

## Sustainable anti-aging skincare with antioxidant hydrogel patches paired with olive pomace extract-loaded phospholipid vesicles

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### ARTICLE INFO

#### Keywords:

Olive pomace  
Polyphenols  
Oxidative stress  
Natural extracts  
Anti-aging  
Skin hydration  
Biocompatibility  
Sustainable skincare  
Agro-industrial byproducts

### ABSTRACT

Skin aging is driven by intrinsic and extrinsic factors, with oxidative stress playing a central role. In this study, a sustainable antioxidant vesicular dispersion was developed by loading an olive pomace extract into phospholipid vesicles. The extract was obtained via a green solvent-based method and was rich in polyphenols (1309.12 mg/kg dry weight), particularly quercetin (639.64 mg/kg dry weight) and hydroxycinnamic acids (299.34 mg/kg dry weight). The extract had strong antioxidant activity in DPPH and FRAP assays. Four formulations, namely liposomes, hyalurosomes, collagen-liposomes, and collagen-hyalurosomes, were produced according to the hydrating medium used and were small ( $\approx 83$  nm), with narrow size distributions ( $PI \approx 0.22$ ), high negative zeta potentials ( $\approx -37$  mV), and high incorporation efficiencies (up to 82%). The systems remained stable for up to 90 days at 4 °C and withstood thermal and mechanical stress conditions. *In vitro* tests on HaCaT keratinocytes confirmed their biocompatibility and their ability to mitigate hydrogen peroxide-induced cytotoxicity, preserving  $\approx 90\%$  cell viability. As skin hydration plays a crucial role in the prevention of skin aging, in parallel, hydrogel patches were developed and used either alone or in combination with the vesicular dispersion to evaluate their contribution to skin moisturization. Their combination resulted effective in enhancing both the superficial skin hydration ( $>100\%$ ) and deep hydration (increased by  $\approx 17\%$ ). These findings support the complementary use of olive pomace extract-loaded vesicular systems and hydrogel patches as a promising, eco-friendly strategy for combating skin aging through topical antioxidant delivery and skin hydration.

### 1. Introduction

Skin aging is a natural yet complex process that results in visible changes such as wrinkles, dryness, and loss of elasticity [1,2]. These changes are driven by intrinsic factors, such as cellular degeneration from oxidative stress, and extrinsic factors, including environmental exposure [3,4]. Natural compounds with strong antioxidant activity, particularly those derived from plants, have garnered attention for their potential to help restore the balance between endogenous antioxidant defenses and reactive oxygen species production, thereby mitigating oxidative stress-related skin aging [5]. In this context, olive pomace, a by-product generated during olive oil production, is an appealing source of bioactive compounds, including polyphenols, triterpenic acids, and other antioxidants [6]. However, the efficient recovery of these

compounds remains challenging, as the quality, composition, and antioxidant potency of the resulting extracts are influenced by various factors [7]. These include the inherent characteristics of the plant source and its geographic origin, as well as handling and storage conditions [8,9]. In addition, the final extract profile is also largely influenced by the extraction technologies and operating conditions [6]. Different extraction techniques, such as solvent extraction, supercritical fluid extraction, and enzyme-assisted extraction, can yield extracts with varying compositions and concentrations of bioactive compounds even when applied to the same starting raw material [10]. Similarly, the choice of solvent, temperature, and extraction time can further modulate the selectivity and efficiency of the process, determining the relative abundance of specific classes of compounds [7]. With a growing emphasis on natural ingredients and sustainable practices in skincare, increasing attention

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<https://doi.org/10.1016/j.jddst.2026.108851>

Received 5 March 2026; Received in revised form 31 July 2026; Accepted 27 August 2026

Available online 27 August 2026

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has been directed toward the use of green solvents, such as ethanol, ethyl lactate, glycerol, and natural deep eutectic solvents, for the recovery of bioactive compounds [11,12]. In this regard, replacing conventional organic solvents with more sustainable alternatives, such as ethanol instead of methanol, represents a promising strategy to reduce environmental impact while preserving extract functionality [13].

Beyond extraction-related challenges, the effective use of olive pomace-derived bioactives in topical formulations also requires addressing formulation-related limitations, including limited stability and suboptimal ability to reach or interact with the target site on the skin. To address these issues, different advanced delivery systems have been formulated and investigated [14]. Among them, phospholipid vesicles, also known as liposomes, have emerged as promising carriers for the loading and delivery of olive pomace-derived bioactive molecules [15]. Owing to their self-assembled phospholipid structures, liposomes can load, protect, and transport sensitive molecules [16]. In this regard, olive-derived extracts have recently been successfully loaded into liposomes using supercritical-assisted processes, resulting in improved protection and delivery of their bioactive components [17].

Although phospholipid vesicles offer clear advantages in terms of loading, protection, and delivery, their low viscosity may limit their residence time on the skin surface when applied as aqueous dispersions. From a cosmetic and topical perspective, this is an important limitation, as an effective product should not only deliver active compounds but also ensure adequate adhesion and persistence at the application site. In this regard, hydrogel-based systems may provide a practical complementary strategy, as their soft texture and ability to conform to the skin surface can facilitate application, improve user acceptability, and prolong contact between the formulation and the treated area [18,19]. In addition, they can create an occlusive microenvironment on the skin surface, reducing water loss, increasing hydration, and promoting the skin penetration of loaded molecules [20] [21].

Based on these considerations, the present study was designed to address the multifaceted challenge posed by skin aging, sustainable extraction, and topical bioactive delivery. The novelty of the present study lies, therefore, in the integrated response proposed to these interconnected challenges. First, an olive pomace antioxidant fraction was obtained using a green solvent-based strategy (ethanol/water extraction), in line with the growing interest in natural ingredients and environmentally responsible processes for skincare applications. Second, this bioactive-rich extract was loaded into various phospholipid vesicular systems using the eco-sustainable direct sonication method, thereby improving its stability, protection, and topical delivery while again avoiding non-green solvents. Third, the optimized vesicular formulation was evaluated either alone or in association with separate hydrating hydrogel patches. This combined regimen was intended to couple the delivery advantages of phospholipid vesicles with the application, residence, and hydration benefits of hydrogel-based systems, without integrating the two components into a single formulation. Overall, the developed systems were investigated through physico-chemical characterization, antioxidant activity assays, *in vitro* biocompatibility studies on human keratinocytes (HaCaT), evaluation of protection against hydrogen peroxide-induced oxidative damage, and *in vivo* assessment of skin hydration after application.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Sodium hyaluronate, marine hydrolysed collagen powder consisting of type I fish collagen peptides, glycerol and other reagents were purchased from Galeno Srl (Italy). Phospholipid S75 was purchased from AVG Srl (Italy). Folin & Ciocalteu phenol reagent, sodium carbonate, gallic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and other reagents of analytical grade were purchased from Sigma-Aldrich (Milan,

Italy). Methanol, acetonitrile, and orthophosphoric acid (85%), were purchased from Carlo Erba (Milan, Italy). In addition, Shikimic acid, gallic acid, hydroxytyrosol, syringic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, 3-hydroxybenzoic acid, caffeic acid, 2,5-dihydroxybenzoic acid, p-coumaric acid, ferulic acid, benzoic acid, rutin, oleuropein, quercetin dihydrate and apigenin were all analytical grade and were obtained from Sigma Aldrich (Milan, Italy). Double-deionized water was produced with a Milli-Q system, showing a conductivity of less than 18.2 M $\Omega$  (Millipore, Bedford, MA, USA). Ethanol and methanol were purchased from Carlo Erba (Milan, Italy).

### 2.2. Olive pomace material and ultrasound-assisted extraction

The olive pomace was kindly provided by a local olive mill, Olearia Medda s.r.l., and derived from olives of the Semidana cultivar processed during the 2024 olive oil harvesting campaign using a two-phase milling process. The sample was dried for 48 h at 40 °C and ground at room temperature (25 °C) to obtain a fine powder. The extraction temperature of 40 °C was selected as a mild condition, in accordance with previous studies on phenolic recovery from olive pomace and olive oil by-products, to improve extraction efficiency while limiting thermal stress [22]. This dry matrix was stored under vacuum, in darkness, at room temperature, until extraction. 50 g of dry matrix was dispersed in 500 mL of a mixture of ethanol and water (50% v/v) and stirred at 40 °C for 4 h. The mixture was then mixed overnight at 25 °C. The following day, the mixture was sonicated in an ultrasonic bath for 10 min. It was then centrifuged at 4000 rpm for 15 min, and the supernatant was used to determine the total phenolic content and the antioxidant activity (Sections 2.3.2 and 2.3.3). The ethanol was removed from the supernatant through low-pressure evaporation in rotavapor (Rotavapor RII, BÜCHI Labortechnik AG, Flawil, Switzerland) coupled to a vacuum pump (Vacuum Pump V-700, BÜCHI Labortechnik AG, Flawil, Switzerland). The remaining water was lyophilized for 48 h (Lyovapor™ L-200, BUCHI) to obtain the solid extract. The extraction yield was calculated as the percentage relative to the dry matrix.

The ethanol/water ratio was selected on the basis of preliminary extraction trials performed at a fixed matrix-to-solvent ratio of 1:10, using different ethanol percentages in water (0, 25, 50, 75, and 100% v/v). The resulting extracts were compared in terms of total phenolic content and antioxidant activity by Folin-Ciocalteu, DPPH, and FRAP assays (Sections 2.3.2 and 2.3.3). The 50% (v/v) ethanol/water mixture was selected because it provided the best compromise in terms of phenolic recovery and antioxidant activity compared with the other ethanol/water ratios tested (data not shown).

### 2.3. Extract characterization

#### 2.3.1. Polyphenols HPLC—DAD analysis

An Agilent HPLC 1100 liquid chromatograph coupled with a Thermo Finnigan CHROMQUEST UV 6000 diode array detector (DAD), controlled by an Agilent ChemStation (Agilent Technologies, Rome, Italy), was used for polyphenol characterization and quantification. The column was a Phenomenex C18 (5  $\mu$ m, 150 mm  $\times$  4.6 mm) working at room temperature. A 10  $\mu$ L volume sample was injected and eluted in 120 min (0.4 mL/min) via a binary gradient mobile phase consisting of phosphoric acid 0.22 M (solvent A) and acetonitrile (solvent B) with a follow gradient elution: 0 min, 95% A–5% B; 30 min 90% A–10% B; 35 min, 85% A–15% B; 70 min, 70% A–30% B; 100 min, 10% A–90% B; 120 min, 100% B. Each time, the column was reconditioned for 15 min. Given the chemical structure of the target phenolic compounds injected, three distinct wavelengths were used: 280 nm, 360 nm, and 520 nm. Peak identification was carried out by

comparing the retention time of each compound and their respective UV spectra with the reference standard. Oleuropein and hydroxytyrosol were below the limit of quantification (LOQ) in our HPLC-DAD analysis. Caffeoylquinic acids were expressed as chlorogenic acid equivalents.

The polyphenol standards were injected either individually or as a mixed solution. A calibration curve

of the mixed solution was prepared in orthophosphoric acid 0.22 M from suitable dilution of the work mixed solution (1000 mg/L in methanol). Correlation values ranged between 0.9990 and 0.9999. The quantification data were reported as mg/kg DW.

### 2.3.2. Total phenolic content

The total phenolic content of the liquid extract was assessed using the Folin-Ciocalteu assay. 100  $\mu$ L of either the extract or blank water was mixed with 500  $\mu$ L of Folin & Ciocalteu phenol reagent. 6.4 mL of water and 3 mL of 20% w/v sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) were added after 5 min, and the samples were then incubated in the dark for 90 min. Using varying concentrations of gallic acid (0.01–1 mg/mL), a 7-point calibration curve was constructed. The absorbance was measured at 765 nm against blank with a microplate reader (Synergy 4 Reader, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). Result was expressed as mg of gallic acid equivalents/mL of extract.

### 2.3.3. Antioxidant activity (DPPH and FRAP assays)

The *in vitro* assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability and ferric reducing antioxidant power (FRAP), were carried out to investigate the antioxidant activities of the extract. In the assay, 10  $\mu$ L of the extract was mixed with a methanolic solution of DPPH (990  $\mu$ L, 40  $\mu$ g/mL) and incubated for 30 min in the dark at room temperature. The absorbance was measured at 517 nm against blank. The antioxidant activity was calculated according to the following equation:

$$\text{Antioxidant activity (\%)} = \frac{ABS_{DPPH} - ABS_{sample}}{ABS_{DPPH}} \times 100$$

The antioxidant activity was also expressed as mg of Trolox equivalents per mL of extract, using a calibration curve prepared with Trolox solutions ranging from 0.05 to 5 mg/mL.

The antioxidant activity was also evaluated as the ability of extract to reduce the ferric complex at acidic pH, using the ferric reducing antioxidant power (FRAP) assay. The reagent was prepared by mixing the following three solutions (10:1:1 v/v): a) 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM hydrochloric acid; b) 300 mM sodium acetate; c) 20 mM Iron(III) chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ). A calibration curve was constructed using Iron(II) sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) at different concentrations (100–2000  $\mu$ M). Each sample was incubated for 15 min with the FRAP reagent and finally the absorbance was measured at 593 nm against blank. The antioxidant activity was expressed as  $\mu$ mol of  $\text{Fe}^{2+}$  equivalents/mL of sample.

## 2.4. Preparation of olive pomace-loaded vesicles

Olive pomace extract-loaded phospholipid vesicles were prepared by direct sonication. Olive pomace extract (20 mg/mL) and phospholipid S75 (120 mg/mL) were dispersed in different hydrating media: water for liposomes, sodium hyaluronate solution at 0.2% (w/v) for hyalurosomes, hydrolysed collagen solution at 0.2% (w/v) for collagen-liposomes, and sodium hyaluronate/hydrolysed collagen solution, both at 0.2% (w/v), for collagen-hyalurosomes. Empty vesicles were prepared under the same conditions with no extract. The terms hyalurosomes, collagen-liposomes, and collagen-hyalurosomes were used operationally to indicate phospholipid vesicles prepared in the presence of sodium hyaluronate, hydrolysed collagen, or both polymers in the hydration media, respectively. The pH of all the hydrating media, measured at 25 °C before sonication (pH 60 Violab, XS Instruments, Italy), was ~6.5. Phospholipid S75 was selected as a biocompatible, naturally derived vesicle-forming component widely used in topical liposomal formulations [23]. The phospholipid concentration, polymer/lipid ratios, and sonication conditions were selected based on previous studies on polymer-modified phospholipid vesicles and

preliminary experiments aimed at obtaining small, homogeneous, and physically stable vesicles with high extract incorporation efficiency [24, 25]. Dispersions (2 mL) were sonicated 8 + 8 cycles (4 s on and 2 s off) with a Soniprep 150 MSE (MSE Scientific Instruments, Crawley, UK) at an amplitude of 14  $\mu$ m. This pulsed sonication protocol, consisting of short on/off cycles, was used to limit temperature increase during vesicle preparation. All samples were stored at 4 °C until further characterization.

## 2.5. Characterization of olive pomace-loaded vesicles

The average size, polydispersity index, and zeta potential of the freshly prepared vesicles were measured using a Zetasizer Ultra (Malvern Instruments, Worcestershire, UK), after dilution (1:100) using water. The same parameters were measured every 30 days for 90 days to assess the long-term stability of samples stored at 4 °C.

Each formulation was purified from the non-incorporated extract molecules by dialyzing the sample (1 mL) against distilled water (2 L) at 25 °C for 2 h, using dialysis tubing (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., Rancho Domingo, USA), changing the medium after 1 h [26]. Measuring the antioxidant activity with DPPH assay of samples before and after dialysis, the incorporation efficiency was calculated and expressed as the percentage of the antioxidant activity of dialysed samples versus those non-dialysed [27].

The vesicle dispersions were subjected to stressful conditions according to a previous study [28]. Briefly, they were subjected to a single sequential stress cycle consisting of 1 h at 4 °C, 1 h at 50 °C, and 30 min at 2500 rpm. Mean diameter and polydispersity index were measured after each step and compared to the corresponding parameters of the freshly prepared samples.

## 2.6. In vitro biocompatibility assay and protective effect against oxidative stress

HaCaT cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin and streptomycin (10,000 units/mL of penicillin and 10,000/mL of streptomycin) in a humidified atmosphere containing 5% carbon dioxide, at 37 °C.

The methods used for the biocompatibility assay and protective effect against oxidative stress were adapted from a previously published work, with slight modifications [23].

After 24 h of incubation, the medium was removed and replaced for testing the biocompatibility, cells were seeded in 96-well plates ( $1 \times 10^4$  cells/well). After 24 h of incubation, medium was removed and replaced with complete DMEM containing olive pomace extract in aqueous dispersion, olive pomace extract-loaded vesicles, or the corresponding empty vesicles, at concentrations equivalent to those used for the loaded formulations (20, 2, 0.2 and 0.02  $\mu$ g/mL). After subsequent 48 h of incubation, medium was removed, replaced with thiazolyl blue tetrazolium bromide (MTT, 100  $\mu$ L/well, 0.5 mg/mL). MTT was removed after 3 h, formazan crystals were dissolved with dimethyl sulfoxide (100  $\mu$ L/well) and absorbance was measured at 570 nm with a microplate reader (Synergy LX, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). Each sample was tested in triplicate and untreated cells were used as negative control (100% viability).

For testing the protective effect against oxidative stress, cells were seeded in 96-well plates ( $1 \times 10^4$  cells/well) and incubated for 24 h. The cells were stressed with hydrogen peroxide (0.5 mM) and simultaneously treated with the olive pomace extract in dispersion or loaded in vesicles diluted in complete DMEM to reach 2  $\mu$ g/mL. Therefore, no pre-incubation with hydrogen peroxide was performed. The cells that were stressed with hydrogen peroxide (0.5 mM) but not treated with samples were used as negative control, while those not stressed with hydrogen peroxide nor treated with samples were the positive control (100%

viability). After 4 h, the viability of cells was evaluated using the MTT assay.

## 2.7. Preparation of the hydrogel patches

To prepare hydrogel patches, gelatine (2 g) was dispersed in distilled water (40 g) and brought to 50 °C, under continuous stirring until complete dissolution. In a different beaker, glycerol (20 g), kaolin (0.5 g), xanthan gum (0.5 g), hydroxyethyl cellulose (1 g), lemon oil (1 g) and sweet almond oil (5 g) were weighted and homogeneously mixed. This mixture was added to the gelatine solution, mixed thoroughly, and poured into 6-well plates to obtain circular hydrogel patches. Each well was filled with 6 g of hydrogel formulation. The filled moulds were placed at 40 °C for 4 h and at 25 °C overnight, until complete solidification. The obtained circular hydrogel patches had an average diameter of 3.5 cm, an average thickness of 6.1 mm, and a weight-per-unit-area of 0.63 g/cm<sup>2</sup>. The hydrogel patches were then ready for use.

## 2.8. Skin moisture measurement

The combined effect of phospholipid vesicles and hydrogel in enhancing skin hydration was evaluated. The experiment was conducted on 30 healthy volunteers, both male and female, aged between 20 and 35 years, after written informed consent was obtained. Before enrolment, all participants were clearly informed about the study objectives, procedures, and the non-invasive nature of the measurements. The study consisted exclusively of non-invasive cosmetic instrumental measurements and, in accordance with COLIPA/Cosmetics Europe, EEMCO, and SCCNFP/European Commission guidance, no mandatory ethics committee approval number is prescribed for this type of non-invasive cosmetic instrumental evaluation [29–32].

Following a 20-min acclimatization period, clinical and instrumental evaluations were conducted in temperature and humidity-controlled conditions (temperature: 22 °C ± 2 °C and relative humidity: 72% ± 5%). Liposomes, hyalurosomes, collagen-liposomes, and collagen-hyalurosomes (100 µL each) were applied to distinct areas of the volunteer forearms, covered with the hydrogel patches, and left in place for 30 min. A hydrogel patch alone was also applied as an additional control. Hydration parameters were measured before and after the 30 min of treatment on the treated areas as well as on a control area where no product was applied. Superficial and deep skin moisturization were measured using non-invasive devices: MoistureMeterSC and MoistureMeterEpiD (Delfin Technologies).

For each treatment and for the control areas, superficial and deep hydration values were collected at baseline ( $t_0$ ) and after 30 min of application ( $t_{30}$ ). Data were first averaged across all volunteers for each condition. To minimize inter-individual variability and environmental effects, hydration values were normalized to the corresponding untreated control area measured at the same time point. Specifically, mean hydration values at  $t_0$  were divided by the mean control value at  $t_0$ , and mean hydration values at  $t_{30}$  were divided by the mean control value at  $t_{30}$ .

Subsequently, the normalized hydration value at  $t_{30}$  was further normalized to the corresponding normalized baseline value ( $t_0$ ). The increase in skin hydration was finally expressed as percentage variation using the following equation:

$$\text{Increase (\%)} = (\text{Normalized value}_{t_{30}} - 1) \times 100$$

The resulting values represent the percentage increase in superficial and deep skin hydration after 30 min of treatment compared to baseline.

## 2.9. Statistical analysis of data

Results were expressed as the means of at least 3 independent

experiments ± standard deviations. Multiple comparisons of means (ANOVA) were used to substantiate statistical differences between groups, while paired Student t-test was used to compare two samples. Significance was tested at the 0.05 level of probability ( $p < 0.05$ ). Data analysis was carried out using the software package XL Statistic for Excel.

## 3. Results

### 3.1. Extract characterization

#### 3.1.1. HPLC-DAD analysis of polyphenols

The extraction yield calculated as the percentage relative to the dry matrix, was  $14.9 \pm 1.9\%$ . The total polyphenol quantity measured by HPLC was 1309.12 mg/kg (Table 1). Among the identified polyphenols, the majority belonged to the class of hydroxycinnamic acids, comprising a total of six compounds, of which four were identified as caffeoylquinic acids. Within this class, caffeic acid was the most abundant, with a concentration of 109.26 mg/kg. Quercetin, a flavonoid, was the most abundant compound overall, its concentration was 639.64 mg/kg. Other detected flavonoids included rutin (341 mg/kg) and apigenin (29.14 mg/kg).

#### 3.1.2. Total phenolic content and antioxidant activity

The antioxidant activity of liquid extract from olive pomace assessed with DPPH was  $86 \pm 1\%$ , corresponding to  $189.94 \pm 1.09$  mg of Trolox equivalents per mL of extract (Table 2), confirming its strong capacity to neutralize free radicals. The FRAP assay indicated an important ferric reducing ability of extract,  $550 \pm 14$  µmol of Fe<sup>2+</sup> equivalents per mL of extract, highlighting its potential to reduce oxidative agents. Furthermore, the total phenolic content determined using the Folin-Ciocalteu method was  $12.7 \pm 2$  mg of gallic acid equivalents per mL of extract, suggesting that phenolic compounds significantly contribute to the antioxidant activity of extract.

### 3.2. Characterization of olive pomace extract-loaded vesicles

The physicochemical characteristics of the freshly prepared formulations were analyzed (Table 3). The average diameter of olive extract loaded vesicles was  $\approx 83$  nm and polydispersity index was  $\approx 0.22$ , with no statistically significant differences among the four samples ( $p > 0.05$ ). The zeta potential of all samples was highly negative ( $\approx -37$  mV,  $p > 0.05$  among different formulations). The incorporation of olive pomace extract did not affect the vesicle size, as empty vesicles were sized  $\approx 79$  nm ( $p > 0.05$  versus loaded vesicles). Moreover, empty vesicles, except the collagen-liposomes that has polydispersity index  $\approx 0.238$  ( $p < 0.05$  versus other empty vesicles), were less homogenous and monodispersed (polydispersity index  $\approx 0.329$ ,  $p < 0.05$  versus loaded vesicles).

The incorporation efficiency of the olive pomace extract varied, as that of liposomes, hyalurosomes and, collagen-hyalurosomes was higher ( $\approx 80\%$ ,  $p > 0.05$ ) while that of collagen-liposomes was the lowest efficiency ( $\approx 66\%$ ,  $p < 0.05$  versus the value of other vesicles).

**Table 1**  
Polyphenols identified by HPLC-DAD analysis.

| Compounds                   | mg/kg of dry weight |
|-----------------------------|---------------------|
| Caffeic acid                | 109.26              |
| Other caffeoylquinic acid   | 165.77              |
| p-coumaric acid             | 24.31               |
| Total Hydroxycinnamic acids | <b>299.34</b>       |
| Rutin                       | 341.00              |
| Quercetin                   | 639.64              |
| Apigenin                    | 29.14               |
| Total flavonoids            | <b>1009.78</b>      |
| Total polyphenols           | <b>1309.12</b>      |

**Table 2**

Antioxidant activity of the extract, measure with DPPH, FRAP and Folin-Ciocalteu assays. Mean values  $\pm$  standard deviations are reported (n = 3).

|                             | DPPH assay |                                        | FRAP assay                                           | Folin-Ciocalteu assay                       |
|-----------------------------|------------|----------------------------------------|------------------------------------------------------|---------------------------------------------|
|                             | Percentage | mg of Trolox equivalents/mL of extract | $\mu\text{mol of Fe}^{2+}$ equivalents/mL of extract | mg of gallic acid equivalents/mL of extract |
| Olive pomace liquid extract | 86 $\pm$ 1 | 189.94 $\pm$ 1.09                      | 550 $\pm$ 14                                         | 12.7 $\pm$ 2                                |

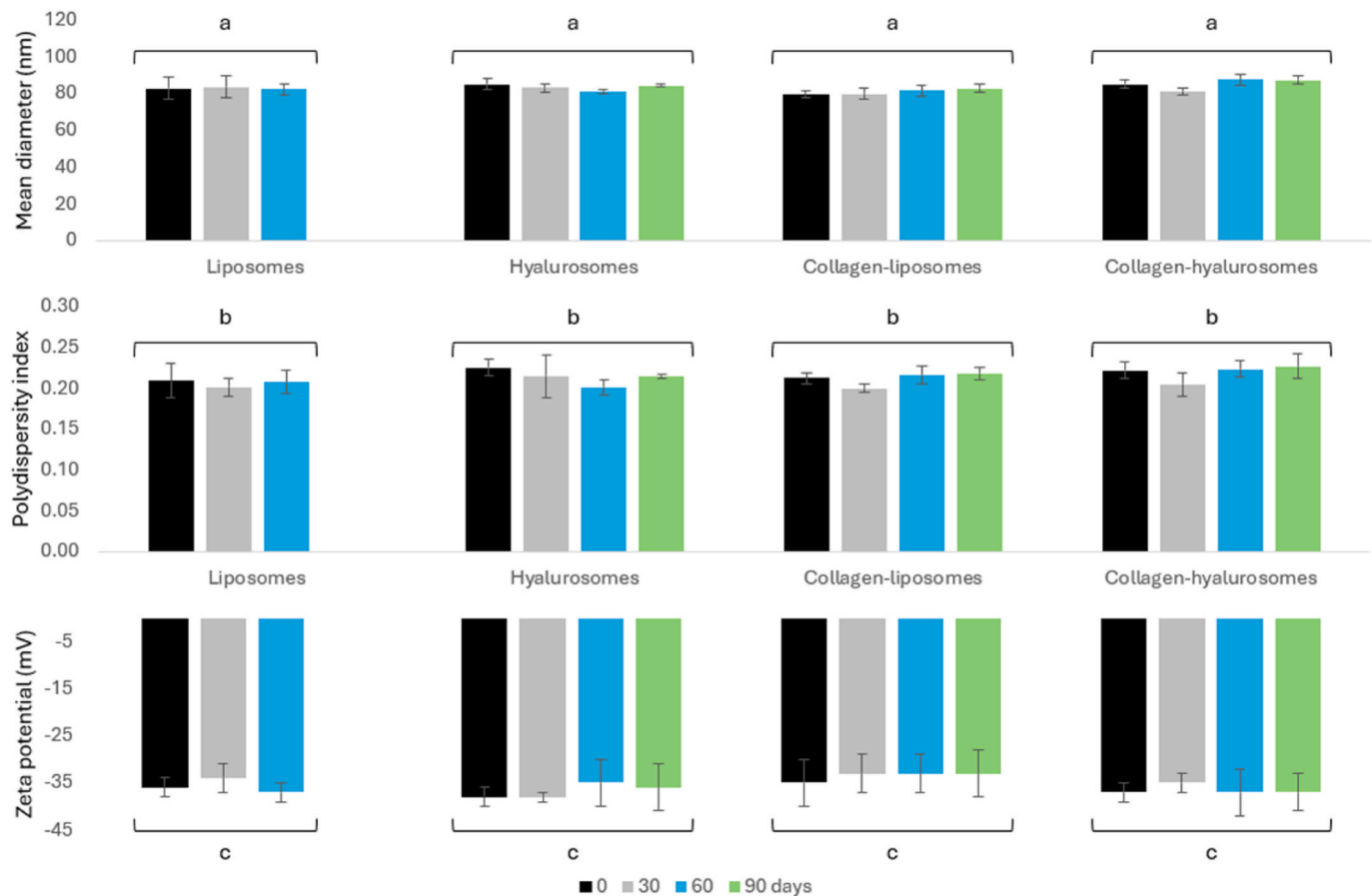
**Table 3**

Mean diameter (MD), polydispersity index (PI), zeta potential (ZP), and entrapment efficiency (EE) of empty and olive pomace extract-loaded vesicles. Each value represents the mean  $\pm$  standard deviation of at least three replicates. The same letter (a, b, c, d, e, f, g) indicates values not statistically different from each other ( $p > 0.05$ ) and different from other values ( $p < 0.05$ ).

|                             | MD (nm)                 | PI                             | ZP (mV)                  | EE (%)                  |
|-----------------------------|-------------------------|--------------------------------|--------------------------|-------------------------|
| Empty liposomes             | 73 <sup>a</sup> $\pm$ 5 | 0.334 <sup>b</sup> $\pm$ 0.012 | -57 <sup>d</sup> $\pm$ 3 | -                       |
| Empty hyalurosomes          | 87 <sup>a</sup> $\pm$ 6 | 0.330 <sup>b</sup> $\pm$ 0.032 | -48 <sup>e</sup> $\pm$ 2 | -                       |
| Empty collagen-liposomes    | 74 <sup>a</sup> $\pm$ 4 | 0.238 <sup>c</sup> $\pm$ 0.015 | -49 <sup>e</sup> $\pm$ 2 | -                       |
| Empty collagen-hyalurosomes | 80 <sup>a</sup> $\pm$ 4 | 0.324 <sup>b</sup> $\pm$ 0.014 | -49 <sup>e</sup> $\pm$ 2 | -                       |
| Liposomes                   | 83 <sup>a</sup> $\pm$ 6 | 0.209 <sup>c</sup> $\pm$ 0.021 | -36 <sup>f</sup> $\pm$ 1 | 82 <sup>g</sup> $\pm$ 5 |
| Hyalurosomes                | 85 <sup>a</sup> $\pm$ 3 | 0.237 <sup>c</sup> $\pm$ 0.011 | -38 <sup>f</sup> $\pm$ 2 | 78 <sup>g</sup> $\pm$ 3 |
| Collagen-liposomes          | 79 <sup>a</sup> $\pm$ 2 | 0.212 <sup>c</sup> $\pm$ 0.012 | -35 <sup>f</sup> $\pm$ 5 | 66 $\pm$ 4              |
| Collagen-hyalurosomes       | 85 <sup>a</sup> $\pm$ 2 | 0.222 <sup>c</sup> $\pm$ 0.010 | -39 <sup>f</sup> $\pm$ 1 | 81 <sup>g</sup> $\pm$ 3 |

The mean diameter, polydispersity index, and zeta potential of olive pomace extract-loaded vesicles were monitored for 90 days to evaluate the stability of the formulations (Fig. 1). The behaviour of hyalurosomes, collagen-liposomes and collagen-hyalurosomes was similar, as their tested parameters did not statistically change over 90 days ( $\approx 83$  nm,  $p > 0.05$ ). Liposomes remained stable for up to 60 days, after which phase separation appeared, making further measurements impossible.

The stability of the formulations under stress conditions was assessed subjecting the samples to one sequential stress cycle, consisting of incubation for 1 h at 4 °C, 1 h at 50 °C, and subsequent centrifugation at 2500 rpm for 30 min. The mean diameter and polydispersity index were measured after each step and compared to the freshly prepared samples. No significant variations were observed in either the mean diameter or the polydispersity index of formulations across all conditions remained not statistically different, indicating that the samples remained stable throughout the experiment (Fig. 2).



**Fig. 1.** Mean diameter, polydispersity index and zeta potential of olive pomace extract-loaded liposomes, hyalurosomes, collagen-liposomes, and collagen-hyalurosomes, stored in the dark at 4 °C for 90 days. Mean values (bars)  $\pm$  standard deviations are reported (n = 6). The same letter (a, b, c) indicates values not statistically different from each other ( $p > 0.05$ ) and different from other values ( $p < 0.05$ ).

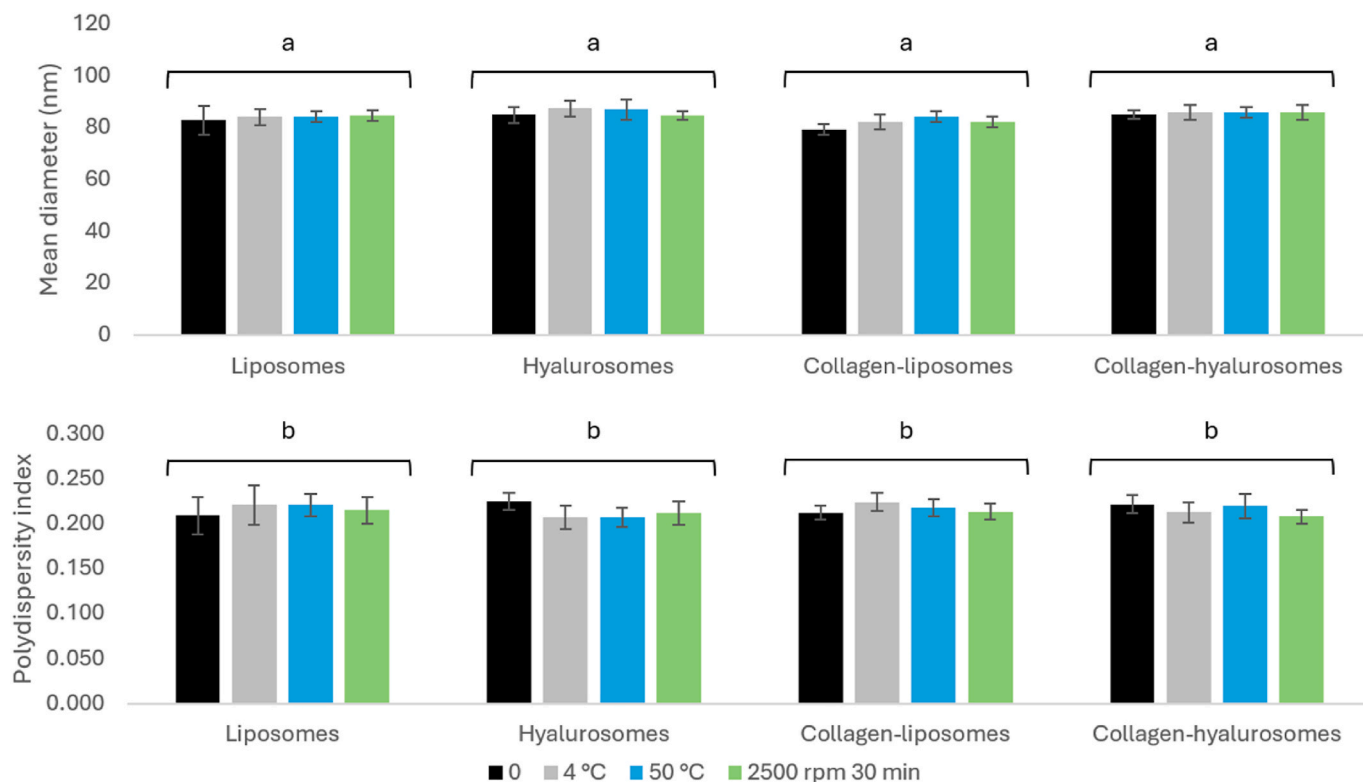


Fig. 2. Mean diameter and polydispersity index of olive pomace extract-loaded liposomes, hyalurosomes, collagen-liposomes and collagen-hyalurosomes, freshly prepared, incubated 1 h at 4 °C, 1 h at 50 °C, and centrifuged 2500 rpm  $\times$  30 min. Mean values (bars)  $\pm$  standard deviations are reported ( $n = 6$ ). The same letter (a, b) indicates values not statistically different from each other ( $p > 0.05$ ) and different from other values ( $p < 0.05$ ).

### 3.3. Biocompatibility assay and protective effect against oxidative stress

The biocompatibility assay on keratinocytes was performed using both olive pomace extract in dispersion and loaded into vesicles diluted to reach different concentrations in cell medium (20, 2, 0.2, and 0.02  $\mu\text{g/mL}$ ). The corresponding empty vesicles were also evaluated and showed cell viability values comparable to the untreated control under all tested conditions ( $p > 0.05$ ). The viability of cells incubated with 20  $\mu\text{g/mL}$  of liposomes was  $\sim 78\%$  and that of cells incubated with 20  $\mu\text{g/mL}$  of collagen-liposomes  $\sim 87\%$ . The two values were significantly lower than that of the positive control (100% viability). However, at lower concentrations, the viability of cells treated with liposomes and collagen-liposomes was comparable to that of the control ( $\sim 100\%$ ). Similarly, all other tested samples at all concentrations exhibited cell viability values comparable to those of the untreated control ( $\sim 100\%$ ), also at the highest concentration (20  $\mu\text{g/mL}$ ), confirming the overall biocompatibility of the formulations (Fig. 3, upper panel).

Based on the biocompatibility result, 2  $\mu\text{g/mL}$  of extract loaded in vesicles was used to evaluate the sample ability to avoid the damage effect of hydrogen peroxide on cells.

Keratinocytes stressed with hydrogen peroxide had  $\sim 74\%$  viability, while those stressed and simultaneously treated with formulations were protected from oxidative damage, maintaining  $\sim 90\%$  viability (Fig. 3, lower panel).

### 3.4. Skin moisture measurement

The extract-loaded phospholipid vesicles were applied on the skin and covered with a hydrogel patch. The vesicles were not dispersed within the hydrogel matrix; rather, the hydrogel patch was used as a hydrating and occlusive support placed over the applied dispersion. A

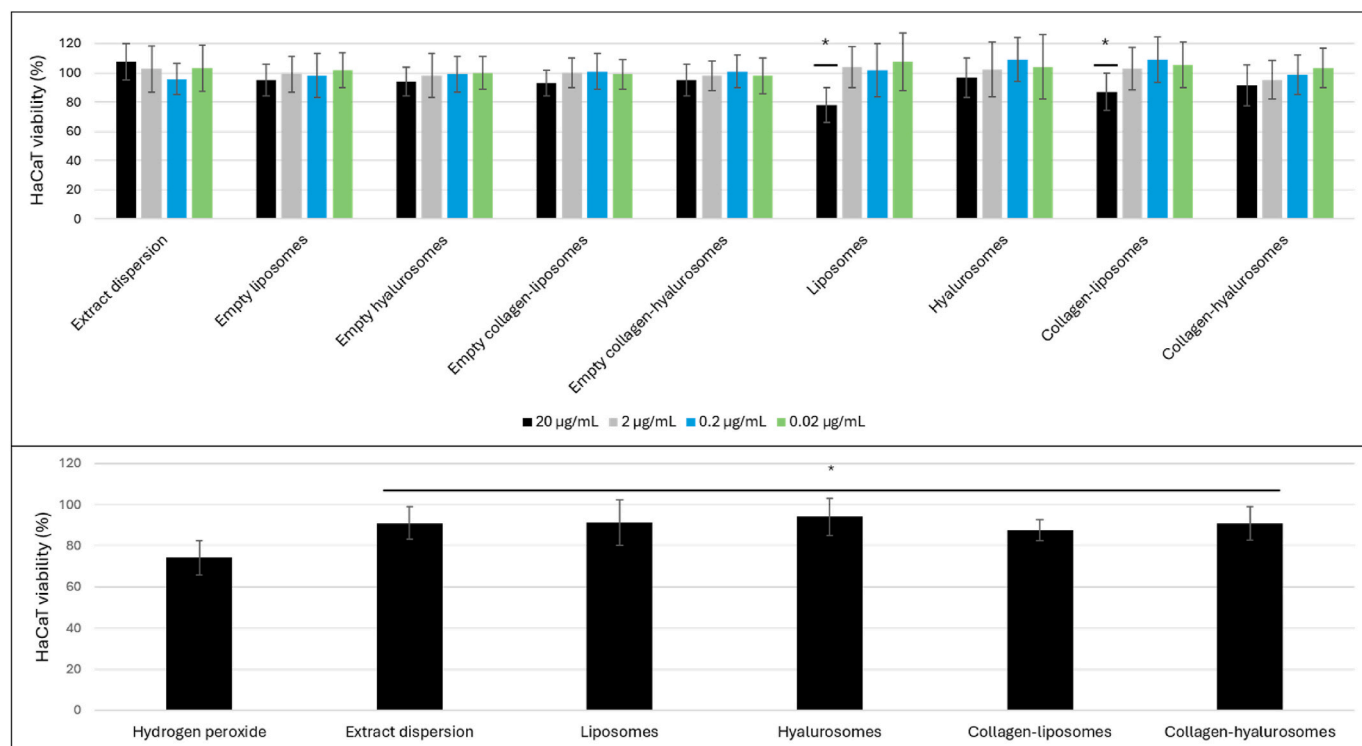
preformed hydrogel patch applied alone, without prior application of vesicular dispersion, was used as comparison. The combined treatment with the vesicular dispersion and the preformed hydrogel patch was more effective than the hydrogel patch applied alone, increasing superficial hydration by  $\sim 100\%$ , compared with  $\sim 73\%$  for the patch alone ( $p < 0.05$ ) (Fig. 4).

However, no statistically significant differences were found in the values obtained using the different formulations (liposomes, hyalurosomes, collagen-liposomes, and collagen-hyalurosomes), probably because after 30 min of a single application, the additional components of vesicles (i.e., collagen and/or hyaluronic acid) were not able to affect the skin hydration, and long-term treatment is needed.

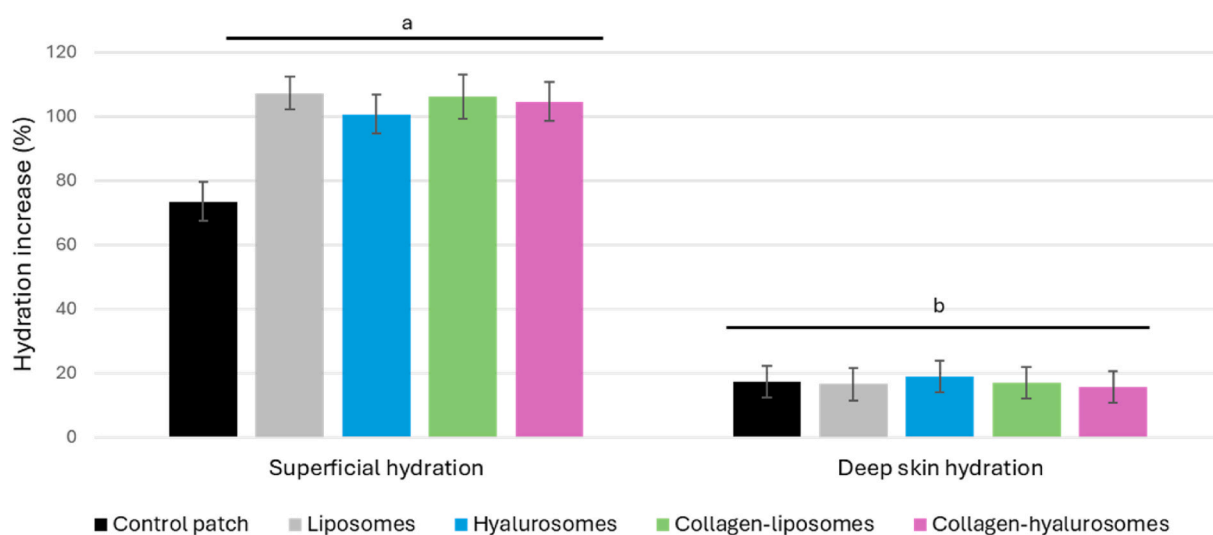
Similarly, the hydration of the deep skin after 30 min of a single application was  $\sim 17\%$  ( $p > 0.05$  across treatments) with the patch alone or in combination with the vesicles, regardless of vesicle composition. Therefore, also in this case, a long-term treatment may be beneficial.

## 4. Discussion

The valorization of olive pomace, the main by-product of olive oil production, could be a key means of supporting the circular economy within the olive oil industry [33]. Despite being traditionally considered waste, olive pomace is a rich source of bioactive compounds, including polyphenols, which have antioxidant, anti-inflammatory, and antimicrobial properties, confirmed in several studies [34]. For this reason, olive oil industry by-products have been increasingly investigated as sustainable cosmetic ingredients, particularly for the development of antioxidant, anti-aging, photoprotective, and skin-protective formulations [35,36]. Previous studies have mainly focused on incorporating olive-derived extracts into conventional topical systems, such as emulsions and creams, or on encapsulation approaches to improve the



**Fig. 3.** **Upper panel:** viability of keratinocytes (HaCaT) incubated for 48 h with olive pomace extract aqueous dispersion or olive pomace extract-loaded vesicles, properly diluted with growth medium up to 20, 2, 0.2 and 0.02 µg/mL. Empty vesicles, following the same dilutions of the extract-loaded vesicles, were used as controls. The mean values (bars) ± standard deviations are reported (n = 3). \*p < 0.05 were considered significantly different compared to the untreated control (100% viability). **Lower panel:** viability of keratinocytes stressed with hydrogen peroxide (0.5 mM) or stressed with hydrogen peroxide (0.5 mM) and simultaneously treated with olive pomace extract aqueous dispersion or olive pomace extract-loaded vesicles, diluted with growth medium up to 2 µg/mL. The mean values (bars) ± standard deviations are reported (n = 3). \*p < 0.05 were considered significantly different compared to cells treated with hydrogen peroxide.



**Fig. 4.** Increase (%) in superficial and deep skin hydration after 30 min of application of the preformed hydrogel patch alone (control patch) or after application of olive pomace extract-loaded vesicles (100 µL) covered with the preformed hydrogel patch. Mean values (bars) ± standard deviations are reported (n = 6). The same letter (a, b) indicates values not statistically different from each other (p > 0.05) and different from other values (p < 0.05).

stability and applicability of phenolic compounds [37]. In this context, the present work aimed to move beyond the simple use of olive pomace extract as an active ingredient by combining green extraction, phospholipid vesicle-based delivery, and a complementary hydrogel patch-based hydration strategy.

In line with sustainable practices, the hydroethanolic extraction

strategy used in this study enabled the recovery of an extract with high phenolic content and marked antioxidant activity, as confirmed by DPPH, FRAP, and Folin-Ciocalteu assays. The use of an ethanol/water mixture is particularly relevant for cosmetic applications, since it enables the extraction of phenolic compounds while avoiding more toxic organic solvents. The phenolic profile of the olive pomace analyzed by

HPLC-DAD in this study was dominated by flavonoids (mainly quercetin and rutin) and hydroxycinnamic acids, whereas oleuropein and hydroxytyrosol were below the limit of quantification (LOQ). Although olive pomace is often reported to contain oleuropein and its degradation products, its phenolic composition is highly variable and depends on several factors, including olive cultivar, fruit ripening stage, and olive oil extraction technology [38]. In the present study, the pomace was obtained from olives of the Semidana cultivar processed using a two-phase extraction system, which may have contributed to the observed phenolic profile. Furthermore, oleuropein is known to be susceptible to enzymatic hydrolysis and oxidative degradation during olive oil processing, which may further reduce its concentration [39]. Therefore, the predominance of hydroxycinnamic acids and flavonoids observed in the present study likely reflects the specific characteristics of the analyzed pomace rather than the phenolic composition typically associated with fresh olive fruits [40]. However, the detected polyphenols are consistent with those reported in the literature, as a recent review by Tapia-Quirós et al. (2022) on the recovery of polyphenols from agri-food by-products highlighted strong similarity between the qualitative and quantitative polyphenolic profiles of the olive pomace extract obtained in this study [34]. Similarly, the concentrations of caffeic acid, caffeoylquinic acids, p-coumaric acid, and rutin were consistent with the ranges reported by Tamasi et al. (2019) [41]. On the other hand, the quercetin content detected in this study (639.64 mg/kg DW) was approximately 18 times higher than that reported by the same authors. This may result from variations in the extraction method. The hydroalcoholic extraction carried out in this study is versatile and suitable for both polar and non-polar compounds. For certain compounds, it can provide higher extraction efficiency than water-based methods while maintaining the advantage of avoiding toxic solvents. The high quercetin content observed in our extract may also be related to the characteristics of the starting material, including cultivar, geographical origin, storage conditions, and drying procedure [42].

Among the compounds identified in this extract, several are well-known for their beneficial effects on skin health. Quercetin, the most abundant compound in the extract, is a powerful antioxidant and anti-inflammatory agent, and a promising molecule for anti-aging applications, as it can protect skin cells from oxidative stress, reduce UV-induced damage, and modulate inflammatory responses [43,44]. Rutin has significant antioxidant and anti-inflammatory properties as well [45]. A rutin nanocrystal gel that significantly improved skin penetration and effectively prevented UV-induced photoaging *in vivo* was developed by Li et al. [46]. In a previous study, rutin was able to attenuate inflammation downregulating the AGE-RAGE signaling pathway, which is particularly relevant in conditions like psoriasis [47]. These findings highlight the potential of this molecule in mitigating skin aging and inflammatory responses. Caffeic acid is a hydroxycinnamic acid that further contributes to the antioxidant and anti-inflammatory potential of the extract [48]. It directly targets the ERK1/2 signaling pathway and was capable of mitigating solar UV-induced carcinogenesis in the skin, suggesting a role in photoprotection and skin cancer prevention [49]. It also enhances the activity of endogenous antioxidant enzymes, such as superoxide dismutase and catalase, thereby reducing oxidative stress [50].

Considering its promising content, the olive pomace extract was loaded into phospholipid vesicles to improve the topical applicability of its main bioactive compounds. Vesicular systems can favour the dispersion and protection of sensitive antioxidant molecules and promote close contact with the skin surface, supporting their use as carriers for topical antioxidant delivery. To further investigate the influence of hydrating polymers on vesicle performance, the formulations were prepared using water, sodium hyaluronate, hydrolysed collagen, or their combination as hydrating media. All formulations had favourable characteristics for topical delivery, as they were small ( $\approx 83$  nm), homogeneously dispersed (polydispersity index  $\approx 0.217$ ), negatively charged ( $\approx -37$  mV), and capable of loading a high amount of extract (ranging from  $\sim 66\%$  to  $\sim 82\%$ ). The absence of significant differences in

mean diameter between empty and extract-loaded vesicles suggests that the extract was accommodated within the vesicular structures without inducing swelling, aggregation, or major bilayer reorganization detectable by dynamic light scattering. This behaviour may be explained by the ability of phenolic compounds to partition within different regions of the phospholipid bilayer or at the lipid–water interface, depending on their polarity and molecular structure [51,52].

The nanosized dimensions and narrow size distribution are advantageous for topical application, as vesicle size is known to influence dermal delivery, while a low polydispersity index indicates a homogeneous vesicle population [53]. In addition, the highly negative zeta potential may contribute to colloidal stability by reducing vesicle aggregation through electrostatic repulsion [54]. These highly negative values can be mainly attributed to the negatively charged phospholipid components. After extract loading, the absolute zeta potential values decreased compared with the corresponding empty vesicles. This reduction may be related to the localization of olive pomace extract components at the vesicle surface or at the lipid–water interface, where phenolic compounds may partially screen or mask the negatively charged polar groups of the vesicles. Nevertheless, all extract-loaded formulations maintained highly negative zeta potential values, indicating sufficient electrostatic repulsion to support colloidal stability.

The high incorporation efficiency confirmed the ability of phospholipid vesicles to accommodate the complex phytochemical fraction of the extract. The lower incorporation efficiency observed for collagen-liposomes may be attributed to possible interactions among hydrolysed collagen, phospholipids, and extract components, which could partially affect vesicle organization and extract retention.

When enriched with polymers, the vesicles stored for 90 days maintained their key physicochemical characteristics, demonstrating long-term stability. Hyaluronic acid has been reported to improve the physical stability of phospholipid vesicles, with hyalurosomes being usually more stable than liposomes [18]. Similarly, the addition of collagen to aqueous solutions has been associated with stabilization of liposomal structures *in vitro* [55]. Consistent with these observations, hyalurosomes, collagen-liposomes, and collagen-hyalurosomes maintained their physicochemical properties throughout storage, whereas conventional liposomes exhibited phase separation after only 60 days. This suggests that the presence of sodium hyaluronate and/or hydrolysed collagen contributed to the physical stabilization of the vesicular dispersions, probably by increasing the organization of the external aqueous phase and limiting vesicle aggregation.

The stability of the vesicle dispersions under stress conditions was evaluated to simulate the temperature fluctuations and mechanical stress that may occur during the transportation and storage of cosmetic products. The samples were sequentially treated at low ( $4^\circ\text{C}$ ) and high ( $50^\circ\text{C}$ ) temperatures, followed by centrifugation. All formulations remained stable, as no significant changes in mean diameter or polydispersity index were observed after any of the stress tests. Overall, the storage and stress stability data indicate that the modified vesicular systems, particularly those containing sodium hyaluronate and/or hydrolysed collagen, may be more suitable than conventional liposomes for the development of stable topical cosmetic products suitable for real-world applications.

The biocompatibility assessment in HaCaT cells showed that the formulations were generally well tolerated under the tested conditions, supporting their potential suitability for topical applications. The empty-vesicle controls confirmed the good cytocompatibility of all carrier systems, with HaCaT cell viability remaining  $\geq 90\%$  across the tested concentration range. Likewise, the non-encapsulated extract did not negatively affect cell viability. Among the extract-loaded formulations, hyalurosomes and collagen-hyalurosomes maintained high viability at all concentrations, whereas liposomes and collagen-liposomes caused a moderate reduction at the highest concentration tested,  $20\text{ }\mu\text{g/mL}$ . Since neither the free extract nor the corresponding empty vesicles produced a comparable decrease, this effect may be

associated with enhanced cellular delivery of the encapsulated compounds rather than with an intrinsic toxicity of the vesicular components [56]. The better tolerability of the hyaluronan-containing formulations may indicate that hyaluronan modulates vesicle-cell interactions, reducing the impact of the loaded extract at high concentrations. At concentrations ranging from 0.02 to 2 µg/mL, all loaded systems maintained viability around or above the control level, demonstrating a broad cytocompatible concentration range. Based on these results, the lower concentration of 2 µg/mL, which was biocompatible for all formulations, was selected for the oxidative stress protection assay. All four formulations were able to protect keratinocytes from oxidative damage caused by hydrogen peroxide when used at 2 µg/mL (Fig. 3, lower panel), which is very important for cosmetic products, as oxidative stress is a major contributor to skin aging [57]. The preservation of cell viability at approximately 90% in stressed cells treated with the formulations suggests that the antioxidant activity of the olive pomace extract was maintained after incorporation into phospholipid vesicles. This finding supports the functional role of the vesicular dispersion not only as a delivery system but also as a protective topical formulation against oxidative stress-related skin damage.

Enhanced superficial hydration was observed by applying the extract-loaded vesicles for 30 min and covering the area with a preformed hydrogel patch. This result aligns with previous studies demonstrating that lipid-based vesicles can improve skin moisture by forming an occlusive layer and enhancing water retention in the stratum corneum [58]. More specifically, the application of the olive pomace extract-loaded vesicular dispersion covered with the preformed hydrogel patch significantly increased superficial skin hydration compared with the hydrogel patch applied alone. This indicates that the vesicular dispersion provided an additional contribution to the hydrating effect of the hydrogel patch, probably through the formation of a lipid-based film on the skin surface and improved water retention in the stratum corneum. Since hydration was measured after a single 30 min application, the acute hydration effect was likely mainly governed by the occlusive contribution of the hydrogel patch and vesicular dispersion, whereas the specific hydrating effect of sodium hyaluronate may require longer exposure times or repeated applications to become measurable. Likely for the same reason, the single 30-min treatment did not improve deep skin hydration or provide an additional benefit over the hydrogel patch alone [59].

## 5. Conclusions

This study demonstrated the feasibility of an integrated and sustainable anti-aging skincare approach based on olive pomace valorization, phospholipid vesicular delivery, and hydrogel-assisted topical application. A phenolic-rich extract with marked antioxidant activity was obtained through hydroethanolic extraction and successfully incorporated into stable, nanosized phospholipid vesicles suitable for topical use. The developed vesicular formulations preserved the antioxidant functionality of the extract, protecting HaCaT cells against hydrogen peroxide-induced oxidative damage at the selected non-cytotoxic concentration. *In vivo*, the vesicular dispersion applied in association with preformed hydrogel patches significantly enhanced superficial skin hydration compared with the hydrogel patch alone, confirming the complementarity between vesicular delivery and hydrogel-based application. Overall, these findings highlight the potential of upcycled agro-industrial byproducts for sustainable and effective skincare applications. Further studies should investigate repeated applications, long-term skin effects, and compound-specific skin permeation to better define the contribution of individual olive pomace bioactives to the observed topical benefits.

## CRedit authorship contribution statement

**Federica Fulgheri:** Writing – review & editing, Writing – original

draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ines Castangia:** Writing – review & editing, Validation, Methodology, Investigation. **Matteo Aroffu:** Writing – review & editing, Validation, Methodology, Investigation. **Matteo Perra:** Validation, Methodology, Investigation. **Alessandro Atzei:** Writing – review & editing, Validation, Methodology, Investigation. **Francesco Corrias:** Validation, Methodology. **Mohammad Firoznejad:** Validation, Methodology. **Alberto Angioni:** Writing – review & editing, Validation, Supervision, Methodology. **Maria Letizia Manca:** Writing – review & editing, Validation, Supervision, Methodology, Data curation. **Maria Manconi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology.

## 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.

## Acknowledgements

We acknowledge financial support under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.5 - Call for tender No.3277 published on December 30, 2021 by the Italian Ministry of University and Research (MUR) funded by the European Union – NextGenerationEU. Project Code ECS0000038 – Project Title e.INS Ecosystem of Innovation for Next Generation Sardinia – CUP F53C22000430001- Grant Assignment Decree No. 1056 adopted on June 23, 2022 by the Italian Ministry of University and Research (MUR).

This work was also funded by the Interreg Italy–France Maritime Programme 2014–2020 – Managing Authority: Tuscany Region (80% European Regional Development Funds, 20% national public funds), within the framework of the research project “Filieri Economiche AgroforeStali circolari per l’Innovazione Eco-sosteniBiLe” (FEASIBLE).

## Data availability

Data will be made available on request.

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