lesion area was expressed as percentage of wound closure:

Wound Closure (%) = $[(a_{t0} – a_{t\Delta}/a_{t0}] \times 100\%$,

where a_{t0} is the wounded area immediately after scratching, and $a_{t\Delta}$ is the wounded area measured at 12, 24, 32 and 48 hours after scratching.

2.8. Statistical analysis of data

Results are expressed as mean ± standard deviation. Analysis of variance (ANOVA) was used to evaluate multiple comparison of means. Student's and Tukey–Kramer t-test were used to substantiate a significant difference between the means of two specific groups using XLStatistics for Windows. The differences were considered statistically significant for $p<0.05$.

3. RESULTS

3.1. Vesicle preparation and characterization

A pre-formulation study was carried out to select homogenous vesicle dispersions, containing stable and small vesicles. Increasing amounts of oleuropein (10, 20, 30, 40 mg/ml) were used in combination with different amounts (from 30 to 240 mg/ml) and kinds of phospholipids (Phospholipon 90G, Phospholipon 90H and Lipoid S75), along with different edge activators (transcutol®, labrasol®, sodium deoxycholate and tween 80) and water co-solvents (ethanol, propylene glycol and glycerol). The energy provided by sonication was varied as a function of the number of cycles (more than 10 and less than 50) and amplitude (from 12 to 15 □). Using Phospholipon 90G, Phospholipon 90H (60, 120 and 180 mg/ml) a precipitate was immediately detectable after sonication irrespective of the used amount of oleuropein. Only when using S75 (180 mg/ml) the dispersions loading 10 and 20 mg/ml of oleuropein were homogenously dispersed at visual inspection and vesicles were sized ~80 nm and the dispersion had a low polydispersity index (0.27). When higher amounts of oleuropein were loaded

a precipitate was observed. The addition of transcutol®, labrasol® or sodium deoxycholate as well as ethanol or propylene glycol led to a significant increase of mean diameter (>250 nm) and polydispersity index (>0.35) of vesicles. The addition of glycerol and tween 80, alone or in association, did not allowed important changes on vesicle features. Finally, the addition of collagen (2 mg/ml) addressed the formation of suitable vesicles: collagen-transfersomes, collagen-glycerosomes and collagen-glytransfersomes (Table 1). Liposomes were prepared as well and used as comparison. The dispersions were directly sonicated to reduce the size of the vesicles and to ensure the formation of homogeneous systems (M Manconi et al., 2003). Empty vesicles, without oleuropein, were also prepared and characterized, to evaluate the effect of the payload on vesicle assembling and features.

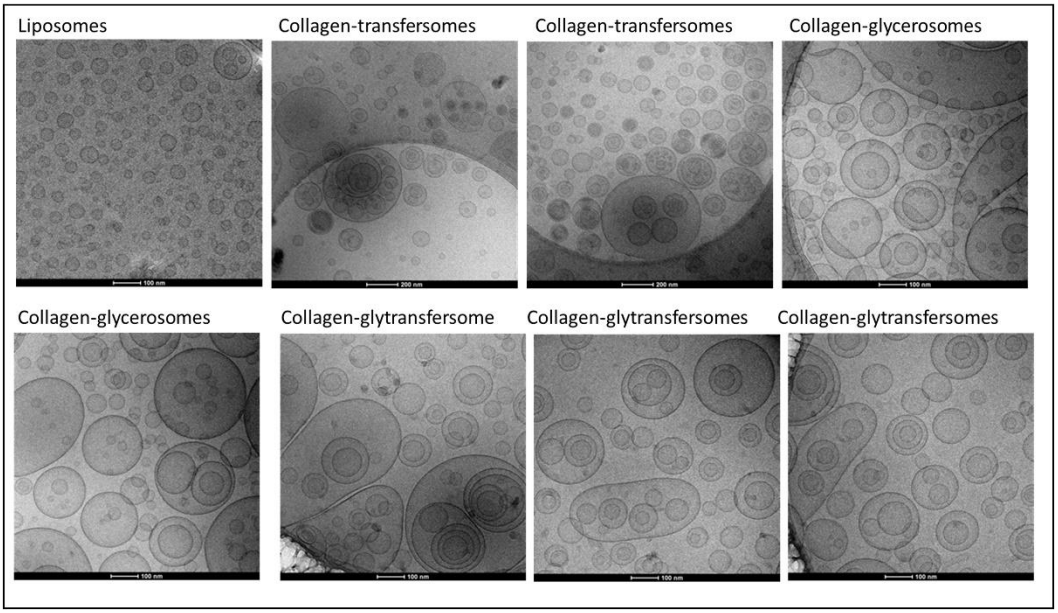

Figure 3. Representative cryo-TEM images of oleuropein loaded liposomes, collagen-transfersomes, collagen-glycerosomes and collagen-glytransfersomes.

Cryo-TEM observation confirmed the actual formation of lamellar vesicles. Liposomes were mostly unilamellar while collagen-vesicles were differently aggregated since a large amount of very small oligolamellar vesicle were observed but simultaneously larger structures were detectable with a peculiar multicompartment organization involving other smaller vesicles inside.

Table 2. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency of empty and oleuropein loaded vesicles. Mean values ± standard deviations are reported (n=6). Each symbol (°, *) indicates the same value.

	MD (nm)	**PI**	**ZP (mV)**	**EE (%)**
Empty liposomes	*79±12	0,27	-77±8	--
Empty collagen-transfersomes	*73±17	0,31	-29±6	--
Empty collagen-glycerosomes	°108±14	0,37	-48±4	--
Empty collagen-glytransfersomes	°100±8	0,29	-36±4	--
Oleuropein liposomes	*83±5	0,27	-42±9	90±2
Oleuropein collagen-transfersomes	°108±5	0,28	-46±4	86±5
Oleuropein collagen-glycersomes	°119±13	0,27	-51±3	94±2
Oleuropein collagen-glytransfersomes	°113±6	0,25	-41±9	92±7

The mean diameter of empty liposomes was around 79 nm and the loading of oleuropein did not allow an increase of vesicle size. Differently, the incorporation of oleuropein caused a slight increase of mean diameter of collagen-transfersomes ($p<0.05$ versus the mean diameter of corresponding empty), while that of collagen-glycerosomes and collagen-glytransfersomes remained unchanged. Oleuropein loaded liposomes were the smallest (around 83 nm) and the addition of collagen along with tween 80 and glycerol individually or in combination, led an increase of mean dimeter (around 108 nm). The polydispersity index value was close to 0.3, irrespective of the formulation, indicating slightly polydispersed samples (Khalil et al., 2012; Phan et al., 2014). The zeta potential was always highly negative, due to the negative group of zwitterionic phosphatidylcholine at this pH (Zhou and Raphael, 2007). All vesicles were able to incorporate high amount of oleuropein as the entrapment efficiency was always higher than 85%, without significant differences among the tested formulations ($p>0.05$; Table 2).

To evaluate the stability of vesicles on storage, dispersions were stored at 25°C in the dark for 4 months and their size, polydispersity index, zeta potential and entrapment efficiency were measured at scheduled times (Figure 2).

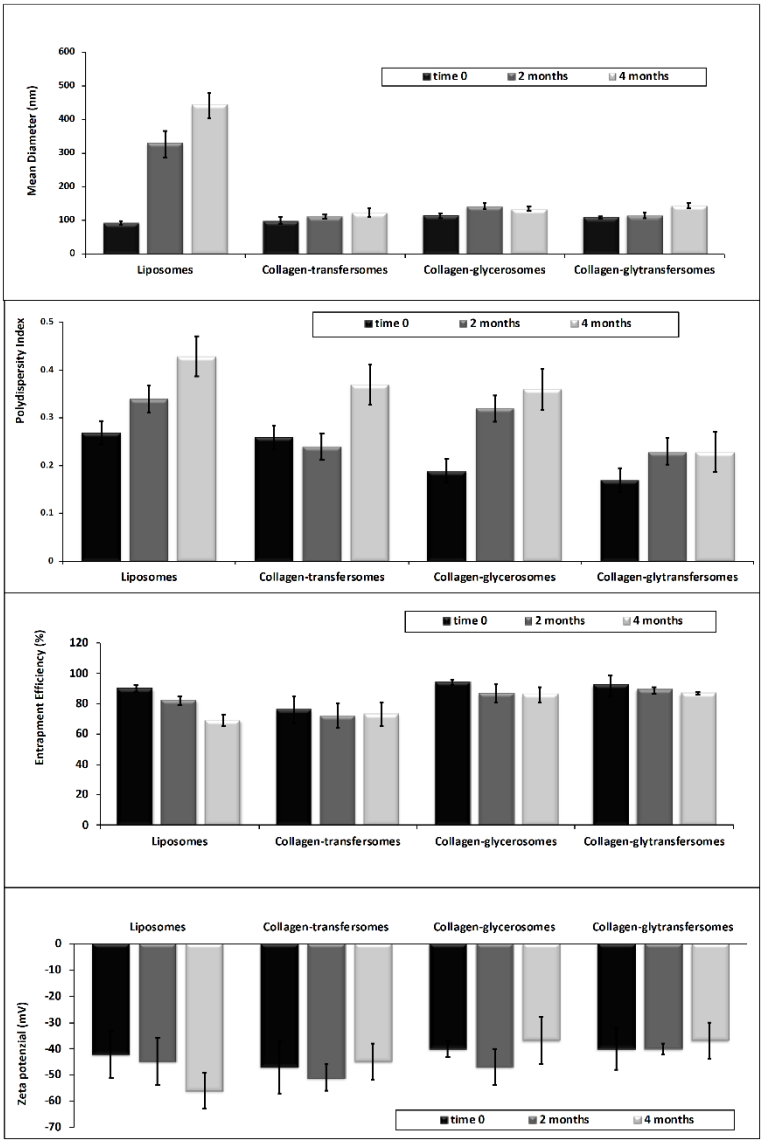

Figure 2. Mean diameter, polydispersity index, entrapment efficiency and zeta potential of vesicles measured during 4 months of storage at 25°C. The mean values ± standard deviations (error bars) are reported (n=6).

The mean diameter of oleuropein loaded liposomes increased up to around 327 nm after two months of storage and further increased up to around 442 nm after four months ($p<0.05$), while the mean diameter of the other vesicles remained almost constant ($p>0.05$) throughout the storage period denoting a positive contribution of collagen, glycerol and surfactant in assembling and stability of vesicle bilayer. Polydispersity index of oleuropein loaded liposomes increased up to around 0.42, that of oleuropein loaded collagen-transfersomes and collagen-glycerosomes slightly increased up to around 0.3 ($p<0.05$, versus the polydispersity index at time zero) while that of oleuropein loaded collagen-glytransfersomes (around 0.25) remained almost constant. In line with the changes of mean diameter, the zeta potential of oleuropein loaded liposomes underwent a modification and became more negative (around -58 mV), confirming a vesicle reassembling. The zeta potential of other

vesicles remained constant and strongly negative (around -44 mV) during the storage, irrespective of the composition of vesicles ($p>0.05$). Closely to zeta potential, the entrapment efficiency did not change either over time, except those of liposomes that undergo a significant reduction of the amount of drug retained (from around 90% to around 69%, $p<0.05$) probably due to the fusion and aggregation phenomena, that caused in turn the increase of mean diameter and the loss of the payload.

3.2. Oleuropein release

The amount of oleuropein released from the different vesicles was measured and compared with that released from its dispersion using a polycarbonate membrane (Figure 1, Supplementary material). The oleuropein release of the dispersion was fast, as around 85% was released at 8 hours and the remaining amount in the following 16 hours. When oleuropein was loaded in vesicles, it was slowly released, and all the tested vesicles provided a similar profile. The oleuropein released was around 55% at 8 hours and reached around 100% only at 96 hours ensuring a controlled and prolonged release over the time.

3.3. Evaluation of the biocompatibility of vesicles

Fibroblasts have been used as they are the most representative cells of the human dermis. They were cultured to allow their growth and then were incubated with oleuropein in dispersion or loaded into the vesicles to evaluate their biocompatibility.

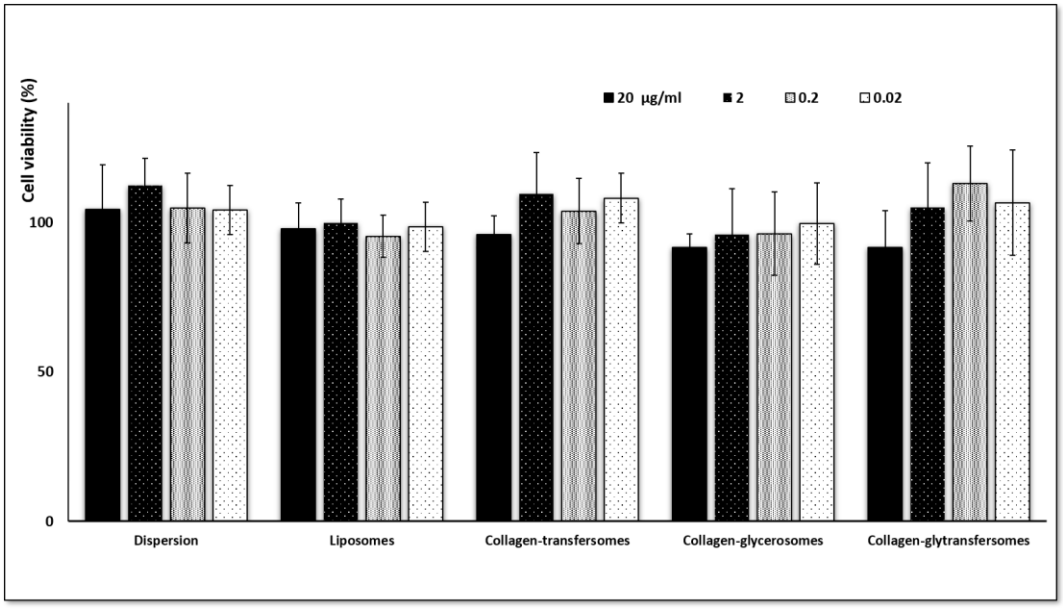

Figure 3. Viability of fibroblasts treated for 48 hours with oleuropein in dispersion or loaded in vesicles. Mean values ± standard deviations (error bars) are reported (n=12).

Using oleuropein in dispersion the viability of fibroblasts was around 100%, at all concentrations tested (20, 2, 0.2, 0.02 .µg/ml), confirming the low toxicity of this natural bioactive. The same trend was observed when oleuropein was loaded in liposomes and collagen-transfersomes (around 95%).

Using collagen-glycerosomes and collagen-glytransfersomes at the higher concentration (20 μg/ml), the viability decreased to around 90% and was higher than 100% when the other concentrations were used. As expected, empty vesicles did not modify the viability (~93%) of cells, as they have been formulated using highly biocompatible ingredients (data not shown).

3.4. Evaluation of the protective effect of the formulations against cell damages caused by oxidative stress

Considering the well-known antioxidant and protective activities of oleuropein, fibroblasts were stressed with hydrogen peroxide (1:50000) and treated with this plant-derived bioactive in dispersion or loaded in vesicles aiming at evaluating its ability to counteract the oxidative damages and prevent cell death, (Figure 4). Formulations were diluted to reach 2 μg/ml of oleuropein, which was a not toxic concentration for fibroblasts after 48 hours of incubation.

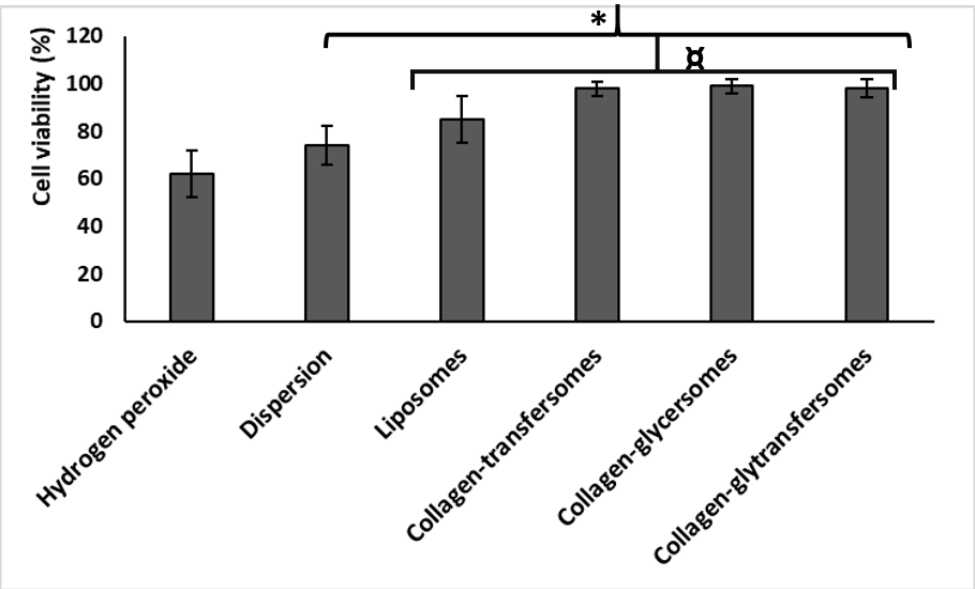

Figure 4. Viability values of cells stressed for 4 hours with hydrogen peroxide and untreated or treated with oleuropein in dispersion or loaded in vesicles. Mean values ± standard deviations (error bars) are reported (n=12). Symbol * indicates values statistically different from those of cells stressed with hydrogen peroxide only. Symbol ¤ indicates values statistically different from those of cells stressed with hydrogen peroxide and treated with oleuropein in dispersion.

After 4 hours of incubation, hydrogen peroxide caused the mortality of around 40% of cells (viability around 60%). The simultaneous treatment of stressed cells with oleuropein in dispersion led to an increase of viability up to around 78% ($p<0.05$ versus the value of stressed and untreated cells). A further increase of the cell viability (around 85%, $p<0.05$ versus the value of cells treated with oleuropein dispersion) was detected protecting the cells with oleuropein loaded liposomes. The

treatment with oleuropein loaded collagen-transfersomes, collagen-glycersomes and collagen-glytransfersomes led the complete restoring of the healthy conditions, reaching around 100% of viability ($p<0.05$ versus the value of vesicles treated with oleuropein loaded liposomes). These results underlined the efficacy of oleuropein against damages caused by oxidative stress and the improved effectiveness of oleuropein when incorporated into collagen-enriched vesicles.

3.5. Inhibition of nitric oxide generation in cells

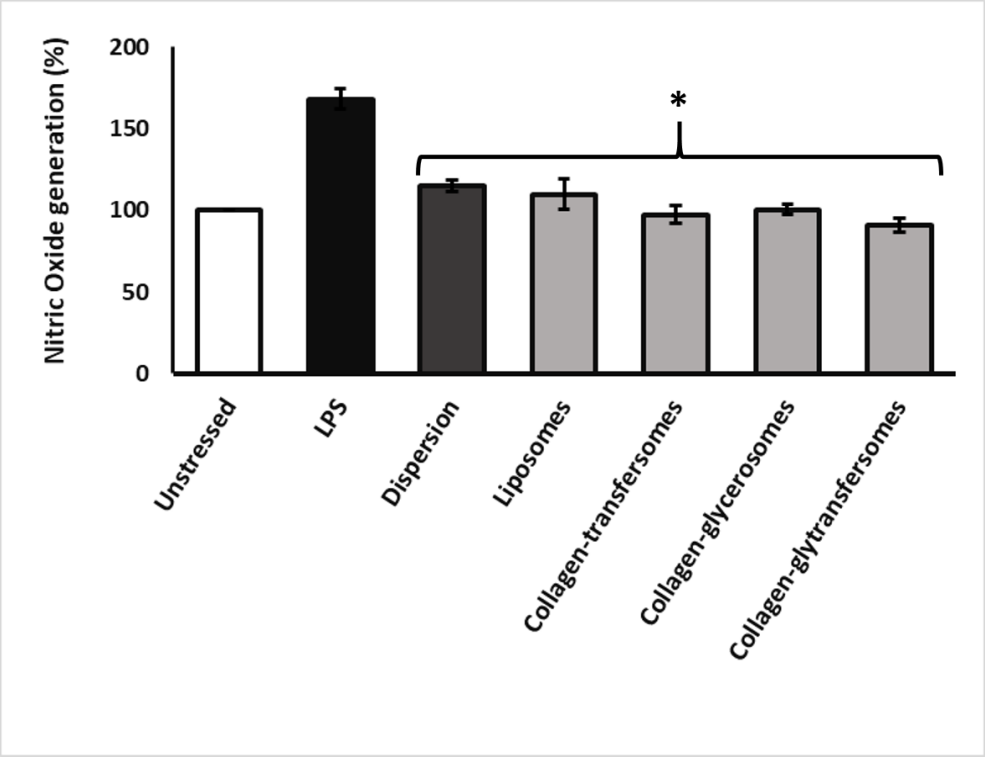

Figure 5. Expression of nitric oxide induced in cells with lipopolysaccharide and modulate by pre-treatment with oleuropein in dispersion or loaded in vesicles. Mean values ± standard deviations (error bars) are reported (n=10). Symbol * indicates values statistically different from that of cells stressed with lipopolysaccharide only.

The nitric oxide production in cells was stimulated with lipopolysaccharide (LPS) and quantified using the Griess reactive (Figure 5). The lipopolysaccharide stimulated the production of nitric oxide in cells up to around 180%. The pre-treatment with oleuropein in dispersion or loaded in liposomes inhibited the nitric oxide release induced by the inflammatory stimulus (around 110%). Interestingly, an additional reduction of nitric oxide release was detected following pre-incubation with oleuropein loaded collagen-vesicles (around 95%) confirming the superior ability of these formulations to promote the protective effect of payload against oxidation and inflammation.

3.6. Scratch wound assay

The ability of the formulations to promote fibroblast proliferation and migration was evaluated by producing a linear wound on a cell monolayer and measuring its closure during 48 hours of

experiment (Figure 6A).

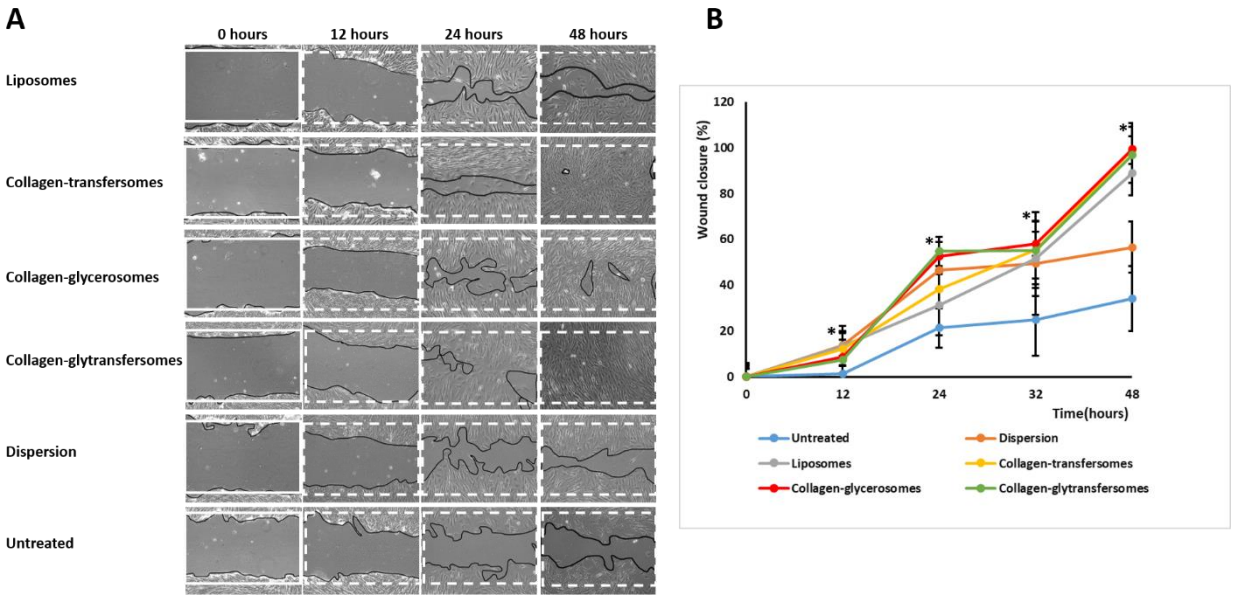

Figure 6. A Representative photomicrographs of wounds performed on cell monolayers untreated or treated with oleuropein in dispersion or loaded in vesicles. Images were taken immediately (0 hour) and 12, 24 and 48 hours after scratching. **B** Graph of wound closure (%) of cell monolayers untreated or treated with oleuropein in dispersion or loaded in vesicles, during 48 hours. Mean values ± standard deviations (error bars) are reported (n=6). Symbol * indicates values statistically different to that of cells treated with oleuropein in dispersion, and to that of untreated cells.

The lesion of untreated cells is representative of their proliferation and migration under physiological conditions, and it did not reach the complete closure at 48 hours (around 40%, Figure 6A and B). The treatment with oleuropein in dispersion promoted in a better extend the closure of the wound at 48 hours achieving around 58%. A further improvement of the wound closure was detected by treating it with oleuropein loaded in vesicles. In particular, using liposomes, the percentage of closure was around 40% at 32 hours but was not completed at 48 hours, as the closure was around 85%, while using collagen-enriched vesicles, the closure of the wounded area reached around 70% at 32 hours and was almost completed at 48 hours (~96%).

Discussion

The findings obtained in this study have confirmed the ability of phospholipid vesicles to stably load oleuropein in high amount, especially when transfersomes and glycerosomes were used and enriched with collagen. Indeed, the collagen was added to support and promote the efficacy of oleuropein in healing the skin lesions, as it makes the skin more elastic and resistant, and stimulates proliferation and motility of fibroblasts (Willershausen et al., 2014). Moreover, collagen is a hygroscopic material, which avoid the loss of water at the epidermis level, that normally occurs in injured skin (Grandoch

et al., 2011; Kim et al., 2018). According to previous studies, tween 80 and glycerol are expected to ameliorate the delivery of oleuropein in the deeper strata of the skin (i.e. dermis), because similarly to monoglyceride and fatty acid, they are capable of perturbing the ordered structure of the skin thus leading the formation of alternative pathway for the bioactive and vesicles, which in turn can reach the dermis (Akinshina et al., 2016; Manca et al., 2016a). In association with phospholipids, tween 80 acts as an edge activator capable of improving the elasticity and deformability of the bilayer (Ahad et al., 2018). Glycerol, added to the dispersing medium, led alterations in phospholipid lamellar bilayer favoring the delivery effectiveness of vesicles, especially in the skin, due to its moisturizing and hydrating properties (Maria Letizia Manca et al., 2014a). As a confirmation of the interaction of both glycerol and tween 80 with the bilayer, liposomes were unilamellar while transfersomes, glycerosomes and glytransfersomes were almost small oligolamellar and large multicompartment vesicles containing smaller ones inside. In a previous study, the multicompartment vesicles have shown optimal carrier performances as they have provided a greater deposition of curcumin in the intestine membrane (Catalan-latorre et al., 2016). This peculiar structure seems to improve the vesicle stability on storage as the size of collagen enriched transfersomes, glycerosomes and glytransfersomes remained constant while that of liposomes increased exponentially over time, as already reported by Kisak et al. (Kisak et al., 2005). In addition, the multicompartment structure of collagen-enriched vesicles strengthened the biological efficacy of oleuropein, as these vesicles were more effective in comparison with dispersion and liposomes in inhibiting hydrogen peroxide damages, nitric oxide production and in promoting the wound healing. Results are in agreement with previous studies performed using ufasomes to deliver oleuropein in the skin (Agrati et al., 2011; Cristiano et al., 2021). The better efficacy of collagen-enriched vesicles can be related to the combination of different positive factors: the antioxidant and anti-inflammatory activity of oleuropein; the carrier performances of transfersomes, glycerosomes and glytransfersomes and the adjuvant efficacy of collagen. Indeed, oleuropein is a natural occurring polyphenol extracted from the olives or the leaves of the olive tree or even from its by-product obtained after the production of olive oil (López-méndez, 2020). In previous studies, it has been tested as bioactive capable of maintaining the body homeostasis, retard aging, mitigate diabetes and its complications and prevent chronic diseases (Aponte et al., 2018; Chondrogianni et al., 2010; Martorell et al., 2016; Zheng et al., 2021). It has been already loaded in liposomes, but in this form was administered orally only as nutraceutical product (Bonechi et al., 2019). Few studies reported its beneficial effect in the skin and in many cases it was used in form of phytocomplexes (e.i. olive oil or extract) (Badiu and Rajendram, 2021; Wanitphakdeedecha et al., 2020). The few studies provided on skin application of oleuropein can be related to its lipophilic nature, which strongly reduced the bioavailability and efficacy after

topical application. The advantages provided by its loading in phospholipid vesicles designed for skin application was not previously explored and its efficacy as raw material or in formulations has not been confirmed yet (Bonechi et al., 2019). Thanks to its beneficial properties and antioxidant power, oleuropein can protect the skin counteracting the overproduction of reactive oxygen species and nitric oxide radicals (Cristiano et al., 2021; Huguet-casquero et al., 2020). Indeed, overproduction and accumulation of reactive oxygen species in the cutaneous tissues can stimulate the release of proinflammatory mediators (i.e. cytokines) thus accelerating cellular senescence and inducing or worsening some inflammatory conditions, which in turn slow-down the healing of cutaneous lesions and facilitate the onset of cutaneous chronic diseases like psoriasis, dermatitis and cancers (Bickers and Athar, 2006). Likewise, an excessive production of nitric oxide, a short-lived radical involved in many biological and pathological functions, drives and stimulates the onset of inflammatory conditions (Aldridge et al., 2008). In addition, previous studies disclosed that nitric oxide radicals can combine under physiological conditions with superoxide species (generated by oxidative stress) to produce a toxic-derived product (peroxynitrite anion), which is considered the most endogenous harmful oxidant, capable of attacking a wide range of biological molecules (Cuzzocrea, 2006). The effectiveness of oleuropein in dispersion in counteracting the production of the reactive oxygen species and nitric oxide radicals has been proved in this work, and its incorporation into collagen-enriched phospholipid vesicles significantly potentiated its beneficial properties. In vitro studies performed using fibroblasts confirmed the ability of oleuropein loaded vesicles to quickly avoid the accumulation of the above-mentioned damaging species suggesting their effectiveness in the skin tissues as well, thus controlling oxidative stress, inflammation and promoting the healing process of skin lesions. Indeed, oleuropein loaded collagen-enriched vesicles accelerated the closure of the lesions performed in a fibroblast monolayer in a better extent than the corresponding liposomes used as reference. The most promising performances of these vesicles may be related to the well-known effect of collagen to promote and speed-up the wound healing (Wang et al., 2020). In particular, collagen-glytransfersomes obtained combining tween 80, as edge activator, glycerol, as stabilizer, and collagen, as healing agent, seemed to be the most promising formulation as they showed the most favourable physicochemical and technological features and high stability along with the best biological activities in vitro.

Conclusion

In this study, the wound healing efficacy of oleuropein loaded in multicompartment phospholipid vesicles enriched with collagen was demonstrated. Indeed, the production of nitric oxide was reduced along with the damages induced by reactive oxygen species, especially when cells were treated with collagen-enriched vesicles. The overall results confirmed that the loading of oleuropein in

phospholipid vesicles enriched with collagen can be considered a suitable strategy for the treatment and/or prevention of skin lesions.

Summary points

- Collagen-enriched vesicles have been specifically tailored for the cutaneous delivery of oleuropein.
- Transfersomes, glycerosomes and glytransfersomes enriched with collagen have been prepared and compared with liposomes, used as reference.
- Collagen-enriched vesicles were small in size, negatively charged and capable of incorporating high amount of oleuropein.
- All vesicles were highly stable on storage as their main physico-chemical properties did not undergo significant variation during 4 months of storage.
- Oleuropein in dispersion or loaded into vesicles was highly biocompatible as confirmed by the high viability of fibroblast, treated for 48h with these formulations.
- Collagen enriched vesicles were capable of counteracting the damages induced by treating fibroblasts with hydrogen peroxide.
- The nitric oxide generation was effectively avoided or slowed-down by treating cells with collagen-enriched vesicles
- Collagen-enriched vesicles, and especially collagen glytransfersomes, were capable of speeding-up the healing of the wounded fibroblast monolayer.

Acknowledgements

This research was supported by the Italian Ministry of Education, University and Scientific Research (MIUR), Grant No. PON a.a. 2017/2018 – cod= DOT1304004.

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Efficacy of topical liposomes loading lentisk oil in the management of wound healing promoted by antioxidant activity and stimulation of proliferation and migration of skin cells

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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. However, many beneficial effects have been associated with the use of fixed oil, which have not deeply investigated yet.

In this study, the lentisk oil has been incorporated into liposomes prepared with natural soy lecithin, using 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 amount of lentisk oil incorporated into the vesicles modulated the accumulation of the bioactives in the whole skin, along with their ability to counteract the damaging effects of hydrogen peroxide in both keratinocytes and fibroblasts and their proliferation and migration, leading an *in vitro* speeding-up of the wound healing.

Overall findings suggested that the incorporation of lentisk oil in liposomes made from soy lecithin can be an alternative and natural approach suitable for the treatment of skin wounds specifically connected with oxidative stress.

Key words: *Pistacia lentiscus*; liposomes; keratinocytes & fibroblasts; wound healing; scratch assay; skin permeation studies, confocal microscopy

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. These traditionally uses and its nutritional properties have been confirmed by both *in vitro* and *in vivo* studies [4–6]. 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 [7,8]. 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 [9].

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 [10].

For the first time in this study, the *P. lentiscus* fixed oil has been loaded into phospholipid nanovesicles, 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) [11,12].

Given that, in the present study, liposomes made with soy lecithin were formulated to incorporate the lentisk oil 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 along with their biological features, such as biocompatibility, protection against oxidative damages and *in vitro* promotion of wound healing in keratinocyte and fibroblast monolayers.

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

Table 1. Composition of lentisk oil loaded liposomes.

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

Samples (1 ml) were freed from the non-incorporated bioactives contained in the oil by dialysing them against water (2.5 liters). Dialysis tubing (Spectra/Por® membranes, 12-14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands) were used and the process was performed at room temperature for 4 hours, replacing the water every hour. The used water (10 litres total) was able to theoretically remove all the bioactives contained in 1 ml of liposome dispersions.

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 [13].

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). Samples were diluted with water (1:100 v/v) before the analyses 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 [14]

Entrapment efficiency, expressed as the percentage of the amount of bioactives initially used versus that found in dialyzed samples, was determined as 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 (n=6 specimens for each formulation), stored at –80°C, was pre-equilibrated in saline at 25°C for 12 hours and then sandwiched between donor and receptor compartments of the 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 □l) on the skin surface. After 4 and 8 hours 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, supplemented with foetal bovine serum, penicillin and streptomycin as culture medium.

The biocompatibility of samples has been evaluated by seeding the cells (keratinocytes and fibroblasts) into 96-well plates and, after 24 hours, treating them for 48 hours with 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).

The protective effect of formulations was evaluated by stressing the cells with hydrogen peroxide (30% diluted 1:30.000 dilution *v/v* with PBS) for 4 hours and simultaneously treating them with the lentisk oil in dispersion or loaded into liposomes, properly 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, cells were washed 3 times with fresh medium and their viability was determined by the MTT [3(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] colorimetric assay, by adding 100 μl of MTT reagent (0.5 mg/ml in PBS, final concentration) to each well. After 2-3 hours, the formed formazan crystals were dissolved in DMSO and their concentration was spectrophotometrically quantified at 570 nm with a microplate reader (Synergy 4, Reader BioTek Instruments, AHSI S.P.A, Bernareggio, Italy). All experiments were repeated at least three times. Results are shown as percent of cell viability in comparison with 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 the 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 hours. 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 hours (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 [15]. 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

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, combined with various amounts of other substances such as triglycerides, fatty acids, and carbohydrates [16]. Soy lecithin is separated from the crude vegetable oil source by using non-toxic solvent and chromatographic procedures with low consumption of energy [17]. The use of lecithin to incorporate lentisk oil allows the production of natural nanotechnological liposomes with promising beneficial activities especially at skin level and by using a simple and easy scalable preparation method. The maximum amount of oil incorporable into the vesicles has been evaluated by means of a pre-formulation study in which increasing amount of oil, starting from 10 mg/ml up to 50 mg/ml, were tested. Unfortunately, the use of high amount of oil (*i.e.* 40 and 50 mg/ml) led the formation of large vesicles (>300 nm), highly polydispersed (polydispersity index >0.5) and instable, as two different phases were detectable in few days after preparation. Given that, 10, 20 and 30 mg/ml of lentisk oil were selected as suitable concentrations to be incorporated into stable and small vesicles, specifically formulated for the treatment of skin lesions. Cryo-TEM images disclosed the formation of small vesicles, homogeneously dispersed and with a spherical and uniform shape (Figure 1).

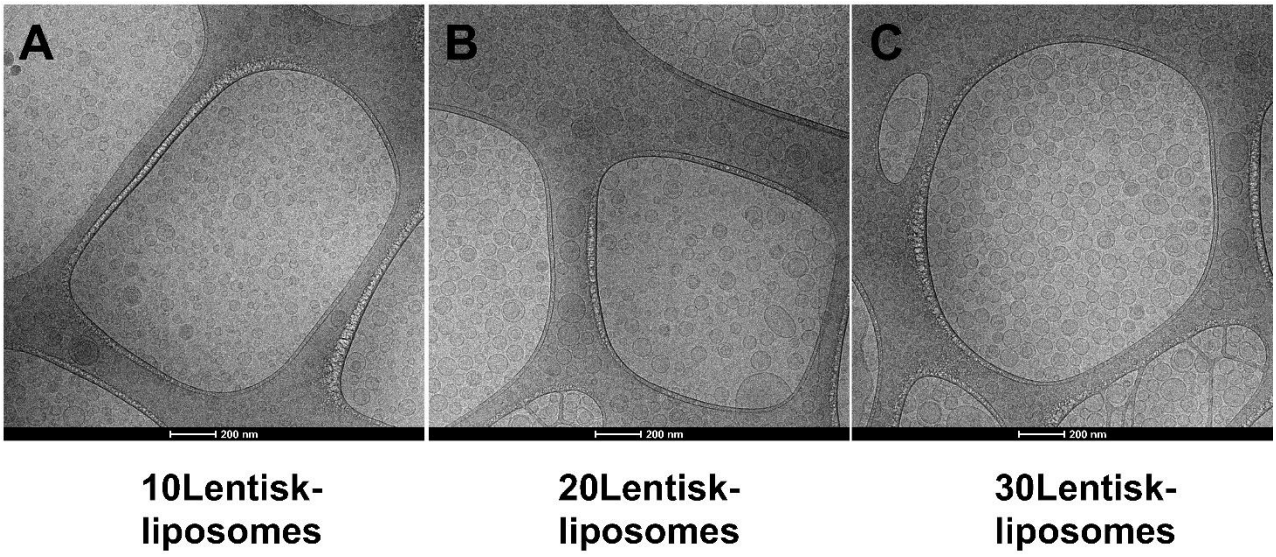

Figure 1. Cryo-TEM images of 10lentisk-liposomes (A), 20lentisk-liposomes (B) and 30lentisk-liposomes (C).

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

Table 2. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency of empty and lentisk oil loaded vesicles. Mean values ± standard deviations are reported (n=6).

	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

In particular, 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 ($p<0.05$ versus other values), 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, $p>0.05$ versus the value of empty liposomes and $p<0.05$ versus other values). 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 [18]. All tested liposomes were able to incorporate 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 (Figure 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 (Figure 2).

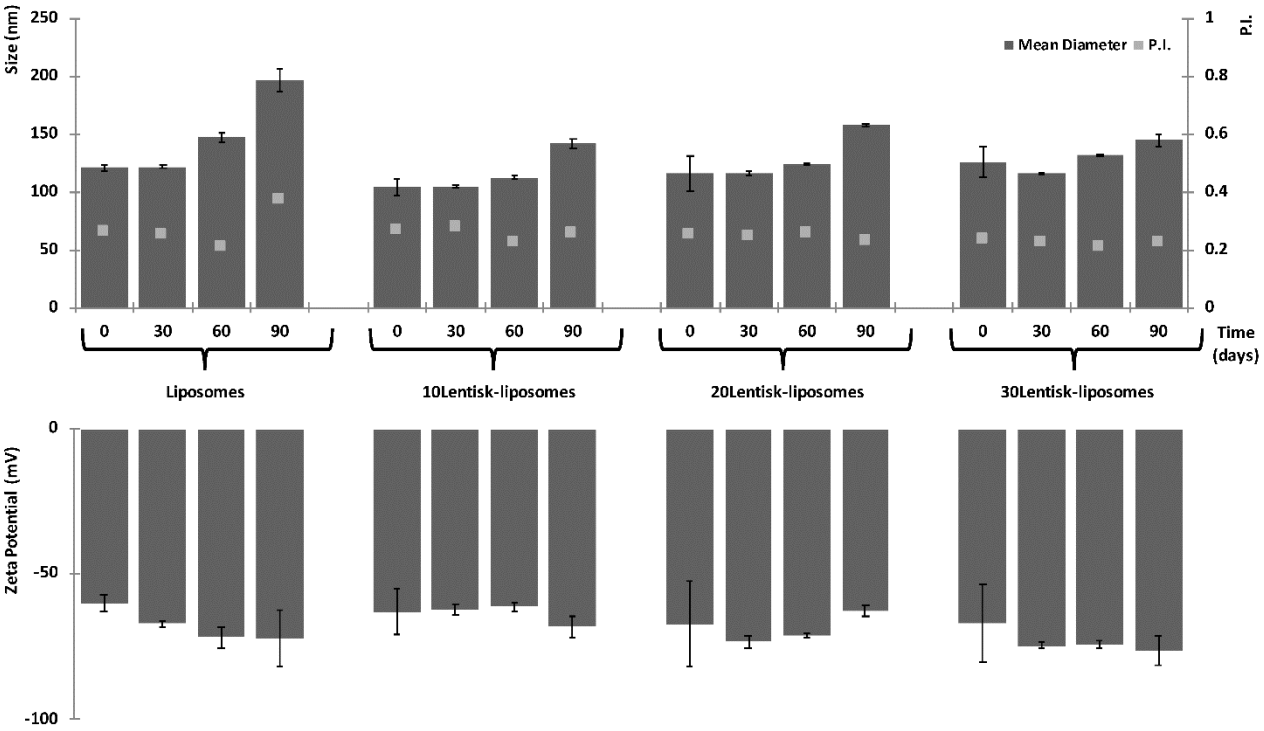

Figure 2. Mean diameter, polydispersity index and zeta potential of vesicles measured during 90 days of storage at 25°C. The mean values ± standard deviations (error bars) are reported (n=6).

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 permitted to visualise the vesicle distribution in the different strata of the skin (Figure 3).

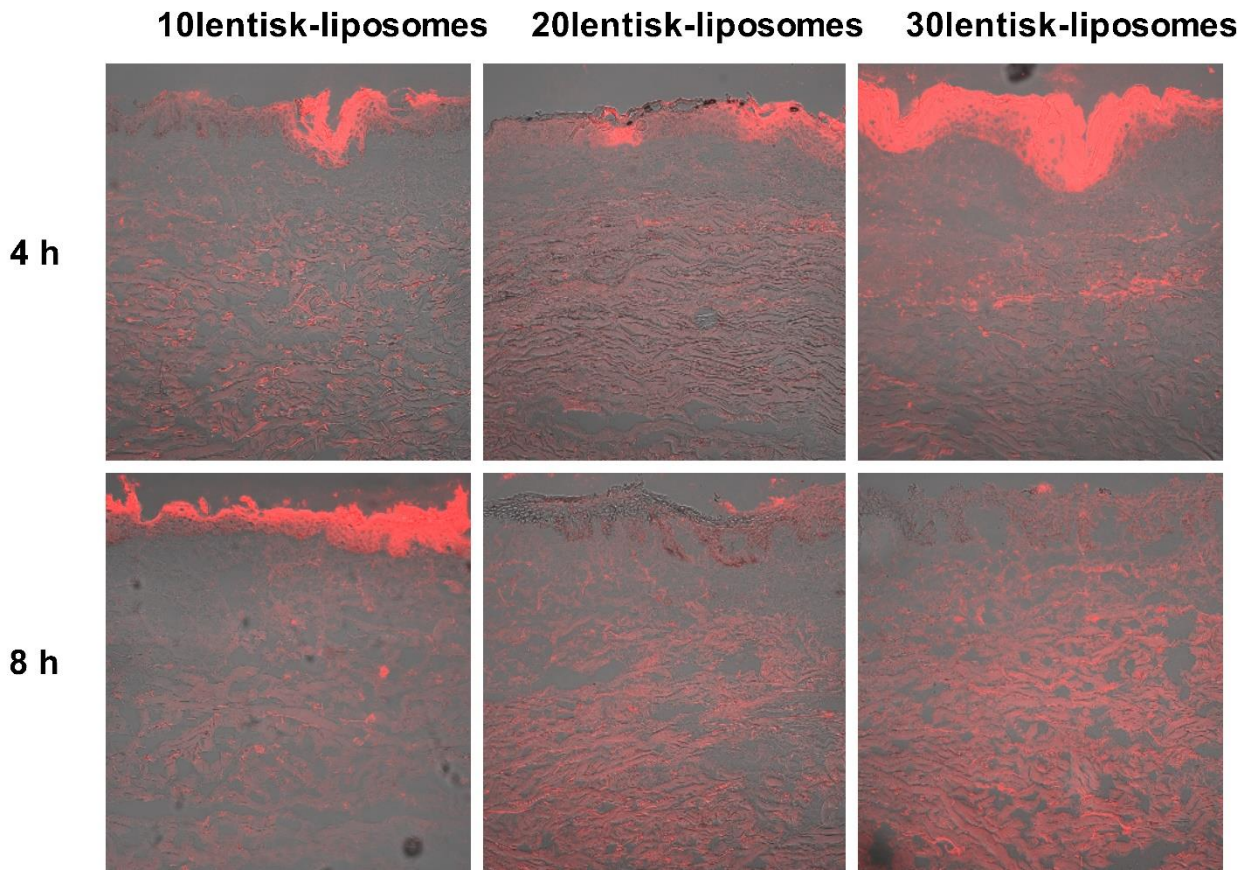

Figure 3. Confocal laser scanning microscopy images of phospho-rhodamine (red) distribution in a skin section (*z* axis) after 4 and 8 hours of treatment with lentisk oil loaded liposomes.

The vesicles pathway was strongly affected by the amount of lentisk oil incorporated. Indeed, 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 hours). The use of higher amount of oil (20 and 30 mg/ml) led to a dipper distribution of the vesicles, especially after 8 hours of treatment. At 4 hours, the red fluorescence was mainly evident on the skin surface (especially for 30lentisk-liposomes) and in the deeper strata was less intense. At 8 hours 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 (Figure 4). Using the oil loaded in liposomes, the viability of keratinocytes was always ≥95% irrespective of the payload concentration and sample dilutions while was slightly

lower (~77%) by using the lentisk oil in dispersion at the lower dilutions, corresponding to 30 and 60 □g/ml of oil. The viability of fibroblasts was always ≥100%, irrespective of both the used sample and the dilution, in addition, cell proliferation (viability ≥120%) was observed by using the vesicles loading 10 and 20 mg/ml of oil, indicating a positive effect of lentisk oil at these concentrations.

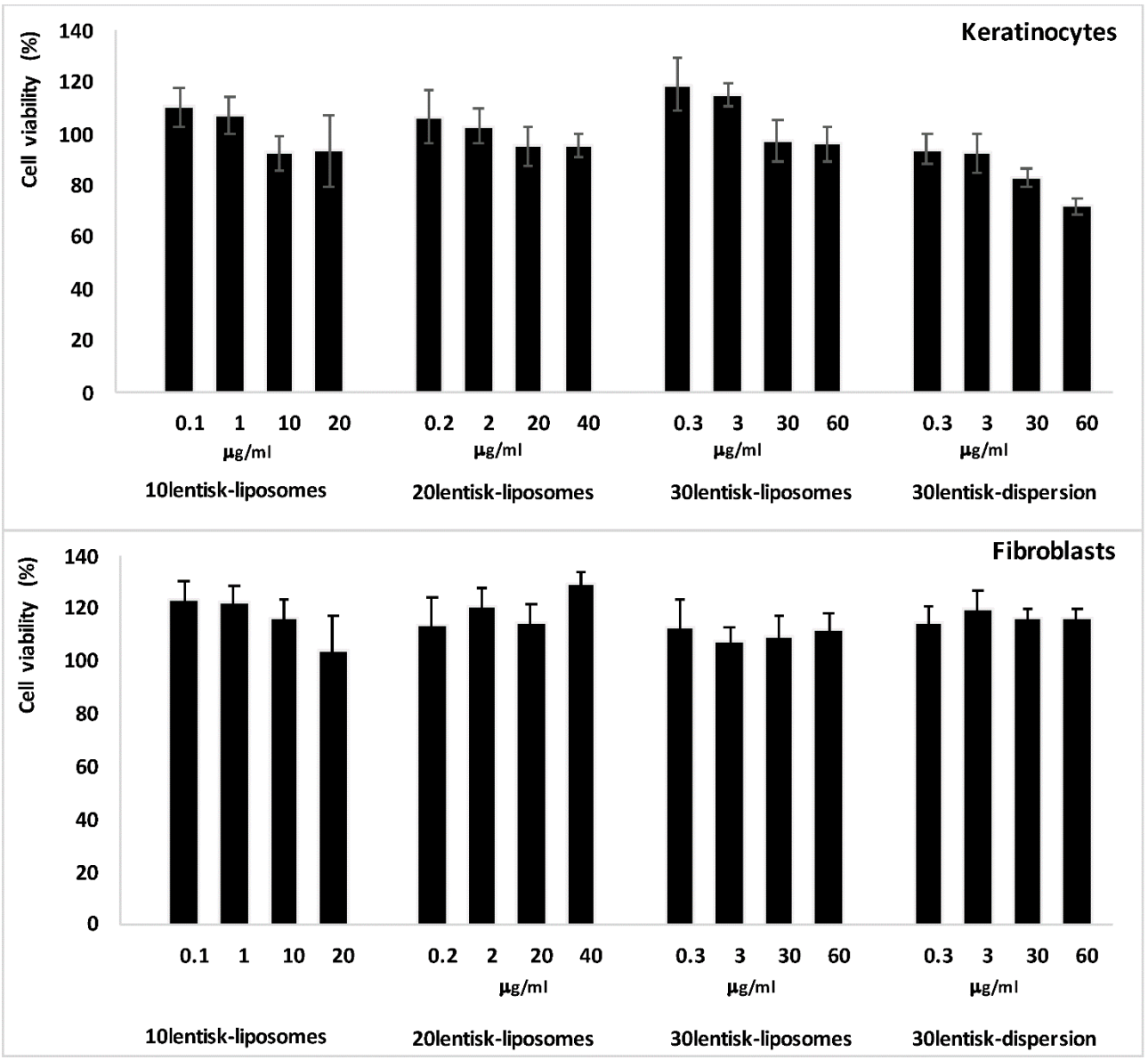

Figure 4. Cell viability of human keratinocytes and fibroblasts treated for 48 hours with different concentrations of lentisk oil. Data are reported as mean values ± standard deviation of cell viability expressed as the percentage of untreated cells (100% of viability).

The ability of formulations to counteract the toxic effect of hydrogen peroxide in cells were evaluated as well (Figure 5). The treatment of cells with hydrogen peroxide led to a significant reduction of keratinocytes and fibroblasts viability, which reached ~58%. The treatment of stressed cells with lentisk oil in dispersion led to an increase of cell viability up to ~77%, while its incorporation into vesicles provided a good protection, which was similar in both keratinocytes and fibroblasts:

irrespective of the used concentration of oil the cell viability was ~106%, suggesting the ability of the tested nanovesicles to totally inhibit the toxic effect provided by the treatment with hydrogen peroxide.

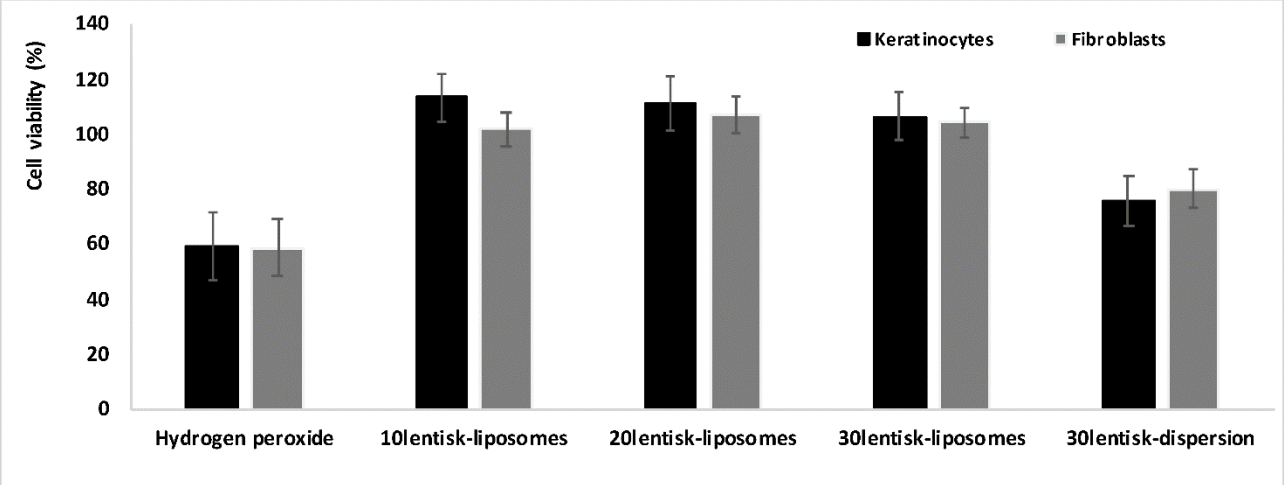

Figure 5. Protective effect of lentisk oil in dispersion or loaded in liposomes against hydrogen peroxide-induced oxidative stress in keratinocytes and fibroblasts. Data are reported as mean values ± standard deviation of cell viability expressed as the percentage of untreated cells (100% of viability).

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 loaded in liposomes of promoting the wound closure. The thickness of lesions was measured at different time points (Figure 6). At 24 hours, the percent closure of untreated cells was ~20% and reached ~40% at 48 hours. When the cells were treated with oil in dispersion or loaded into 10lentisk-liposomes, the behaviour was slightly better: ~20% at 24 hours and ~60% at 48 hours. A significant reduction 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 hours and almost complete (~100% closure), at 48 hours, disclosing an optimal potential of these formulations in accelerating the healing of a wound performed in a cell monolayer.

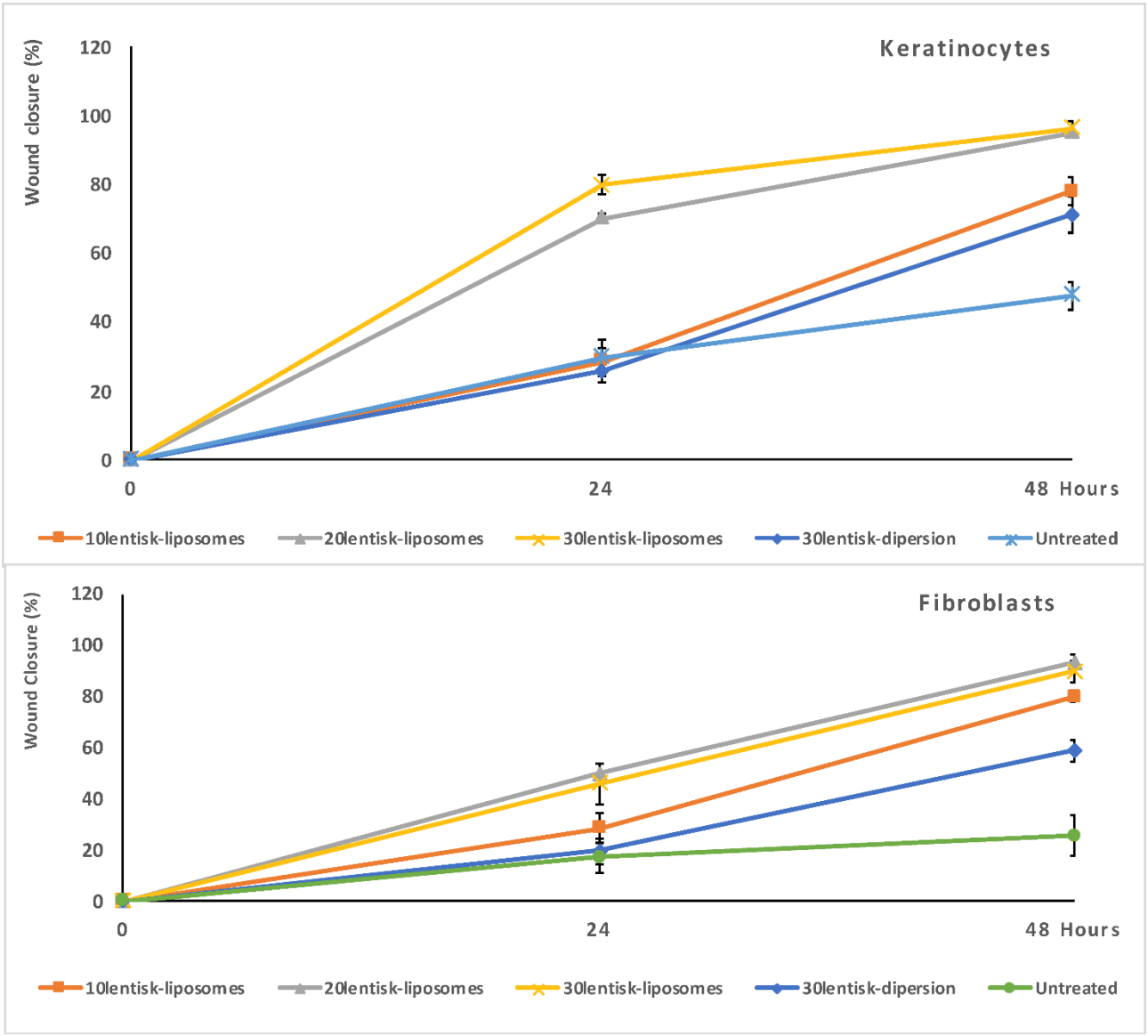

Figure 6. Closure of a wound performed in a monolayer of keratinocytes and fibroblasts untreated or treated with lentisk oil in dispersion or loaded in vesicles, as a function of the time. Mean values ± standard deviations (error bars) are reported (n=6).

4. Discussion

Pistacia lentiscus is a resilient plant capable of growing under hard climatic conditions characterized by nutrient and water scarcity and long-term exposures to extensive solar radiation and high temperatures [19]. To resist to these conditions, it produces several secondary metabolites, which can positively affect their curative and beneficial effects [20]. 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 [21]. Moreover, several phenolic compounds are contained in the oil and they are responsible for oil stability and antioxidant properties [22]. Thanks to this content, lentisk oil, like to other natural oils, has a positive impact on wound-healing process promoting cell proliferation, collagen synthesis, dermal reconstruction, and repair of

lipid barrier function [23]. In addition, in this study, it was confirmed that the suitable loading of lentisk oil into liposomes can improve its topical efficacy in the treatment of skin lesions, due to a synergic effect on the improvement of skin lipid barrier, reduction of oxidative stress and inflammation, and promotion of proliferation and migration of keratinocytes and fibroblasts [23,24]. It was reported that, oils and butters can mostly express emollient action and avoid transdermal water loss from the injured skin, substantiating tissue hydration and promoting/restoring its barrier function [25]. This important effect was improved by the incorporation of oil into liposomes, probably because the phospholipids, as the main components of cell membranes, are potent regulators of epithelial barrier function [26]. The obtained formulations were totally natural derived, being both lentisk oil and soy phosphatidylcholine obtained from plants [27].

Initially, liposomes were prepared by using increasing amounts of lentisk oil, from 10 to 50 mg/ml, but 40 and 50 mg/ml led the formation of large vesicles, highly polydispersed and instable, while those loading 10, 20 and 30 mg/ml of lentisk oil were small in size, homogeneously dispersed and highly negatively charged. They 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 deposition of vesicles in the dipper skin strata, indeed liposomes loading the higher amount of lentisk oil (30 mg/ml) accumulated especially in the dermis, which is the skin strata mainly involved in the regeneration during the wound healing process. In addition, lentisk oil loaded vesicles counteracted the toxic effect of hydrogen peroxide guarantying the restoring of the healthy condition, thanks to its high content of polyphenols, which by scavenging the free radicals avoided cell damages and death [23].

Overall finding suggested the high potential of lentisk oil loaded liposomes for 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 substances led the formation of a natural nanotechnological formulation, ideal for the treatment of skin wound. 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 guaranties 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.

Acknowledgements

The authors also 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.

Conflict of interest statement

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 access statement

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

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La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
Fondo Sociale Europeo, Azione I.1 "Dottorati Innovativi con caratterizzazione Industriale"

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