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Control of skin damages caused by oxidative stress using mangiferin and naringin co-loaded in phospholipid vesicles

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ABSTRACT

Mangiferin and naringin, two naturally occurring antioxidant molecules, were co-loaded in phospholipid vesicles designed for skin delivery. Ultradeformable-liposomes containing tween 80 as edge activator, were used as basic formulation, which was modified adding glycerol (glycerosomes) or a mixture of glycerol and ethanol (et-glycerosomes) and further enriched with a polymer, sodium hyaluronate (glycerohyalurosomes and et-glycerohyalurosomes), to evaluate the role of vesicle composition on their features and performances. Mean dimeter, polydispersity index and zeta potential of prepared vesicles were measured along with their stabilitcay on storage for 90 days, rheological behavior and suitability as systems for the delivery of these active molecules into and through the skin. Vesicles enriched with sodium hyaluronate were the most stable and the smallest and favored the deposition of both mangiferin and naringin in the whole skin, in a better extent than those without polymer. All the vesicles were highly biocompatible and capable of protecting fibroblasts against hydrogen peroxide-induced oxidative damages *in vitro*. Once more, glycerohyalurosomes and et-glycerohyalurosomes where those which improved the most the beneficial effect of mangiferin and naringin, as they were capable of effectively counteracting the formation of skin lesion, or even promoting the wound healing, thanks to their greater ability to inhibit both myeloperoxydase activity and oedema formation *in vivo* in a model mouse in which wound was induced using phorbol acetate.

Statement of significance

In this study, biocompatible nanovesicles co-loaded with nutraceuticals (natural antioxidants) has been considered as a promising alternative therapy in preventing or counteracting pathological conditions linked to skin diseases, such as psoriasis or atopic dermatitis. Compared with the traditional ultradeformable-liposomes, glycerohyalurosomes and et-glycerohyalurosomes showed an improved ability to favour the deposition of both mangiferin and naringin in the whole skin, in counteracting inflammation, myeloperoxydase activity, and especially oedema formation. These findings highlight that the association of these beneficial molecules in the mentioned nanoformulations can be a reinforcement for the traditional treatment of damages induced in the skin by oxidative stress and contribute to the maintenance of the human health.

1. Introduction

Oxidative stress is mainly connected with an excessive accumulation of reactive oxygen species (ROS), such as free radicals, molecules or molecular fragments characterized by one or more unpaired electrons [1]. They are normally produced by the cellular metabolism and

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contribute to regulate normal cellular processes such as intracellular signal transduction, metabolism, proliferation, and apoptosis and if their amount exceed the normal one, it is generally compensated by the endogenous antioxidant systems [2]. However, when external or endogenous stress addresses their overproduction, a deregulation and accumulation occur, which in turn mediates a substantial number of uncontrolled biological responses like DNA modification, lipid peroxidation, and production of inflammatory cytokines. These chronic conditions contribute to the pathogenesis and progression of many important inflammatory skin disorders [3]. Indeed, uncontrolled inflammation of skin may be linked to the development and progression of multiple diseases and unhealthy conditions such as visible skin aging, fibrotic skin, psoriasis, dermatitis, lupus and diabetic foot ulcers [4]. These finding corroborate the importance of controlling the oxidative stress and inflammation that may occur in the skin, not only to avoid early aging but especially to prevent and/or at least control more serious diseases. Among skin diseases associated/connected with oxidative stress, psoriasis and atopic dermatitis are the most common chronic and recurrent inflammatory disorders [5,6]. Their hallmark is the sustained inflammation that leads to: i) infiltration of inflammatory cells, ii) intensified vascularization, iii) uncontrolled keratinocyte proliferation and iiii) dysfunctional differentiation [7,8]. The most common therapeutic protocols, used to control these skin diseases, are mainly based on the use of topical corticosteroids, which seemed to be the most promising in avoiding or reducing the tissue inflammation [9]. However, these drugs are often associated with secondary undesired effects that in turn reduce the patience's compliance and led to an unwanted failure of the therapy.

As an alternative, effective formulation of phytochemicals capable of ensuring care of the skin and protection against oxidative and inflammatory processes is the new challenge of the modern pharmaceutical and cosmeceutical technologies, which could enable a daily protection of the skin avoiding or at least reducing the redox imbalance and the occurrence of chronic inflammations. According to this, several efforts have been done to select effective antioxidant and anti-inflammatory natural molecules, which have been tested for the treatment of skin diseases [10,11]. Among others, phenolic compounds are those explored the most thanks to their well-known beneficial effects, also confirmed by several scientific studies [12,13]. They are a large and heterogeneous group of secondary metabolites naturally occurring in fruits and vegetables, which have at least an aromatic ring with one or more hydroxyl substituents, including functional derivatives like esters, methyl ethers, and glycosides [14]. In nature, they may vary from simple phenolic molecules to complex polymerized compounds. Irrespective of their chemical structure and conformation, overall results validate their significant antioxidant and anti-inflammatory effects that contributes to the preservation and protection of human health against various diseases associated with oxidative damages [15]. A part from the effectiveness of natural phenolic compound, a key role is also played by the formulation, especially the nano-formulation, which may be responsible of the improvement of stability, bioavailability and *in vivo* efficacy of these payloads [16]. In particular, phospholipid vesicles have demonstrated to be an ideal carrier for the delivery of phytochemical or phytocomplexes into and through the skin, since the natural origin of their main components, their high biocompatibility and high versatility [17]. In a previous study, baicalin, a natural flavonoid isolated from Scutellaria baicalensis Georgi, when incorporated into phospholipid vesicles properly formulated for skin delivery, was able to reduce the skin damage induced by phorbol ester (TPA), in a better extent than the free molecule in dispersion and dexamethasone both used as references [18]. Similarly, mangiferin, a natural compound isolated from Mangifera indica L [19–21], was incorporated in different kinds of phospholipidic vesicles (glycerosomes, ethosomes and glycethosomes) [22–24]. These vesicles were capable of incorporating high amount of the bioactive and promoting the healing of the wound induced in mice using TPA, confirming their potential application for the treatment of psoriasis or other topical inflammatory disorders [25]. In a further study, Allaw et al. demonstrated that mucin-glycoltransfersomes, obtained combining the moisturizing properties of glycerol, the penetration enhancing capability of propylene glycol and the bioadhesive properties of mucin, were ideal carrier capable of effectively potentiate the beneficial properties of mangiferin in the treatment of skin lesions [26]. Even, naringin, another natural compound, has also shown interesting anti-inflammatory and antioxidant activities [27,28], which have been potentiated by its incorporation in ultradeformable-liposomes. These systems were considered as a promising formulation for the treatment of topical inflammatory diseases [29]. The optimal effects obtained loading different phytochemicals in phospholipid vesicles were connected to the higher penetration of payload into the skin, its controlled release, and the promoted internalization inside the cells [30].

Considering these promising finding and aiming at improving the local treatment of inflammatory skin diseases, mangiferin and naringin, with promising beneficial properties, were co-loaded in phospholipid vesicles specifically designed modifying ultradeformable-liposomes, used as reference, with the addition of three key components: glycerol, ethanol and sodium hyaluronate. Vesicles were fully characterized in terms of size, homogeneity of the system and surface charge, which have been also constantly monitored during the overall storage period. Moreover, the rheological behavior and the skin penetration and accumulation performances have been evaluated along with the biocompatibility and ability of formulations to counteract oxidative damages induced *in vitro* in fibroblasts. Finally, their efficacy in preventing or repairing skin injuries was assessed *in vivo* in an animal model.

2. Materials and methods

2.1. Materials

Lipoid® S75, soybean lecithin containing lysophosphatidylcholine (3 % maximum), phosphatidylcholine (70 %), phosphatidylethanolamine (10 %), fatty acids (0.5 % maximum), and triglycerides (3 % maximum), were purchased from Lipoid Gmbh (Ludwigshafen, Germany). Sodium hyaluronate low molecular weight (200–400 kDa) was purchased from DSM Nutritional Products AG Branch Pentapharm (Aesch, Switzerland). Naringin, glycerol, Tween 80, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), TPA (12-O-tetradecanoyl-phorbol-13-acetate) and all the other reagents and solvents of analytical grade were purchased from Sigma-Aldrich/Merck (Milan, Italy). Mangiferin was purchased from Carbosynth Limited (Compton, UK). Cell medium, fetal bovine serum, penicillin, streptomycin and all the other reagents used for cell studies were purchased from Thermo-Fisher Scientific Inc (Waltham, MA, US).

2.2. Analytical method

The quantitative analyses were performed by high-performance liquid chromatography (HPLC) using a PerkinElmer® Series 200 equipped with a photodiode array UV detector and a C18 reverse-phase column (Teknokroma®Brisa "LC2" 5.0 μm, 150 × 4.6 mm). The isocratic mobile phase consisted of a mixture of hydrochloric acid (pH 4.0) and methanol (50:50, v/v), the flow rate was 0.8 mL/min, and the injection volume was 20 μL. The detection wavelength was set at 280 nm for both molecules, mangiferin and naringin.

2.3. Vesicle preparation

Ultradeformable-liposomes were prepared weighting in a glass vial S75 (180 mg/mL), tween 80 (5 mg/mL), mangiferin (10 mg/mL) and naringin (10 mg/mL) and dispersing them in water. Glycerosomes were obtained dispersing the same components in a mixture of water and glycerol (1:1) and glycerohyalurosomes adding 0.5 mg/mL of sodium hyaluronate to the mixture of water and glycerol (1:1) used as hydrating

medium. Et-glycerosomes and et-glycerohyalurosomes were obtained hydrating the above specified components with a blend of glycerol, ethanol and water (2:1:2). The dispersions were sonicated for 4 min using a Soniprep 150plus ultrasonic disintegrator (MSE Crowley, London, UK) to obtain homogeneous systems with small vesicles. Empty vesicles and mangiferin and naringin water dispersion were prepared as well, and used as controls.

2.4. Vesicle characterization

Dynamic and Electrophoretic Light Scattering technique has been used to measure mean diameter, polydispersity index, and zeta potential of the vesicles using a Zetasizer nano-ZS (Malvern Panalytical, Worcestershire, UK). These parameters have been timely monitored as well over time (monthly for 90 days) to evaluate the stability of the vesicular dispersion on storage at 4 °C.

The non-incorporated phytochemicals have been removed from the vesicular dispersions by dialysis. 1 mL of each sample has been loaded in Spectra-Por membranes with 12–14 kDa MW cut-off, 3 nm pore size (Spectrum Laboratories Inc., DG Breda, The Netherlands) and dialyzed in 2 L of water for 2 h, refreshing the water after 1 h. The scavenging activity of phytochemicals before and after the purification process was measured by means of the DPPH (2,2′-diphenyl-1-picrylhydrazyl) colorimetric test. 10 μL of each sample () were dissolved in 1990 μL in a methanolic solution of DPPH (4 mg/100 mL) and incubated for 30 min at room temperature, in the dark. After incubation the absorbance (ABS) was measured at 517 nm against blank [31]. All the experiments were performed in triplicate. The scavenging activity was calculated according to the formula: ($ABS_{DPPH} - ABS_{sample}/ABS_{DPPH}$) x 100. The entrapment efficiency was calculated as the percentage of the antioxidant activity of dialyzed versus non-dialyzed samples [32,33].

The interactions between the component of the vesicles and mangiferin and naringin were evaluated by means of Fourier transform infrared (FTIR) analyses performed at room temperature from 500 to 4000 cm^{-1}, using an Agilent Cary 630 FTIR spectrometer (Waldbronn, Germany).

2.5. Rheological measurements

A controlled stress rheometer (Haake RheoStress 1, ThermoFisher Scientific Inc (Waltham, MA, US) equipped with control and data logging software (RheoWin 4.0.1) and a Haake K10 thermostatic bath for constant control of the temperature, was used for the rheological measurements. After loading, samples were allowed to rest for at least 300 s to ensure stress relaxation and temperature equilibrium. A cone-plate sensor (2°, 35 mm diameter) was used to measure flow and small amplitude oscillation sweeps. All measurements were performed in triplicate, at 25 °C. Step flow curves were performed in controlled stress mode (30 s each step in logarithmic distribution). The viscosity results were fitted to the simplified Carreau Equation (1):

$$\eta = \frac{\eta_0}{\left(1 + \left(\frac{\dot{\gamma}}{\dot{\gamma}_c}\right)^2\right)^s} \quad (1)$$

where η_0 is the zero-shear viscosity, $\dot{\gamma}_c$ is the critical shear rate, and s the shear thinning index.

2.6. In vitro cytocompatibility and antioxidant effect of the vesicles

Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal bovine serum, 1 % penicillin and streptomycin, was used to culture 3T3 mouse fibroblasts (ATCC collection, Manassas, VA, US) in an incubator set at 100 % humidity and 5 % carbon dioxide at 37 °C. MTT (3, (4,5-dimethylthiazol-2)2,5 difeniltetrazolium bromide) colorimetric test was used to measure cell viability [34]. The cells (7.5×10^{-3} cells/well) were seeded into 96- well plates, cultured for 24 h, and exposed for 48 h to the phytochemical in dispersions or loaded in vesicles diluted with medium to achieve the desired concentrations (10, 1, 0.1, 0.01 μg/mL of mangiferin and naringin). The MTT solution (0.5 mg/mL final concentration) was added to each well, removed after 3 h, and replaced with dimethyl sulfoxide. The absorbance of the solubilized formazan crystals was read at 570 nm using a microplate reader (Multiskan EX, Thermo Fisher Scientific Inc., Waltham, MA, US). Cell viability was calculated as a percentage of live cells versus untreated control cells (100 % viability).

To evaluate the protective effect of mangiferin and naringin, dispersed In water or loaded in vesicles, against oxidative damages induced using hydrogen peroxide, the cells, seeded into 96-well plates, were firstly incubated for 24 h and thenstressed with hydrogen peroxide (1:50,000 dilution of water solution 30 %; Sigma- Aldrich/Merck, Milan, Italy) and simultaneously treated with phytochemicals (1 μg/mL of mangiferin and naringin). After 4 h of treatment, the cells were washed with phosphate buffer, and the viability was measured by means of the MTT assay as reported above. Untreated cells (100 % viability) were used as positive control, and cells exposed to hydrogen peroxide only were used as negative control.

2.7. In vitro skin delivery studies

Skin penetration and permeation experiments were performed using the dorsal skin of new-born pigs (~1.5 kg) died for natural causes, provided by a local slaughterhouse. The skin was pre-equilibrated in saline prior to the experiments. Full-thickness skin specimens (n = 6) were placed between the donor and receptor compartments of Franz vertical cells (effective diffusion area of 0.785 cm^2). The receptor compartment was filled with aqueous solution containing 1 % of tween 80, thermostated at 37 ± 1 °C and maintained under stirring. during the whole experiment. The donor compartment was filled with the samples (200 μL) being in contact with the skin surface. At scheduled time point and up to 24 h, the receiving solution was withdrawn, refreshed, and analyzed by HPLC to quantify the amount of both mangiferin and naringin in the receptor compartment. After 24 h, the skin surface was dried with filter paper and the stratum corneum was removed by stripping with adhesive tape (Tesa® AG, Hamburg, Germany) [35]. Epidermis was separated from dermis with a surgical scalpel. The tape strips and the skin strata were cut, placed each in a glass vial with methanol and sonicated for 2 min to ensure the complete extraction of both phytochemicals. The obtained dispersions were finally filtered and both mangiferin and naringin content was measured by HPLC.

2.8. In vivo assay

Female CD-1 mice (5–6 weeks old, 25–35 g) were provided by Envigo laboratories (Barcelona, Spain) and acclimatized for one week in the animal facility of the University of Valencia, prior to the experiments. The studies were performed in accordance with the European Union regulations for the handling and use of laboratory animals. The protocols were approved by the Institutional Animal Care and Use Committee of the University of Valencia (code 2021/VSC/PEA/0178).

A short-term topical TPA treatment was performed following a previously reported method [29]. Briefly, one day after shaving, TPA dissolved in acetone (3 μg/20 μL) was applied on the dorsal skin of mice. After 3 h, 200 μL of each formulation was topically applied on the shaved area treated with TPA (n = 5 per group). This procedure was repeated twice per day for 3 consecutive days. Mice were finally sacrificed on day 4 by cervical dislocation. Oedema formation and myeloperoxidase activity were measured. The treated dorsal skin area of mice was excised and weighed to evaluate oedema formation. Myeloperoxidase assay was carried out as previously detailed [22]. Briefly, skin biopsies were homogenized and centrifuged, the supernatant was incubated with a mixture of sodium phosphate buffer (pH 5.4), phosphate buffer (pH 7.4), hydrogen peroxide and 3,3′,5,

5′-tetramethylbenzidine dihydrochloride, and then the absorbance was measured spectrophotometrically at 450 nm and the myeloperoxidase activity calculated from the linear portion of a standard curve.

Dorsal skin specimens of treated mice were used for histological assessment as well. The skin specimens were fixed in 0.4 % formaldehyde, embedded in paraffin, and sectioned at 5 mm. The sections were counterstained with hematoxylin and eosin. The tissues were observed under a light microscope (DMD 108 Digital Micro-Imaging Device, Leica, Wetzlar, Germany).

2.9. Statistical analysis of data

Statistical differences were determined by one-way ANOVA test and Tukey's test for multiple comparisons with a significance level of $p < 0.05$. All statistical analyses were performed using IBM SPSS statistics 28.0.1.1 for Windows (Valencia, Spain). Data are shown as means ± standard deviations.

3. Results

Vesicles capable of simultaneously loading mangiferin and naringin at high concentration (10 mg/mL each) were firstly selected afterwards a preformulation study performed using various amounts and types of phospholipid and surfactants. Finally, ultradeformable-liposomes composed of S75 (180 mg/mL) and tween 80 (5 mg/mL) as edge activator were selected based on their promising features and used as basic formulation. They were improved adding glycerol to the water phase (ratio 1:1) to obtain glycerosomes or the association of 0.5 mg/mL of sodium hyaluronate and glycerol to the water phase (ratio 1:1) to obtain glycerohyalurosomes. Both formulations were further modified using a blend of glycerol, ethanol, and water (2:1:2) as water phase thus obtaining et-glycerosomes and et-glycerohyalurosomes.

3.1. Vesicle characterization

Ultradeformable-liposomes were sized ~149 nm, polydispersed (polydisperity index 0.34) and negatively charged (~-48 mV) (Table 1). The addition of glycerol in the water phase did not modify the mean diameter (~157 nm, $p > 0.05$ versus ultradeformable-liposomes) and zeta potential (~-50 mV) but addressed a decrease of the polydispersity index up to ~0.22, which indicated the formation of a more homogeneous system. The addition of both ethanol and glycerol to obtain et-glycerosomes led a further increase of mean diameter and polydispersity index of vesicles up to ~172 nm and 0.28 while the zeta potential remained unchanged (~-45 mV). The enrichment of vesicles with sodium hyaluronate addressed a reduction of both size (~131 nm) and polydispersity index (0.21) for glycerohyalurosomes, which was even more evident for et-glycerohyalurosomes (~94 nm and 0.15).

The entrapment efficiency was ~87 % irrespective of the composition of the formulations.

The stability of the formulations was evaluated measuring, at scheduled time intervals, their mean diameter, polydispersity index and zeta potential for 3 months (Fig. 1). Vesicles were almost stable as the measured parameters remained constant, except for et-glycerosomes, which underwent a progressive increase of the three parameters: size ~398 nm, polydispersity index 0.39 and the zeta potential ~ -28 mV.

Fourier Transform Infrared studies were performed to better understand the interactions among the vesicle components (Fig. 2). Characteristic absorption bands at 3373 cm^{-1} (hydroxyl group); 2933 cm^{-1} (C–H asymmetric stretching), 1255 cm^{-1} (–C–O–) and 1093 cm^{-1} (–C–O–C) were observed In the spectrum of mangiferin. Similarly, bands at 1080 cm^{-1} (-C–O-), 1641 cm^{-1} (-C═O-) and 1357 cm^{-1} (–C–O–C) were found in the spectrum of naringin, which are all attributable to flavonoids. By comparing the spectra of the raw flavonoids with those of the flavonoids co-loaded in the vesicles, the majority of changes were observed in the region between 1800 and 500 cm^{-1}. Sodium hyaluronate displayed a peak at 1040 cm^{-1} (skeletal vibrations involving the C–O–C bridge), due to its saccharide structure [36]. According to other authors, glycerol spectrum had a peak at 1045 cm^{-1} associated to the stretching of the C–O linkage in C_1 and C_3 [37]. The characteristic peak of glycerol at 1045 cm^{-1} was shifted to 1060 and 1063 cm^{-1} in et-glycerohyalurosomes and glycerohyalurosomes, respectively, confirming an interaction between the biopolymer and glycerol.

Table 1
Mean diameter (MD), polydispersity index (PDI), zeta potential (ZP) and entrapment efficiency (EE) of phospholipid vesicles co-loading mangiferin and naringin. Each value represents the mean value ± standard deviation of at least three replicates. The same symbol (*,§,#) indicates values not statistically different from each other ($p > 0.05$) and different from other values ($p < 0.05$).

Formulation	MD (nm)	PDI	ZP (mV)	EE (%)
Ultradeformable-liposomes	*149 ± 6	0.34	§-48 ± 4	#85 ± 2
Glycerosomes	*157 ± 6	0.22	§-50 ± 4	#86 ± 3
Glycerohyalurosomes	131 ± 6	0.21	§-54 ± 3	#88 ± 2
Et-glycerosomes	172 ± 7	0.28	§-45 ± 2	#88 ± 2
Et-glycerohyalurosomes	94 ± 8	0.15	§-56 ± 4	#88 ± 2

3.2. Rheological measurements

The correlation between viscosity and shear rate of vesicle dispersions was evaluated (Fig. 3).

A pseudoplastic behavior was observed for all vesicle dispersions as at lower shear rates the viscosity remained constant without any flowing of the systems until the shear limit was overcome, which corresponded to the flow of the system, the decrease of the viscosity and the increase of the shear rate. The viscosity of glycerohyalurosomes was the highest followed by that of glycerosomes. The viscosity of both vesicles containing ethanol was lower, probably because of its lower viscosity in comparison with that of both water and glycerol. This behavior well-fitted ($r > 0.99$) to Carreau model, which permitted to calculate the zero-shear viscosity ($\eta_{0,}$ Pa s), the critical shear rate ($\dot{\gamma}_{c,}$ s^{-1}), the shear thinning index(s) and the correlation coefficient (R) (Table 2). The calculated values confirmed that glycerohyalurosomes had the highest viscosity (~59,813 Pa s), around ten-folds higher than that of glycerosomes (~6241 Pa s). The addition of ethanol led a decrease of dispersion viscosity (ten-folds) but, also in this case, the viscosity of et-glycerohyalurosomes (~550 Pa s) was five-folds higher than that of et-glycerosomes (~116 Pa s). The critical shear rate showed that both glycerosomes and glycerohyalurosomes were the most structured among the tested systems as their critical shear rate values were lower compared to that of et-glycerosomes and et-glycerohyalurosomes. The shear thinning index of all the formulation was similar. However, glycerohyalurosomes showed an index value proximately 20 % lower compared to the other systems, indicating that these vesicles had the more pseudoplastic behavior.

3.3. In vitro transdermal delivery

The amount of mangiferin (Fig. 4, upper panel) and naringin (Fig. 4, lower panel) delivered by the vesicles in the skin layers (stratum corneum, epidermis, dermis) and capable of reaching the receptor compartment was measured and compared with the amount accumulated or diffused using the phytochemicals in water dispersion. The amount of the two phytochemicals accumulated in the different skin strata and receptor compartment was very similar. Using either water or liposomal dispersions the main deposition was found in the stratum (~3.5 %) followed by epidermis and dermis ≤1.4 %, while was negligible or even zero in the receptor compartment. Using glycerosomes and et-glycerosomes the accumulation was similar, ~5.1 % in the stratum corneum, ~2.5 % in the epidermis and ~1.0 % in the epidermis, denoting a deeper penetration of payloads versus that provided by ultradeformable-liposomes and dispersion, due to the moisturizing

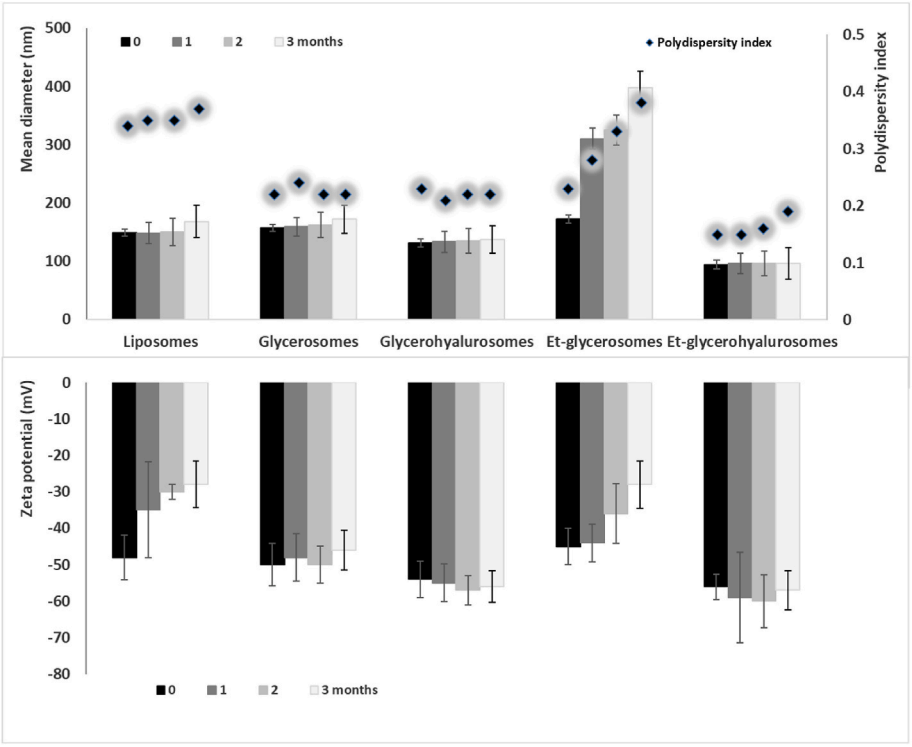

Fig. 1. Mean diameter, polydispersity index and zeta potential values of vesicles co-loading mangiferin and naringin and stored for 3 months at 4 °C. Data are reported as mean values ± standard deviations (error bars) ($n = 3$).

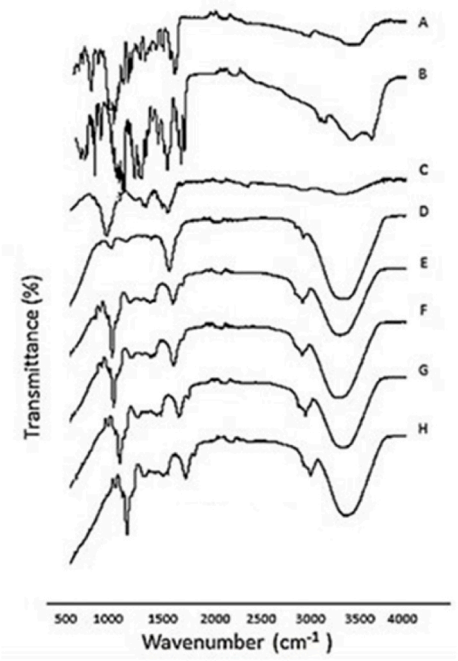

Fig. 2. FT-IR spectra of A) naringin; B) mangiferin; C) sodium hyaluronate; D) ultradeformable-liposomes; E) glycerosomes; F) et-glycerosomes; G) glycerohyalurosomes; H) et-glycerohyalurosomes.

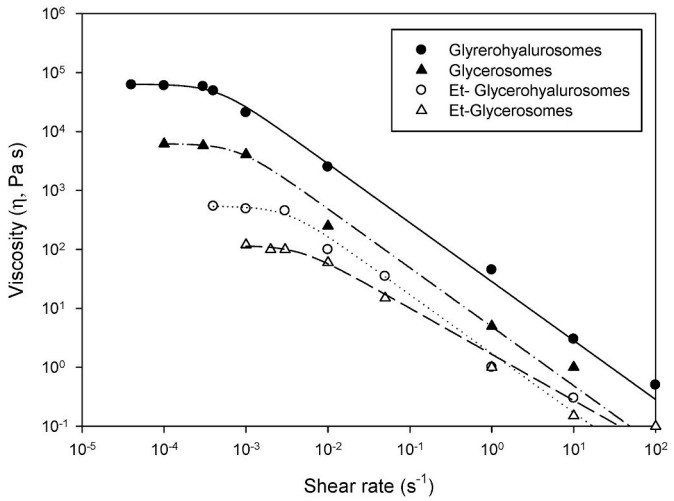

Fig. 3. Viscosity (Pa s) of glycerosomes, glycerohyalurosomes, et-glycerosomes and et-glycerohyalurosomes as a function of shear rate (s^{-1}) fitted to Carreau model.

effect of glycerol. Using glycerohyalurosomes and et-glycerohyalurosomes the penetration of payloads was improved especially at epidermis and dermis level, and they even reached the receptor compartment. Specifically, using et-glycerohyalurosomes the amount of mangiferin found in the stratum corneum was ~5 %, ~9 % in epidermis and ~4 % in dermis, the accumulated naringin in the stratum corneum was ~9 %, ~7 % in epidermis and 2 % in dermis. Using glycerohyalurosomes, the amount of mangiferin found in the stratum corneum was ~3 %, 7 % in epidermis and 2 % in dermis, and that of naringin was ~6 % in stratum corneum, ~5 % in epidermis and ~1 % in dermis.

3.4. In vitro cytocompatibility of vesicles and aility to protect the cells against oxidative damage

The *in vitro* cytocompatibility of mangiferin and naringin co-loaded in vesicles was measured and compared with that of the phytochemicals dispersed in water (Fig. 5A, upper panel). The viability of cells treated either with the dispersion or with the vesicles were especially

Table 2
Rheological parameters values obtained to fit experimental data of glycerosomes, glycerohyalurosomes, et-glycerosomes, et-glycerohyalurosomes to Carreau model. Mean values and standard deviations were reported.

	η_0 (Pa s)	$\dot{\gamma}_c$ (s^{-1})	S	$R >$
Glycerosomes	6241 ± 105	0.0008 ± 0.0001	0.47 ± 0.07	0.999
Glycerohyalurosomes	59,813 ± 4719	0.0005 ± 0.0001	0.40 ± 0.28	0.998
et-glycerosomes	116 ± 4.64	0.004 ± 0.001	0.49 ± 0.21	0.996
et-glycerohyalurosomes	540 ± 34	0.003 ± 0.001	0.50 ± 0.21	0.990

affected by the sample dilutions as the higher viability was found treating cells with the lower concentration of phytochemicals. The higher viability was found for cells treated with mangiferin and naringin co-loaded in glycerosomes, glycerohyalurosomes, et-glycerosomes and et-glycerohyalurosomes (~95 %), without any significant differences among both samples and concentrations tested, indicating their good biocompatibility.

The prevention and/or slowing down of damages and death caused by hydrogen peroxide in fibroblasts, by treating them with mangiferin and naringin co-loaded in vesicles, was evaluated measuring the cell viability upon treatment with either hydrogen peroxide only or the combination of hydrogen peroxide and vesicular dispersions (Fig. 5B, lower panel). The viability of cells stressed with hydrogen peroxide decreased up to ~59 % and slightly increased (~70 %) treating them with the phytochemicals in aqueous dispersion, ($p > 0.05$ versus the viability of untreated cells). The co-loading of phytochemicals in vesicles, significantly improve their beneficial properties as the viability increased up to ~95 % ($p > 0.05$ among the values of cells treated with all the vesicles), suggesting an almost complete protection of cells against oxidative damages.

3.5. *In vivo assays*

Topical application of TPA, twice per day, was capable of inducing inflammation and skin lesions in dorsal skin of mice (Fig. 6, upper panel). These lesions were treated with the most promising formulations: mangiferin and naringin co-loaded glycerohyalurosomes and et-glycerohyalurosomes, and mangiferin-naringin in aqueous dispersion or co-loaded in ultradeformable-liposomes, which were used as references. The skin with TPA only appeared dried, crustose, and damaged and similar to that treated with the phytochemicals in dispersion. The treatment with ultradeformable-liposomes led a slight reduction of damages as the skin appeared less dried and squamous. The best results were obtained treating the skin with glycerohyalurosomes and et-glycerohyalurosomes, indeed in these cases the skin was still thin but rosy and hydrated, without visual lesions, especially when glycerohyalurosomes were used. Visual inspection was confirmed by the histological analysis of the skin specimens as in the skin of animals damaged with TPA and untreated with the samples the whole epidermis was lost and the dermis was strongly inflamed (Fig. 6, upper panel). The stratum corneum of the skin treated with mangiferin and naringin in dispersion, was damaged but still present and both epidermis and dermis were still inflamed. Only a partial damaging of the stratum corneum was detectable after treatment of the skin with mangiferin and naringin co-loaded in ultradeformable-liposomes, without any inflammatory process detectable at epidermis and dermis level. The features of the skin treated with glycerohyalurosomes and et-glycerohyalurosomes, were the most promising as so damages at stratum corneum level were observed and the epidermis and dermis were not inflamed.

The values of MPO inhibition (%) and oedema inhibition (%) of the skin of mouse treated either with phytochemicals in dispersion or loaded in vesicles were measured (Fig. 7). The obtained results confirmed the visual inspection, i.e., mangiferin and naringin, when loaded in glycerohyalurosomes and et-glycerohyalurosomes, provided ~90 % of the oedema and MPO inhibition confirming that they were more effective when loaded in ultradeformable vesicles in comparison with the aqueous dispersion used as reference.

4. Discussion

Mangiferin is a natural occurring polyphenol with strong antioxidant and anti-inflammatory activities, which has been proposed as an

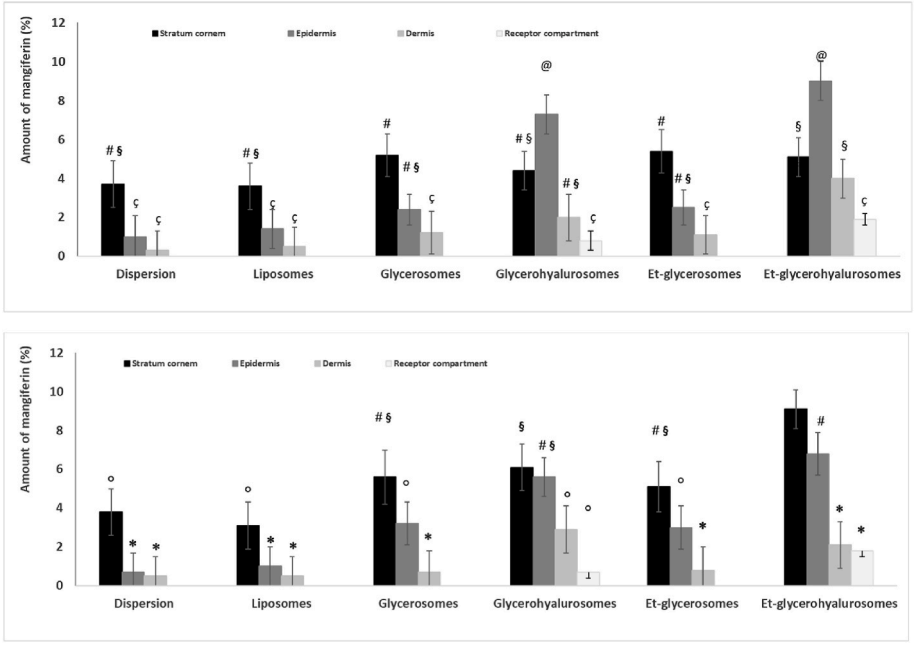

Fig. 4. Amount of mangiferin (upper panel) and naringin (lower panel) accumulated in stratum corneum, epidermis, dermis and receptor compartment after 24 h of treatment with payloads in dispersion or loaded in vesicles. Mean values ± standard deviations are reported ($n = 6$). The same symbol indicates not statistically different values ($p > 0.05$), which are instead statistically different from values marked with different symbols ($p < 0.05$).

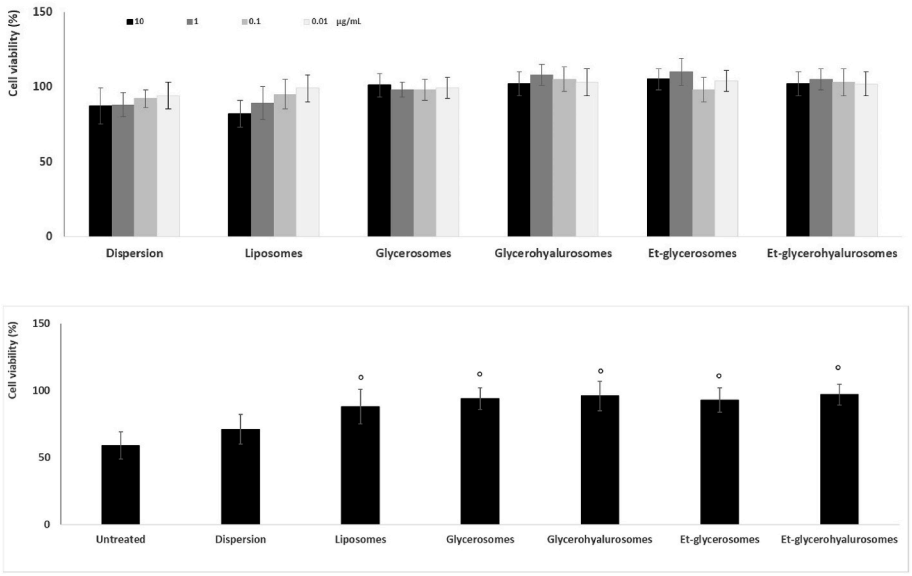

Fig. 5. Viability of 3T3 cells incubated for 48 h with mangiferin and naringin in dispersion or loaded in vesicles and diluted to reach different concentrations (upper panel). Viability of 3T3 stressed with hydrogen peroxide and simultaneously treated with mangiferin and naringin (10 μg/mL), in dispersion or co-loaded in vesicles (lower panel). Data are reported as mean values (bars) ± standard deviations. Symbol ° indicates values not statistically different from each other ($p > 0.05$) and statistically different from that of cells stressed with hydrogen peroxide and untreated ($p < 0.05$).

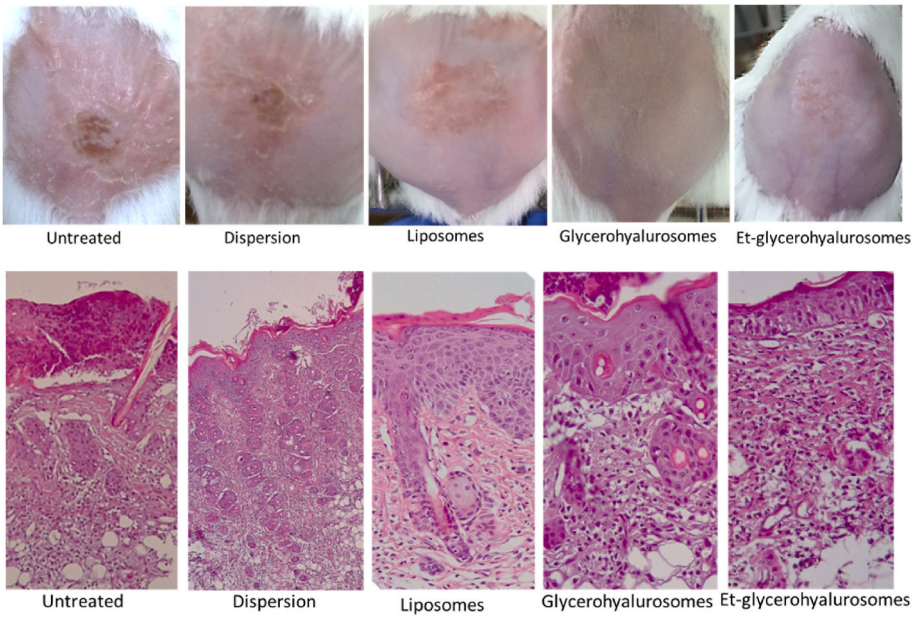

Fig. 6. Upper panel: Representative images of macroscopic appearance of the skin of mice damaged with TPA and untreated or treated with mangiferin and naringin in dispersion or loaded in ultradeformable-liposomes, glycerohyalurosomes and et-glycerohyalurosomes. Lower panel: Representative histological images of skin lesions of mice treated with TPA only or with mangiferin and naringin in dispersion or loaded in ultradeformable-liposomes, glycerohyalurosomes or et-glycerohyalurosomes, stained with hematoxylin-eosin and observed under an optical microscope.

affective agent for the treatment of cutaneous disorders and/or to promote skin regeneration [38,39]. Naringin is another natural antioxidant, principally used to protect the skin from the damaged and apoptosis caused by UV exposition, mainly exerted inhibiting ROS production, *COX*-2 over-expression and inflammatory reactions [40]. Considering their complementary effects, they were previous tested alone as photostabilizing and photoprotective agents in sunscreens formulations to prevent the formation of reactive oxygen species [41]. To the best of our knowledge, they were never used in association for the care of skin diseases and were never co-loaded in nanocarriers. To fill this gap, in the present study, they were successfully co-loaded in improved phospholipid vesicles tailored for skin delivery. The first goal was the loading of both phytochemicals at high concentration (10 mg/mL each), which was achieved formulating ultradeformable-liposomes containing soy phosphatidylcholine at high concentration (180 mg/mL) and tween 80 (5 mg/mL) as edge activator, that facilitate their co-loading and improve the vesicle deformability and the skin delivery performances [18]. Ultradeformable-liposomes were then enriched with glycerol, ethanol and sodium hyaluronate to further improve their performances upon topical application. Sodium hyaluronate has been chosen as it is a natural biopolymer capable of immobilizing the vesicles and improving their stability and skin delivery [42]. Additionally, it has a well-known

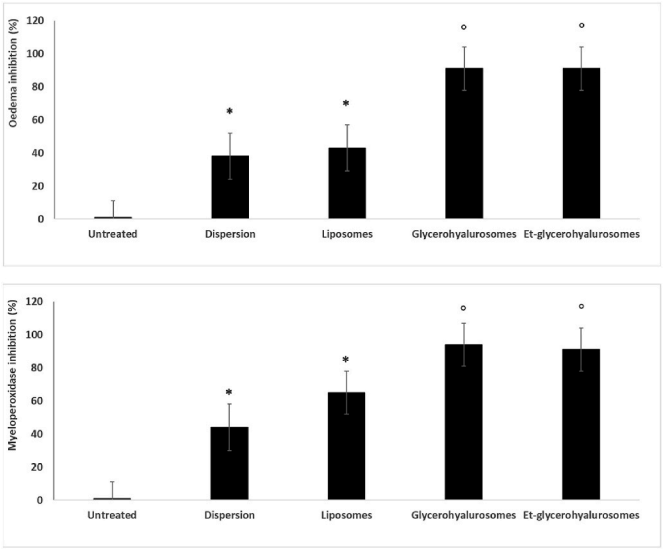

Fig. 7. Inhibition of myeloperoxidase (%) and oedema (%) measured in the mice skin damaged with TPA and treated with mangiferin and naringin co-loaded in vesicles or dispersed in water. Mean values ± standard deviations are reported. Symbol (°) indicates values, are not statistically different from each other ($p > 0.05$) but different from values detected in skin damaged wit TPA and untreated ($p < 0.01$); symbol (*) indicates values, not statistically different from each other ($p > 0.05$) but different from values detected in skin damaged wit TPA and untreated ($p < 0.05$).

role in skin wound healing and promotes skin regeneration [43]. Its association with glycerol in phospholipid vesicles is promising as demonstrated by optimal results obtained in previous studies [17,44]. According to Casula et al. glycerohyalurosomes and et-glycerohyalurosomes were smaller than the corresponding ultradeformable-liposomes probably due to an ideal interaction among phospholipid, payloads, glycerol and sodium hyaluronate [45]. The prepared vesicles, irrespective of the addition of glycerol and hyaluronan, were stable, only ethanol in glycerosomes had a destabilizing effect, which was prevented and/or avoided adding the hyaluronan since it probably promotes a better assembling of vesicles along with their immobilization in dispersion [46]. The zeta potential of vesicles was highly negative and remained unchanged over time, between −50 and −60 mV, ensuring vesicle repulsion in dispersion thus avoiding aggregation and fusion phenomena. Moreover, negatively charged vesicles are widely used as transdermal drug delivery thanks to their ability to promote the penetration of various payloads through the stratum corneum and their higher biocompatibility in comparison with positively charged vesicles, as previously reported by different authors [47–49]. In addition, FTIR spectrum confirmed the actual incorporation of mangiferin and naringin in the vesicles as a significant reduction of the intensity of the peaks was observed. According to previous results [46,50], the rheological analysis (Table 2) indicated that the addition of sodium hyaluronate strongly affected the dispersion viscosity as that of glycerohyalurosomes was around ten-folds higher than that of glycerosomes and that of et-glycerohyalurosomes was around five-folds higher than that of et-glycerosomes. Differently, the addition of ethanol addressed more fluid or less viscous dispersions. Anyways, the system viscosity did not directly affect the skin delivery performances of vesicles, since glycerohyalurosomes, which had the highest viscosity, did not provide the highest deposition of the payloads. The skin delivery seems to be more affected by the presence of hyaluronan in the formulation, irrespective of the dispersion viscosity, as the glycerohyalurosomes and even more the et-glycerohyalurosomes (having much lower viscosity), addressed the higher skin deposition and, additionally, allowed the transdermal flux of both, mangiferin and naringin [43]. Results can be explained by the high hygroscopic nature of hyaluronan that might enhance stratum corneum hydration, facilitating the passage of intact vesicles [46]. Probably, the positive effect of hyaluronan was strengthened by the presence of glycerol, which is another hygroscopic and viscous component, as their synergic effect ensure the higher delivery performance of vesicles, as previously confirmed [51]. The prepared vesicles were highly biocompatible irrespective of both vesicle surface charge and concentration of phytochemicals tested (Fig. 5, upper panel), confirming the safety of highly negatively charged systems [47,49] and suggesting their effective suitability as topical products. They were also capable of protecting the cells from the damages and death caused by hydrogen peroxide, re-establishing, almost completely, the healthy condition (~95 % of viability). The same protection was not ensured using the phytochemicals in aqueous dispersion as the cell viability was ~70 % (Fig. 5, lower panel), denoting the important role played by the vesicle as carrier not only in promoting the skin accumulation and penetration into and through the skin, but also in favouring the interaction with cells [30].

Considering the promising physico-chemical characteristics and *in vitro* biocompatibility and efficacy of mangiferin and naringin co-loaded in vesicles and aiming at reducing the number of animals only ultradeformable-liposomes, glycerohyalurosomes and et-glycerohyalurosomes were tested *in vivo*, and results were compared with those of the phytochemicals in dispersion. The dorsal skin of animals was damaged by topical applications of TPA to the dorsal skin of mouse, which is a consolidate model to induce acute inflammation, oedema, epidermal hyperplasia, dryness but also skin ulceration and severe panniculitis [52]. Despite the topical application, twice per day, of mangiferin-naringin in aqueous dispersion, there was still a loss of epidermis and a moderate accumulation of inflammatory cells in dermis, while the application of ultradeformable-liposomes reduced in a better extent than the dispersion, the infiltration of the inflammation mediators at dermal level. As expected, the skin treated with glycerohyalurosomes and et-glycerohyalurosomes showed a further reduction of the inflammation, and epidermis and dermis were especially preserved. In this sense, results confirmed that the loading in specifically designed vesicles is essential to ensure the success of treatment, indeed, only the treatment with mangiferin and naringin co-loaded in glycerohyalurosomes and et-glycerohyalurosomes addressed the complete repair and healing of the skin lesion.

Findings of visual and histological inspection were confirmed by the measurements of the two biomarkers involved in the inflammation process (oedema and MPO). The goal was surely strengthened by the hyaluronan itself, which promotes wound healing [53]. These findings are consistent with the results reported by Taléns-Visconti et al. [54] and confirm the effectiveness of these vesicles as topical systems for the treatment of skin damages.

5. Conclusions

This research reveals that the co-loading of mangiferin and naringin in glycerohyalurosomes with or without ethanol can be a promising and effective alternative for the treatment of inflammatory conditions at skin level. Further work should be performed to confirm the therapeutic potential of the proposed nanoformulations.

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CRediT authorship contribution statement

Maria Pleguezuelos-Villa: Investigation, Methodology, Writing – original draft. **Ines Castangia:** Investigation, Writing – original draft. **Octavio Diez-Sales:** Validation, Writing – review & editing. **Maria**

Letizia Manca: Conceptualization, Investigation, Supervision, Writing – review & editing. **Maria Manconi:** Conceptualization, Data curation, Supervision, Writing – review & editing. **Amparo Ruiz Sauri:** Methodology. **Raquel Taléns-Visconti:** Formal analysis, Supervision, Writing – review & editing. **Amparo Nácher:** Conceptualization, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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