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Extraction of the antioxidant phytocomplex from wine-making by-products and sustainable loading in phospholipid vesicles specifically tailored for skin protection

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ABSTRACT

The present study is aimed at valorizing grape pomace, one of the most abundant winery-making by-products of the Mediterranean area, through the extraction of the main bioactive compounds from the skin of grape pomace and using them to manufacture innovative nanoformulations capable of both avoiding skin damages and promoting skincare. The phytochemicals were recovered through maceration in hydroethanolic solution. Catechin, quercetin, fisetin and gallic acid, which are known for their antioxidant power, were detected as the main compounds of the extract. Liposomes and phospholipid vesicles modified with glycerol or Montanov 82® or a combination of both, were used as carriers for the extract. The vesicles were small (~183 nm), slightly polydispersed (PI $\geq$ 0.28), and highly negatively charged (~−50 mV). The extract was loaded in high amounts in all vesicles (~100%) irrespective of their composition. The antioxidant activity of the extract, measured by using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) test, was 84 ± 1%, and slightly increased when loaded into the vesicles (~89%, $P < 0.05$). The grape pomace extract loaded vesicles were highly biocompatible and able to protect fibroblasts (3T3) from the oxidative stress induced by hydrogen peroxide.

1. Introduction

With more than 60 million tons per year, grapes (*Vitis vinifera* L., Vitaceae) are the second world's largest fruit produced [1]. About 80% of the total crop is used in winemaking, 13% is sold as table grapes, and the remaining share is used for raisins, juice, and other products [2]. During the winemaking process, a solid waste known as grape pomace is generated [3], which accounts for approximately ~20% of the processed grapes [1]. It is mainly composed of peels and seeds, often mixed with stalks, and is still rich in bioactive compounds that are only partially extracted during the winemaking process, and, if recovered, could be exploited in the food, cosmeceutical, pharmaceutical, agricultural fields, as well as for energy recovery [3]. Indeed, this waste has elicited growing interest over the last years as it is considered an eco-friendly and economical source to obtain nutraceuticals, cosmeceuticals, and pharmaceutical products [4–6].

The possibility of recovering from waste products characterized by a high value on the market is consistent with the integration of waste management activities in the context of the market economy [7,8]. Concerning organic residues, this desirable perspective supposes a radical change in the technical approach to their management. Up to now, the main objective has been to limit the environmental impacts

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deriving from incorrect management of organic waste. This objective was, and still is, pursued in the form of biostabilization, which is obtained by applying treatments aimed at reaching a drastic demolition and mineralization of the original organic substance, accompanied at most by the recovery of energy (e.g., through methanization) or an indistinct plurality of organic compounds to be used as soil improver in agriculture (e.g., compost). The principles of sustainability and circular economy require, on the other hand, that treatments aimed at a mild breakdown of the original structure are preliminarily applied to safeguard the integrity of compounds of commercial interest [9]. Only after completion of this action of selective recovery, the management is completed with the application of more invasive treatments of the exhausted biomass. Ambitious recovery objectives compatible with production activities must be supported by the characteristics of high and constant quality, an aspect that makes waste from agro-industrial activities preferable to heterogeneous mixtures such as the organic fractions of municipal solid waste [10].

The bioactive compounds contained in grape pomace, such as anthocyanins, stilbenes, or gallate derivatives, among others, have various activities such as antioxidant, anti-bacterial, anti-inflammatory and anti-aging [2,11–14]. Their biological activities mainly rely on their radical scavenging activity, which allows them to prevent the oxidative damage caused by ROS (Reactive Oxygen Species) [15]. ROS are commonly produced during physiological processes and neutralized by the endogenous antioxidant systems [16]. When oxidative species are excessively generated an imbalance occurs in favor of the ROS and oxidative stress ensues. Oxidative stress is a phenomenon that is related to the development of many pathological conditions [17]. Several research studies have underlined the importance of external antioxidants in controlling the endogenous oxidative process as they are capable of interrupting the propagation of free radicals or inhibiting the formation of free radicals thus reducing oxidative damage [18].

In previous studies, grape pomace extract disclosed a great potential in scavenging free radicals and counteracting oxidative stress and tissue damages [19]. In addition, it was demonstrated that the therapeutic potential of grape pomace extracts depends on their phenolic content along with the composition of their formulation [6]. Indeed, the adequate dosage-form formulation is a key parameter, which can modify the pharmacokinetic parameters of both natural and synthetic drugs [20]. In the last century, research on nanomedicine ameliorated the efficacy of several drugs by using nanocarriers. Given that, a new horizon is aimed at obtaining natural drugs or phytocomplexes from sustainable and widely available biomasses such as waste, by using eco-friendly methods and improving their effectiveness by applying the nanotechnologies already used in nanomedicine for synthetic drugs [21,22]. The extraction and application of a phytocomplex obtained from grape pomace fully responds to the overarching purpose of finding new safe and effective formulations by combining the sustainable exploitation of natural resources with pharmaceutical nanotechnology. Grape pomace is the main by-product of the winery sector, rich in natural bioactives, but it is also a contaminant if incorrectly disposed of, thus its sustainable extraction and ad hoc formulation may foster its environmentally sound management within a bioeconomy context [15, 23,24].

Among the different nanocarriers developed for different actives, liposomes and nanovesicles specifically modified represent the preferred choice for skin delivery, as they are composed of biocompatible and safe ingredients [25]. Various studies displayed the ability of both liposomes and modified-liposomes to improve the effectiveness and bioavailability of drugs and natural bioactive compounds at skin level [5,26,28].

In this context, the activity of a multidisciplinary research group including researchers active in the fields of botany, biotechnology applied to pharmaceuticals, nutraceuticals and cosmetics, and valorization of organic waste, is framed. The research activities span from the characterization of specific types of pomace to the application of waste treatments aimed at valorizing the exhausted biomass resulting from the extraction of marketable compounds.

The present manuscript is focused on the part of the activities aimed at the extraction of the main bioactive compounds from the skins of pomace and their use to manufacture innovative nanoformulations.

The main aim of the research activity was to develop, through winery-making by-products valorization, grape pomace extract containing nano-delivery systems based on phospholipid vesicles as an alternative antioxidant system against skin oxidative stress. To achieve this goal, the grape skin was separated from pomace and pre-treated with water to eliminate sugars and then macerated in a water-ethanol mixture. The antioxidant activity and total phenolic content of the extract were measured and the extract with the highest antioxidant potential was loaded into liposomes and modified liposomes. The main physico-chemical properties (size, zeta potential, and ability to incorporate the extract) were evaluated. The ability of the formulations to prevent oxidative damage induced in 3T3 cells by hydrogen peroxide, as well as their biocompatibility, were evaluated and compared with that of the extract in dispersion.

2. Materials and methods

2.1. Materials

Lipoid S75 (S75), a mixture of soybean phospholipids (~70% phosphatidylcholine, 9% phosphatidylethanolamine and 3% lysophosphatidylcholine), triglycerides and fatty acids, was kindly provided by AVG S.r.l. (Garbagnate Milanese, Milan, Italy), local supplier for Lipoid GmbH (Ludwigshafen, Germany). Montanov® 82 was kindly supplied by Gatfossè (Milano, Italy). Trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, ethanol, and all other reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). For mobile phase preparation, water was purified by a Milli-Q system from Millipore (Bedford, MA, USA), formic acid was provided by Sigma-Aldrich (Steinheim, Germany) and LC-MS-grade acetonitrile was purchased from Fisher Chemicals (Waltham, MA, USA).

Cell medium, foetal bovine serum, penicillin, and streptomycin, and all other reagents for cell studies, were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, US).

2.2. Plant material

The extract was obtained from Carignano pomaces skins, kindly supplied by Cantina Santadi (SW Sardinia, Italy). The grape skins were manually separated from seeds and aliquots of 100 g of samples were stored at −80 °C until use. Grape skins were lyophilized, grinded to obtain a fine powder, and kept under vacuum in the dark and at 25 °C until the extraction (not pre-treated skins, NPT). Moreover, a further sample of 50 g of skins was suspended in 2 l of distilled water and maintained under constant stirring, at 25 °C for 24 h. After that time, the sample was wrung out and filtered to remove the solvent, lyophilized, grinded, and stored under vacuum and in the dark at 25 °C until the extraction (pre-treated skins, PT). The size of the obtained powder particles was measured with a laser diffraction particle size analyzer, Malvern Mastersizer 3000 (Malvern Panalytical Ltd, Worcestershire, UK), according to the wet dispersion method and distilled water as a dispersant.

2.3. Extraction method

A modified solid-liquid extraction procedure was adopted. Briefly, 30 g of pre-treated and not pre-treated grape skins were suspended in 970 ml of a mixture of ethanol:water (70:30 v/v, density 0,88556 g/ml). The suspension was shaken in the dark at 25 °C for 48 h. At scheduled times (0,1, 2, 3, 4, 6, 8, 24 h), samples were sonicated for 1000 s (200 cycles, 5 on, 5 off, 15 μm of probe amplitude) by using a high-intensity ultrasonic disintegrator (Soniprep 150, MSE Crow- ley, London, UK), to

enhance extraction [26].

At the end of the extraction process, the dispersion was centrifuged two times (30 min, 8000 rpm) to separate the solid and liquid phases. Ethanol was eliminated from the extractive solution 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), while water was removed by lyophilization, thus obtaining a hygroscopic purple powder that was maintained in the dark and under vacuum until use.

2.4. HPLC-ESI-TOF-MS analysis of grape pomace skins extracts

Pre-treated and non-pre-treated extracts were analyzed by High Performance Liquid Chromatography coupled with an electrospray ionization Time Of Flight mass spectrometry (HPLC-ESI-TOF-MS). HPLC analysis was performed by using an RRLC 1200 series (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, autosampler, a binary pump, and a DAD detector. For chromatographic analysis, extracts were prepared at a concentration of 5 μg/ml and 2 μg/ml by dissolving the appropriate amount of the extract in a ethanol: water blend (70:30 v/v). The analytical column used was a 150 mm × 4.6 mm id, 1.8 μm Zorbax Eclipse Plus C18 (Agilent Technologies, Palo Alto, CA, USA). The mobile phases were water with formic acid (0.1%) as eluent A, and acetonitrile as eluent B. The flow rate was 0.5 ml min^{-1}. Total run time was 65 min using the following multistep linear gradient: 0 min, 5% B; 55 min 95% B; 60 min 5% B; 65 min 5% B. The initial conditions were held for 5 min. The injection volume was 10 μl and the separation of the compounds was carried out at room temperature.

The HPLC system was coupled to a TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an orthogonal electrospray (ESI) interface (model G1607 from Agilent Technologies, Palo Alto, CA, USA) operating both in negative and in positive ionization mode. The effluent from the HPLC column was reduced using a "T" type splitter before being introduced into the mass spectrometer (split ratio 1:3), so that the flow to the ESI-TOF-MS detector was 125 μl min^{-1}. The detection was carried out considering a mass range of 50–1000 *m/z*. The optimum values of the source parameters for negative ionization mode were: capillary voltage + 3.5 kV; drying gas temperature, 210ºC; drying gas flow, 9 l min^{-1}; and nebulizing gas pressure, 2.3 bar. The values of the transfer parameters were: capillary exit −120 V; skimmer 1, −40 V; hexapole 1, −23 V; RF hexapole, 80 Vpp; and skimmer 2, −22.5 V. For the positive ionization mode the optimum values were: capillary voltage, +4 kV; drying gas temperature, 190ºC; drying gas flow, 9 L min^{-1}; and nebulizing gas pressure, 2.0 bar. The values of the transfer parameters were: capillary exit, +120 V; skimmer 1, +40 V; hexapole 1, +23 V; RF hexapole, 100 Vpp; and skimmer 2, +22.5 V.

The instrument was calibrated externally with a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) that was directly connected to the interface and contained a 10 mM sodium formate cluster solution. The mixture was injected at the beginning of each run and all the spectra were calibrated before compound identification.

The accurate mass data of the molecular ions were processed through the software Data Analysis 4.0 (Bruker Daltonics), which provided a list of possible elemental formulas by using Generate-Molecular Formula Editor. The Generate Molecular Formula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum and maximum elemental range, electron configuration and ring-plus double bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma Value) for increased confidence in the suggested molecular formula.

2.5. Folin-Ciocalteu and DPPH assay

The total phenolic content of skin extracts was measured according to the Folin–Ciocalteu colorimetric assay by using a UV spectrophotometer (Lambda 25, PerkinElmer, USA). The ethanolic extract solution (100 μl, 1 mg/ml), the Folin–Ciocalteu reagent (100 μl) and Na_2CO_3 aqueous solution (800 μl, 20% w/v) were mixed, and the absorbance was read at 765 nm after 30 min of incubation in the dark, at 25 °C. The total phenolic content was calculated by using the calibration curve of gallic acid (0–0.100 mg/ml) and expressed as mg of gallic acid equivalent/g of dry extract. The experiments were carried out in triplicate.

The antioxidant activity of extracts was assessed by measuring their ability to scavenge the DPPH radicals. The ethanolic solutions of extracts (20 μl, 1 mg/ml) were mixed with 1980 μl of DPPH methanolic solution (40 μg/ml), and incubated for 30 min at room temperature, in the dark. Then, the absorbance was measured at 517 nm against blank.

The antioxidant activity (AA) was calculated according to the following formula (Eq. 1):

$$AA\% = [(ABS_{DPPH} - ABS_{sample})/ABS_{DPPH}] \times 100 \text{ [29]}. \quad (1)$$

A calibration curve using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) at different concentrations (0–0.010 mg/ml) was built and used as reference and expressed as mg of Trolox equivalent/g of dry extract. All the experiments were performed in triplicate.

2.6. Vesicle preparation

S75 (150 mg/ml) and the pre-treated grape pomace skins extract (30 mg/ml) were weighed in a glass vial and hydrated with 2 ml of bidistilled water to obtain liposomes [30]. Montanov® 82 (10 mg/ml) was added to lipid and extract to obtain montanov-liposomes. Glycerosomes were prepared by hydrating S75 and grape pomace extract with a mixture of glycerol:water (1:4 v/v), and montanov-glycerosomes were obtained by adding Montanov® 82 (10 mg/ml) to glycerosomes. The dispersions were sonicated (10 +5 + 3 cycles, 5 s on and 2 s off, 13 μm of probe amplitude, allowing the sample cooling between each sonication), by using a Soniprep 150 ultrasonic disintegrator (MSE Crowley, London, UK), to obtain homogeneous systems with small particles. Empty formulations were also prepared and used as references.

2.7. Vesicle characterization

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

The average diameter, polydispersity index (a measure of the width of size distribution) and zeta potential of vesicles were determined by dynamic and electrophoretic light scattering by using a Zetasizer Ultra (Malvern Panalytical Ltd, Worcestershire, UK). Samples ($n \geq 3$) were diluted with water (1:100) and analyzed at 25 °C.

The samples (2 ml) were purified from the non-incorporated components by dialysis (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, Netherlands) against water (2 l) for 2 h, refreshing the water each hour. The antioxidant activity of the samples (see Section 2.6 for the method used) was measured before and after dialysis, and the entrapment efficiency was calculated as the percentage of the antioxidant activity of dialyzed samples versus non-dialyzed samples (Eq. 2).

$$EE\% = \frac{AA\%_{dialyzed}}{AA\%_{non-dialyzed}} \times 100 \quad (2)$$

2.8. Biocompatibility of formulations

Primary mouse embryonic fibroblasts (3T3) (ATCC collection, Manassas, VA, USA) were grown as monolayers in 75 cm^2 flasks, incubated with 100% humidity and 5% CO_2 at 37 °C. Phenol red-free Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 10% foetal bovine serum, penicillin and streptomycin, was used as culture medium. The cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well, and after 24 h of incubation were exposed for 48 h to the extract in dispersion or loaded in vesicles. Samples were diluted with cell medium to reach different concentrations of extract (30, 3, 0.3, 0.03 μg/ml). At the end of the experiment, the cell viability was assessed by performing the 3(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) test. MTT (100 μl, 0.5 mg/ml, final concentration) was added to each well and incubated at 37 °C for 2–3 h. The formazan crystals formed by the mitochondrial dehydrogenase activity of vital cells were dissolved in DMSO, and the absorbance at 570 nm was measured by using a microplate reader (Synergy 4, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). All the experiments were repeated at least three times and in triplicate. The results are expressed as a percentage of viable over untreated cells (100% viability) [31].

2.9. Protective effect of formulations against oxidative stress in 3T3 cells

3T3 cells were seeded in 96-well plates and incubated at 37 °C for 24 h, then treated for 4 h with hydrogen peroxide (1:50.000), and simultaneously with the different formulations properly diluted with the medium to obtain three different concentrations (30, 3, 0.3 μg/ml). At the end of the experiment, the cells were washed with PBS and the viability was measured by the MTT assay, as reported above (see Section 2.8). Untreated cells and cells treated with hydrogen peroxide were used as negative and positive controls, respectively.

2.10. Statistical analysis of data

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

3. Results

3.1. Extraction of bioactive compounds

The skins separated from grape pomace were pre-treated with distilled water to reduce the content of sugars and, in turn, the hygroscopicity of the final extract. Pomace not pre-treated with water was also used to extract the phytocomplex. After freeze-drying processing, samples (not pre-treated and pre-treated) were grinded to obtain a powder with a higher specific surface area and capable of promoting the interaction between particles and extractive medium (Table 1). When the water pre-treatment was not performed, the pomace resulted to be very hygroscopic, and after grinding formed large particles with a mean diameter of ~682 μm, as compared to the smaller size (~82 μm) of the particles derived from the pre-treated pomace.

Table 1
Particle size and extraction yield (%) of not pre-treated and pre-treated grinded pomace.

Material	Particle size (μm)			Extraction Yield (%)
	D_{10}	D_{50}	D_{90}	
Not pre-treated	120 ± 6	682 ± 17	1640 ± 50	7 ± 1
Pre-treated	5 ± 1	82 ± 4	426 ± 13	5 ± 1

Pomace maceration was performed in a hydroethanolic solution (70:30 v/v) to ensure the extraction of both polar and low polar phytochemicals. The observed extraction yields were not particularly high: ~7% for not pre-treated and ~5% for pre-treated grape pomace skins, respectively (Table 1). The obtained not pre-treated and pre-treated extracts appeared as homogeneous powders with small particles and highly hygroscopic. The pre-treated extract was light and vivid purple powder (Fig. 1A), while the not pre-treated extract was heavy and purple-brown colored (Fig. 1B).

3.2. Identification of recoverable phytochemical compounds

The base peak chromatograms obtained by HPLC–ESI–TOF–MS analysis showed high background signals and extracted ions chromatograms were obtained for not pre-treated extract (Fig. 2 A and B) and pre-treated extract (Fig. 2 C and D).

The performed analysis allowed the detection of 55 different compounds in both the extracts, 45 were detected in negative mode while 10 were observed in positive mode. Among these, 45 compounds were identified (Table 2).

The majority of the 45 detected compounds were found in both the pre-treated and not pre-treated extracts and they have been previously described in grape or wine-making by-products [34,39]. The identified compounds belonged to different chemical classes that included organic acids, phenolic compounds (benzoic acids, flavonoids, stilbenes, anthocyanin and proanthocyanidin), triterpenes and fatty acids.

3.2.1. Organic acids

According to the mass spectroscopy analysis and HPLC elution profile, a total of five compounds were identified as organic acids. Tartaric, malic, citric acids and 2-isopropylmalic acid (peaks 3,4,5 and 7), which are commonly present in grapes or their by-products [34]. While gluconic acid (peak 2), was previously associated with other biomasses, to the best of our knowledge this is the first time that it is detected in grape pomace skin [32,33]. All the organic acids were detected in the not pre-treated extract, and only two of them were observed in the pre-treated extract (peaks 2 and 3).

3.2.2. Phenolic compounds

The adopted method made possible the identification of 25 different phenolic compounds: one benzoic acid, seventeen flavonoids, four stilbenes, one anthocyanin and two proanthocyanidins.

3.2.2.1. Benzoic acids. Syringic acid (peak 12) was the only benzoic acid to be detected, and only in the not-pre-treated extract. To the best of our knowledge, this compound has been detected in grape pomace skin for the first time in this study [35,36].

3.2.2.2. Flavonoids. Results revealed the presence of 17 flavonoids in the extract, including nine aglycons and eight derivatives. The aglycons detected have been all previously described in grapes or grape by-products. In the present study, catechin isomers (peaks 8, 10, a and c), fisetin (peaks 23 and i), quercetin (peaks 20 and f) and myricetin (peak 19) have been detected in both extracts [38,39,41]. Among the derivatives, only epicatechin gallate (peak 14) was found in the pre-treated extract and myricetin hexoside (peak 11) in the not pre-treated one [38,39]. Conversely, quercetin hexoside (peaks 13 and e), syringetin hexoside (peak 11), quercetin-3-methyletere (peaks 24 and j) and apigenin-6,8-di-C-arabinoside (peak d) were detected in both extracts, and apigenin-6,8-di-C-arabinoside for the first time in this study [38,39,42,51].

3.2.2.3. Stilbenes. ε-Viniferin and isomers were found in both extracts (peaks 18,21 and g), whilst α-Viniferin was detected only in the pre-treated one (peak h). The presence in grapes of all the stilbenes

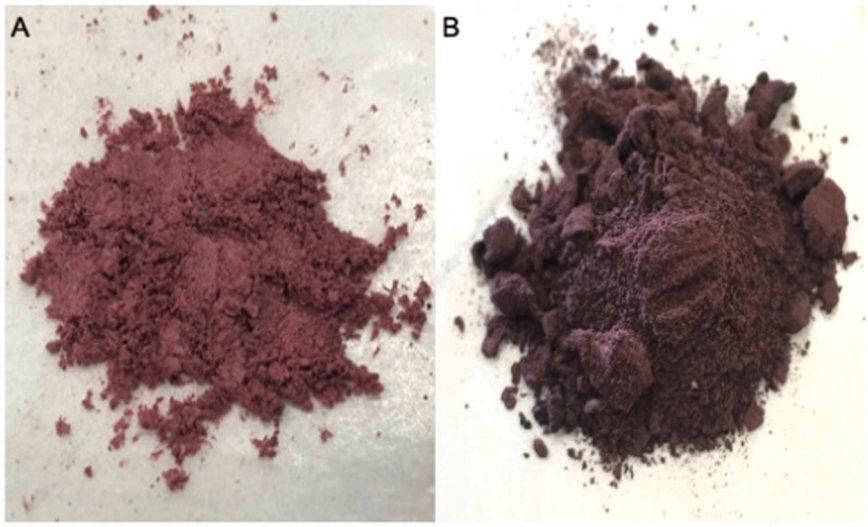

Fig. 1. Images of the obtained pre-treated (A) and not pre-treated (B) extracts of grape skins from pomaces.

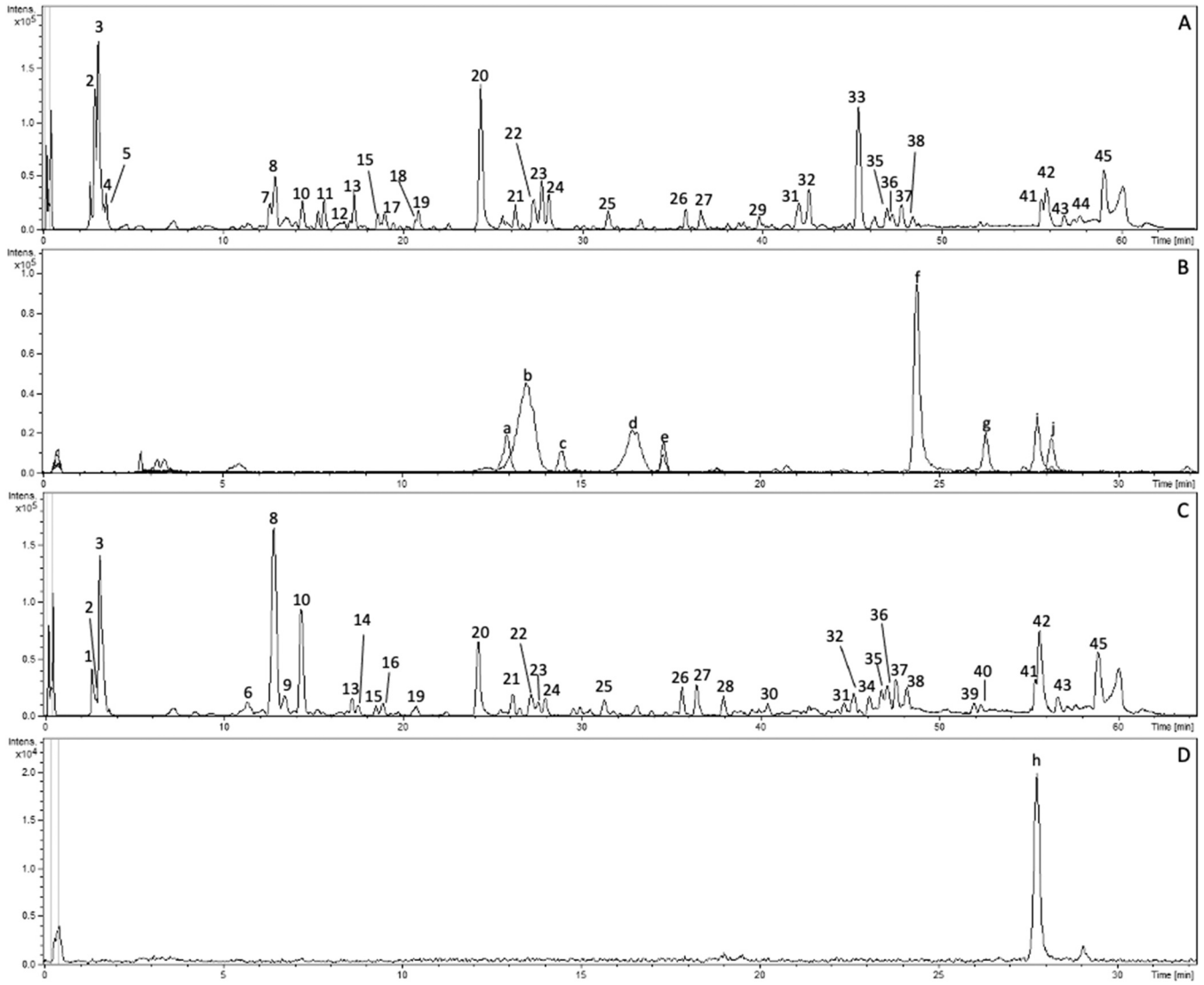

Fig. 2. Base Peak Chromatograms of not pre-treated (A) and pre-treated (C) extract obtained by HPLC-ESI-TOF-MS analysis in negative mode, and Extracted Ions Chromatogram of not pre-treated (B) and pre-treated (D) extract obtained by HPLC-ESI-TOF-MS analysis in positive mode.

Table 2
Compounds found in not pre-treated (NPT) and pre-treated (PT) extracts. Retention time, molecular formula, experimental and theoretic *m/z* ([M-H]$^-$ or [M-H]$^+$), error, mSigma, extract in which the compounds were detected and bibliographic references.

Peak	RT (min)	Molecular Formula	*m/z* exp.	*m/z* theoric	Error (ppm)	mSigma	Compound	Extract	Reference
[M-H]$^-$									
Organic acids									
2	2.9	$C_6H_{12}O_7$	195.0527	195.0510	-8.4	3.3	Gluconic acid	NPT, PT	[32,33]
3	3.1	$C_4H_6O_6$	149.0107	149.0092	-10.5	2.4	Tartaric acid	NPT, PT	[34]
4	3.4	$C_4H_6O_5$	133.0155	133.0142	-9.1	22.7	Malic acid	NPT	[34]
5	3.6	$C_6H_8O_7$	191.0216	191.0197	-9.9	7.3	Citric acid	NPT	[34]
7	12.7	$C_7H_{12}O_5$	175.0614	175.0612	-1.2	2.1	2-Isopropylmalic acid	NPT	[34]
Benzoic acids									
12	17.1	$C_9H_{10}O_5$	197.0460	197.0455	-2.5	8.8	Syringic acid	NPT	[35,36]
Flavonoids									
8	12.9	$C_{15}H_{14}O_6$	289.0724	289.0718	-2.0	1.7	Catechin isomer	NPT, PT	[32,37,38]
10	14.5	$C_{15}H_{14}O_6$	289.0705	289.0718	4.5	3.5	Catechin isomer	NPT, PT	[32,37,38]
11	15.7	$C_{21}H_{20}O_{13}$	479.0814	479.0831	3.7	20.2	Myricetin hexoside	NPT	[39]
13	17.3	$C_{21}H_{18}O_{13}$	477.0689	477.0675	-2.9	6.3	Quercetin hexoside	NPT, PT	[39]
14	17.5	$C_{22}H_{18}O_{10}$	441.0828	441.0827	-0.3	20.9	Epicatechin gallate	PT	[38]
15	18.6	$C_{23}H_{24}O_{13}$	507.1172	507.1144	-5.5	18.8	Syringetin hexoside	NPT, PT	[39]
19	20.9	$C_{15}H_{10}O_8$	317.0328	317.0303	-7.8	22.9	Myricetin	NPT, PT	[40]
20	24.3	$C_{15}H_{10}O_7$	301.0375	301.0354	-7.1	3.4	Quercetin	NPT, PT	[38]
23	27.7	$C_{15}H_{10}O_6$	285.0413	285.0405	-2.8	10.9	Fisetin	NPT, PT	[41]
24	28.1	$C_{16}H_{12}O_7$	315.0519	315.0510	-2.9	9.5	Quercetin-3-methyletere	NPT, PT	[42]
Stilbenes									
18	20.7	$C_{28}H_{22}O_6$	453.1372	453.1344	-6.2	11.0	ε-Viniferin or isomer	NPT	[39,43]
21	26.3	$C_{28}H_{22}O_6$	453.1364	453.1344	-4.4	18.4	ε-Viniferin or isomer	NPT, PT	[39,43]
Pronthocyanidins									
6	11.3	$C_{30}H_{26}O_{12}$	577.1361	577.1351	-1.6	9.3	Proanthocyanidin b2 isomer	PT	[37,44,45]
9	13.4	$C_{30}H_{26}O_{12}$	577.1361	577.1351	-1.7	24.2	Proanthocyanidin b2 isomer	PT	[37,44,45]
Triterpenes									
27	36.5	$C_{30}H_{48}O_4$	471.3519	471.3480	-8.4	6.3	Maslinic acid	NPT, PT	[46]
43	56.7	$C_{30}H_{48}O_3$	455.3543	455.3531	-2.7	21.0	Oleanolic acid isomer	NPT, PT	[46–48]
44	57.6	$C_{30}H_{48}O_3$	455.3555	455.3531	-5.2	20.7	Oleanolic acid isomer	NPT	[46–48]
Fatty acids									
22	27.3	$C_{18}H_{34}O_5$	329.2339	329.2333	-1.6	7.3	9,10,13-Trihydroxy-11-octadecenoic acid	NPT, PT	[49]
28	37.9	$C_{18}H_{34}O_4$	313.2389	313.2384	-1.6	2.4	12-Hydroperoxy-octadecadienoic acid	PT	[32]
29	39.8	$C_{18}H_{32}O_4$	311.2248	311.2228	-6.6	16.0	13-Hydroperoxy-octadecadienoic acid	NPT	[32]
31	42.0	$C_{18}H_{30}O_3$	293.2135	293.2122	-4.3	5.5	Hydroxy-octadecatrienoic acid isomer	NPT, PT	[32,37]
32	42.5	$C_{18}H_{30}O_3$	293.2124	293.2122	-0.8	1.7	Hydroxy-octadecatrienoic acid isomer	NPT, PT	[32,37]
33	45.2	$C_{18}H_{32}O_3$	295.2281	295.2279	-0.9	1.4	Hydroxy-octadecadienoic acid	NPT	[32,37]
35	46.9	$C_{18}H_{30}O_3$	293.2133	293.2122	-3.6	5.3	Hydroxy-octadecatrienoic acid isomer	NPT, PT	[32,37]
36	47.2	$C_{18}H_{30}O_3$	293.2148	293.2122	-9.0	10.6	Hydroxy-octadecatrienoic acid isomer	NPT, PT	[32,37]
37	47.7	$C_{18}H_{30}O_3$	293.2144	293.2122	-7.4	12.4	Hydroxy-octadecatrienoic acid isomer	NPT, PT	[32,37]
38	48.3	$C_{18}H_{30}O_3$	293.2146	293.2122	-8.0	17.8	Hydroxy-octadecatrienoic acid isomer	NPT, PT	[32,37]
41	55.4	$C_{18}H_{30}O_2$	277.2182	277.2173	-3.2	7.2	Linolenic acid	NPT, PT	[32,47,50]
45	58.9	$C_{18}H_{32}O_2$	279.2337	279.2330	-2.8	6.3	Linoleic acid	NPT, PT	[32,47,50]
Unknowns compounds									
1	2.7	$C_{20}H_{26}N_2O_{19}$	597.1091	597.1057	-5.7	9.3	UK 1	PT	
16	18.9	$C_{33}H_{26}N_4O_{10}$	637.1608	637.1576	-5.0	22.6	UK 2	PT	
17	19.0	$C_{26}H_{30}N_4O_{15}$	637.1622	637.1635	2.0	7.8	UK 3	NPT	
25	31.4	$C_{13}H_{18}O_3$	221.1183	221.1183	0.0	5.0	UK 4	NPT, PT	
26	35.7	$C_{25}H_{44}N_4O_2$	431.3403	431.3392	-2.6	5.5	UK 5	NPT, PT	
30	40.4	$C_{26}H_{35}N_7O_2$	476.2807	476.2779	-5.8	21.7	UK 6	PT	
34	46.0	$C_{31}H_{42}N_4$	469.3358	469.3337	-4.4	12.0	UK 7	PT	
39	51.8	$C_{26}H_{46}N_8O_3$	517.3588	517.3620	6.2	23.1	UK 8	PT	
40	52.2	$C_{16}H_{30}O_3$	269.2141	269.2122	-7.1	5.3	UK 9	PT	
42	55.7	$C_{16}H_{32}O_3$	271.2293	271.2279	-5.5	2.7	UK 10	NPT, PT	
[M-H]$^+$									
Flavonoids									
a	12.9	$C_{15}H_{14}O_6$	291.0853	291.0863	3.6	2.0	Catechin isomer	NPT, PT	[34,37,38]
c	14.5	$C_{15}H_{14}O_6$	291.0865	291.0863	-0.6	6.3	Catechin isomer	NPT, PT	[34,37,38]
d	16.4	$C_{25}H_{26}O_{13}$	535.1480	535.1446	-6.3	7.4	Apigenin-6,8-di-C-arabinoside	NPT, PT	[51]
e	17.3	$C_{21}H_{18}O_{13}$	479.0832	479.0820	-2.5	7.5	Quercetin hexoside	NPT	[39]
f	24.4	$C_{15}H_{10}O_7$	303.0491	303.0499	2.7	4.7	Quercetin	NPT, PT	[38]
i	27.7	$C_{15}H_{10}O_6$	287.0539	287.0550	4.1	11.6	Fisetin	NPT, PT	[41]
j	28.1	$C_{16}H_{12}O_7$	317.0677	317.0656	-6.8	19.3	Quercetin-3-methyletere	NPT, PT	[42]
Anthocyanins									
b	13.5	$C_{23}H_{24}O_{12}$	493.1310	493.1341	6.1	17.6	Malvidin glucoside	NPT, PT	
Stilbenes									
g	26.3	$C_{28}H_{22}O_6$	455.1463	455.1489	5.8	29.8	ε-Viniferin	NPT, PT	[39,43]
h	27.7	$C_{42}H_{30}O_9$	679.1960	679.1963	0.4	25.8	α-Viniferin	PT	[39]

highlighted by the adopted characterization method is confirmed by Flamini et al. [39].

3.2.2.4. Anthocyanins and proanthocyanidins. Malvidin hexoside (peak b) was the only anthocyanin compound detected in both extracts. On the other hand, two peaks detected in the pre-treated extract (6 and 9) were identified as proanthocyanidin B2 isomers, consistently with other studies [44,53].

3.2.3. Triterpenes

Three triterpenes, maslinic acid (peak 27) and oleanolic acid isomers (43 and 44) were identified, and maslinic acid, a triterpenoid found in both the extracts, for the first time in this study as a component of grapes or grapes by-products [46].

3.2.4. Fatty acids

Twelve fatty acids were observed in the two extracts, 10 of them, trihydroxy-octadecenoic acid (peak 22), hydroxy-octadecatrienoic acid or isomers (peaks 31, 32, 35, 36, 37 and 38), hydroxy-octadecadienoic acid (peak 33), linolenic acid (peak 41) and linoleic acid (peak 45), were detected in both pre-treated and not-pre-treated extracts. Hydroperoxy-octadecadienoic acid (peak 29) was present only in the not-pre-treated extract, while compound number 28 was only found in the pre-treated one. Among these compounds, only linolenic acid and linoleic acid have been previously described as components of grapes [47].

3.3. Evaluation of total phenolic content and antioxidant activity of the extracts

The total phenolic content was determined according to the Folin-Ciocalteau assay, which provides a quick estimation. The phenolic content of in pre-treated extract (23.8362 mg GAE/g of dry extract) was higher than in the not pre-treated extract (15.43298 mg GAE/g of dry extract), possibly as a consequence of the pre-treatment with water that removed several hydrophilic molecules, like sugars, from the grape pomace skins. This claim seems to be supported by the observed higher antioxidant activity of the pre-treated extract, quantified with the DPPH assay and referred to Trolox (31.88% and 4.33677 mg TE/g) as compared to that of the not pre-treated one (18.60% and 2.56276 mg TE/g).

Given that, the pre-treated extract was considered more suitable for the aim of the present study, and thus loaded into specifically tailored phospholipid vesicles and used for the further technological and biological assessments.

3.4. Characterization of the vesicles containing the extract of grape pomace skins

Vesicles were prepared by using Phospholipon S75 as the main phospholipid and filled with the pre-treated extract. The solid components were hydrated with water (liposomes) or water and glycerol (glycerosomes). Furthermore, Montanov® 82, a nonionic alkylpolyglucoside emulsifier of natural origin, was added to the formulations to increase the stability of the vesicles and their permeation ability

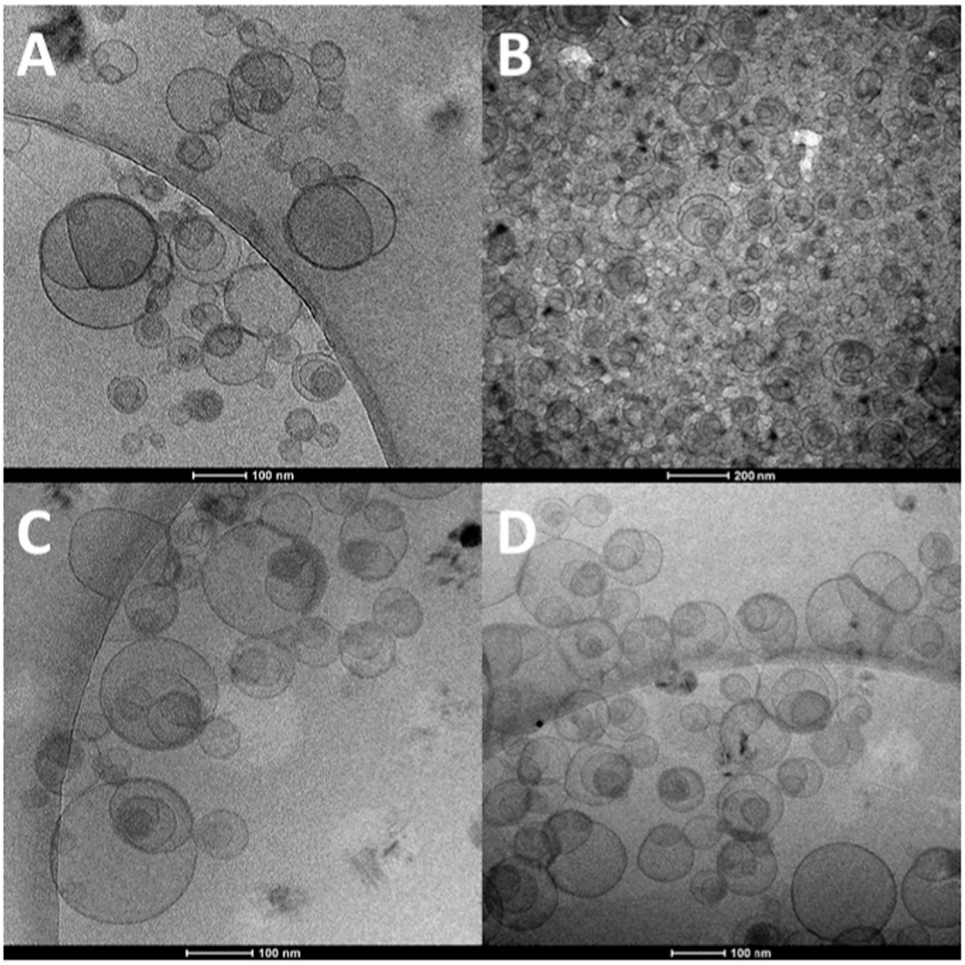

Fig. 3. Representative cryo-TEM images of liposomes (A), montanov-liposomes (B), glycerosomes (C) and montanov-glycerosomes (D).

(montanov-liposomes and montanov-glycerosomes).

The structure and morphology of the vesicles (Fig. 3) were evaluated by cryo-TEM. All vesicles were spherical, uni- or bi-lamellar and sometimes multi-compartment, especially when glycerol was used.

The average diameter, polydispersity index and surface charge of vesicles were measured and compared as a function of the used components. Empty vesicles were used as reference.

Empty vesicles were the smallest (~55 nm) and characterized by a negative surface charge (~−78 mV), and highly polydispersed (polydispersity index ~0.56) (Table 3). The incorporation of the extract into vesicles caused a significant increase in size, probably due to the intercalation of lipophilic molecules in the bilayer, which allowed a modification of phospholipid packing, an enlargement of the bilayer thickness and different vesicle assembling. The size of extract loaded liposomes and glycerosomes was around 190 nm, and the presence of Montanov® 82 caused a further increase up to ~214 nm for liposomes and ~279 nm for glycerosomes, confirming an interaction between the surfactant Montanov® 82 and bilayer. Although Montanov® 82 caused an increase of the vesicle size, its addition led to an improvement of the homogeneity of the system as the polydispersity index slightly decreased (0.30 for liposomes and 0.28 for glycerosomes, Table 3). The surface charge of all the extract loaded vesicles was ~−47 mV and always lower than that of the corresponding empty vesicles, probably due to the positive charge of some extract components found on the vesicle surface.

All the formulations proved to be capable to retain the grape pomace extract in high amount as the entrapment efficiency was always ~100% for all the formulations, irrespective of their composition (Table 3).

3.5. Evaluation of the antioxidant activity of the extract in methanolic solution or loaded in vesicles

Quercetin, catechin isomers, ε and α viniferin and myricetin were the main components of the PT extract and the expected antioxidant activity was assessed by performing the DPPH colorimetric assay (Table 4), which gives a measure of the ability of the extract to scavenge free radicals. The antioxidant activity of the extract in ethanolic solution (30 mg/ml as in the formulations) was 84% ± 1 and slightly increased up to 89% ± 1 after loading into the vesicles with the notable exception of montanov-glycerosomes whose activity did not benefit from the use of vesicles and confirmed values equal to those observed in solution (86% ± 2). These results show that the loading of the extract into the vesicles did not affect its antioxidant capability.

3.6. Biocompatibility of formulations

The biocompatibility of the extract in aqueous dispersion or loaded into vesicles was evaluated by using fibroblasts (3T3), which represent the main cells of the dermis. To assess the biocompatibility and select non-toxic concentrations, the cells were incubated for 48 h with formulations properly diluted with medium to reach four different concentrations of the extract (0.03, 0.3, 3 and 30 μg/ml). The cell viability was measured by using the MMT assay (Fig. 4).

The viability of 3T3 cells treated with the extract in aqueous dispersion was ~100% irrespective of the used concentration. At the highest concentration, the viability of cells treated with the extract loaded in vesicles was lower except for montanov-liposomes. In particular, the viability of the cells incubated with the extract loaded glycerosomes was the lowest, 71%± 8 ($p < 0.05$). Conversely, when exposed to the lower concentrations the cell viability was ~100% regardless of the composition of the formulations ($p > 0.05$).

Table 3
Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (E) of empty and grape pomace extract loaded vesicles. Mean values ± standard deviations are reported (n ≥ 3). Same symbols (*, #, §) indicate same values ($p > 0.05$).

	MD (nm)	PI	ZP (mV)	E (%)
Empty liposomes	*, #57 ± 5	0.52	-80 ± 6	–
Empty montanov-liposomes	#61 ± 7	0.47	-83 ± 3	–
Empty glycerosomes	*48 ± 7	0.60	-74 ± 2	–
Empty montanov-glycerosomes	*,#49 ± 5	0.46	-86 ± 12	–
Extract liposomes	§183 ± 24	0.33	-52 ± 12	101 ± 1
Extract montanov-liposomes	§214 ± 19	0.30	-51 ± 5	100 ± 1
Extract glycerosomes	§194 ± 25	0.37	-39 ± 3	99 ± 2
Extract montanov-glycerosomes	279 ± 22	0.28	-52 ± 6	99 ± 1

Table 4
Antioxidant activity (AA%) of the extract from grape pomace skin in ethanolic solution or incorporated into vesicular formulations. Mean values ± standard deviations are reported (n ≥ 3). Same symbols (*, #, §) indicate same values ($p > 0.05$).

	Antioxidant Activity (AA%)
Dispersion	*84 ± 1
Liposomes	#90 ± 1
Montanov-liposomes	#89.5 ± 0.1
Glycerosomes	#, §88 ± 1
Montanov-glycerosomes	*, §86 ± 2

3.7. Protective effect of the formulations against damages induced by hydrogen peroxide in 3T3 cells

The capability of the extract in aqueous dispersion or loaded in vesicles to protect the cells against oxidative stress was evaluated in vitro by stressing 3T3 cells with hydrogen peroxide, the most widely used apoptosis inducer capable of causing cell death in a time- and concentration-dependent manner [54]. The exposure to hydrogen peroxide significantly reduced the cell viability (~50%, Fig. 5). The simultaneous treatment of the stressed cells with the extract in aqueous dispersion partially prevented the oxidative damage, as the viability increased up to ~70% ($p < 0.05$ versus hydrogen peroxide). The incorporation of the extract into liposomes and montanov-glycerosomes at the lower concentration (3 μg/ml and 0.3 μg/ml, respectively) and into glycerosomes at the lowest concentration (0.3 μg/ml) further enhanced the protection of the cells, as the viability was ≥83%. These results confirmed that the proposed formulations represent a valuable approach for the safe and effective delivery of the extract into cells impaired by oxidative stress Fig. 5.

4. Discussion

In the present study, valuable bioactive molecules still contained in grape by-products were extracted and used to obtain alternative cosmeceutical products [55]. The valorization of grape by-products can avoid environmental issues caused by inappropriate management, and the recovery of high-value products is consistent with a circular economy approach and promotes the sustainability of the winery sector [56].

In view of the application of the results obtained, some considerations need to be made. Considering the large diversity and metabolite wealth of Sardinian vascular flora, the pomace obtained from a local cultivar (Carignano) of Sardinia was used [57–59]. In this respect, the specific characteristics of the area of cultivation can improve the metabolite concentration in the grapefruits [60]. The dried powder directly obtained from the Carignano grape pomace skins does not represent a valuable matrix with high potential as it is, because, though rich in fibbers and antioxidant molecules, it cannot be as effective as the isolated phytocomplex in terms of biological potential, antioxidant capability and protective efficacy [61]. Therefore, extraction is necessary, and the marketability of the obtained extracts mainly depends on the sustainability of the process. Indeed, significant efforts are made to develop affordable extraction and environmentally friendly techniques involving a reduced consumption of solvents and energy and characterized by high extraction efficiency [62–64]. Accordingly, a relatively

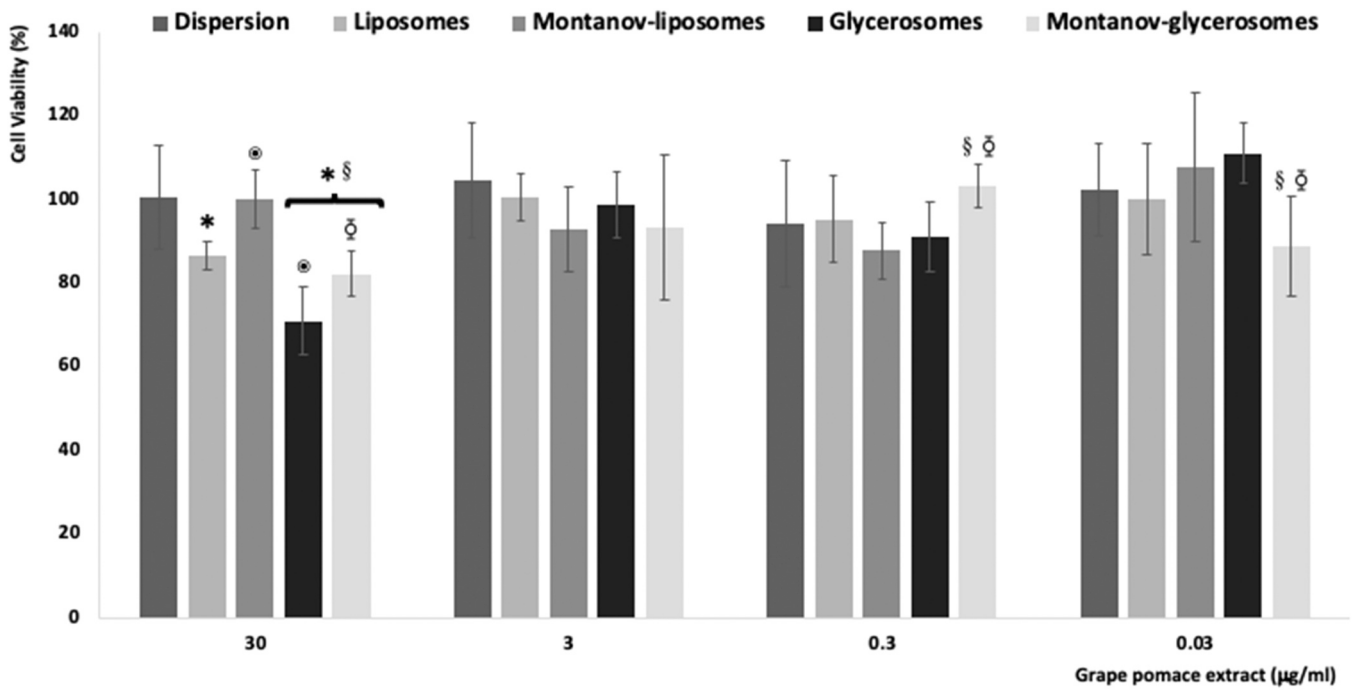

Fig. 4. Viability of 3T3 cells incubated for 48 h with the extract in dispersion or loaded in liposomes, montanov-liposomes, glycerosomes and montanov-glycerosomes. Mean values ± standard deviations are reported. The symbol * indicates values that were statistically different from the extract in dispersion; the symbol ⊙ indicates values that were statistically different from liposomes; the symbol § indicates values that were statistically different from montanov-liposomes and the symbol ♀ indicates values that were statistically different from glycerosomes ($p < 0.05$).

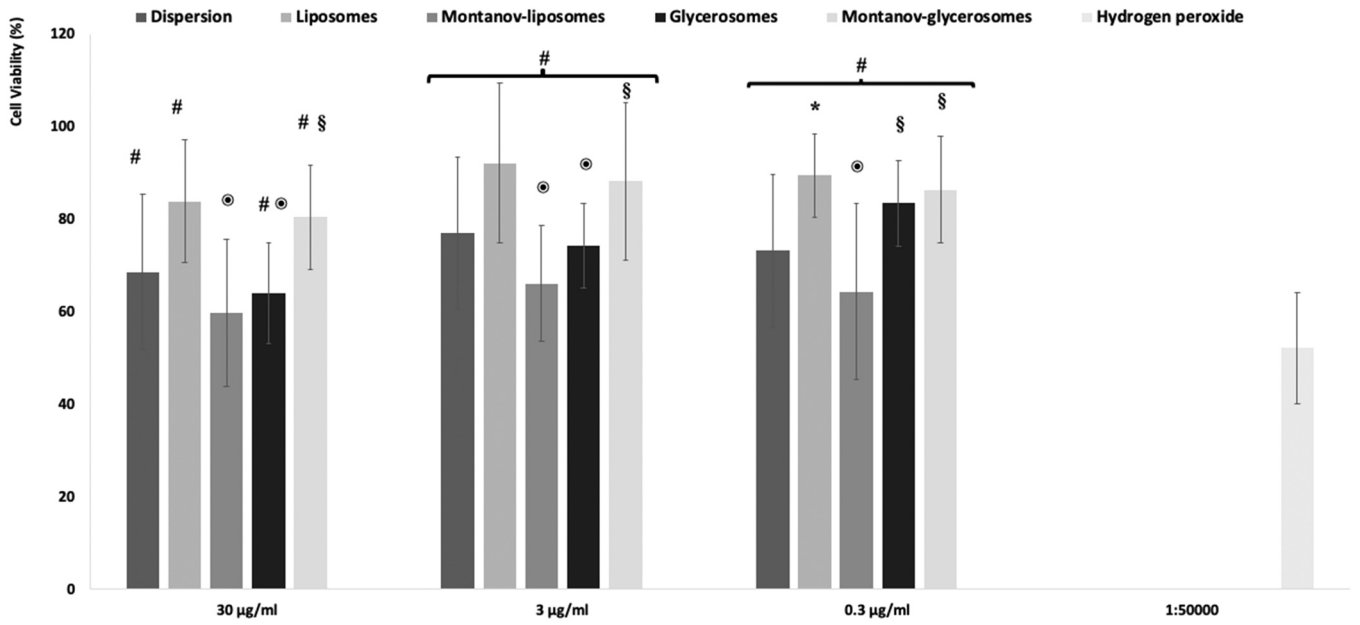

Fig. 5. Viability of 3T3 cells exposed to hydrogen peroxide and treated with the extract in dispersion or loaded into liposomes, montanov-liposomes, glycerosomes and montanov-glycerosomes. Mean values ± standard deviations are reported. The symbol # indicates values that were statistically different from hydrogen peroxide; the symbol * indicates values that were statistically different from extract dispersion; the symbol ⊙ indicates values that were statistically different from liposomes; the symbol § indicates values that were statistically different from montanov-liposomes ($P < 0.05$).

simple method has been tested to extract the bioactive components still present in the grape skin found in the pomace produced during wine-making [23]. The pre-treatment of grape skins with water allowed for the removal of residual soluble sugars and obtaining a more concentrated extract with higher phenols content and, in turn, antioxidant capability. Moreover, the reduction of the sugar presence made possible the production of a less sticky and hygroscopic extract.

Extraction methods based on traditional treatments such as mechanical agitation can be inefficient on the share the phenolic compounds that are bound or trapped within proteins, polysaccharides, on cell walls [65]. In this study, the alternate ultrasound treatment, performed in a mixture of ethanol and water (70:30 v/v) as extractive medium, facilitated the break of plant structures promoting the extraction and the recovery of a considerable amount of phenolic compounds.

In a framework of challenging search for extraction technologies characterized by reduced operating costs and demand for chemicals and energy, along with low water consumption, ultrasound treatment represents one of the most promising options. Process parameters can be easily arranged to match the objectives, it can be combined with other treatments and replace mechanical stirrers to provide effective agitation. From an industrial point of view, ultrasound systems are easy to scale up, install and maintain, and are characterized by competitive energy costs, in particular in the case of extraction of bioactive compounds from plant and animal sources [66–68].

The use of a mixture of two solvents such as water and ethanol characterized by different properties is more effective than a single-solvent approach [69,70]. Indeed, according to previous studies the ethanol-water blend is effective in extracting polar and mid-polar compounds, leading to a high content of phenols and antioxidants in the extracts [71]. Due to both the polar and slightly polar nature of the phenolic grape metabolites, they are easily solubilized in protic media such as hydroalcoholic solutions, as the water presence (30%) softens the organic nature of ethanol and may facilitate the extraction of phenolic compounds with large and small molecular structure [70,72]; this is confirmed by studies performed on other plant biomasses by using ethanol-water blends containing at least 50–60% of ethanol [73,74]. Finally, the use of ethanol (70%) allows solvent recovery with less energy, therefore the process is more suitable for a real scalable application.

A total of 45 compounds were detected in the extracts, most of which were already known to be present in grape and by-products [34,39]. Although 30 over 45 compounds were detected regardless of whether a pretreatment had been performed, some of great interest such as proanthocyanidin b2 isomer and epicatechin gallate were found only in the pre-treated extract.

Recently, several studies have reported the antioxidant potential of extracts obtained from wine-making by-products [14,75]. The present study highlighted that loading the Carignano grape skin extract in ad hoc formulated phospholipid vesicles (liposomes, glycerosomes and montanov-glycerosomes) improved the bioavailability of phytochemicals in both skin and intestine, confirming the results obtained in previous studies [76–78]. The produced formulations enhanced the grape extract capability to protect the cells from oxidative stress-induced by hydrogen peroxide as the hydrogen peroxide-induced ROS production and lipid peroxidation was effectively suppressed [79,80]. Moreover, the presence of glycerol and the nonionic alkylpolyglucoside surfactant (montanov) promotes the penetration ability of vesicles into and through the skin, as previously observed [81,82].

5. Conclusion

The grape skin sorted from the pomace of Carignano cultivar (Sardinia) proved to be a suitable matrix to obtain a phytocomplex rich in bioactive compounds characterized by high antioxidant capability. The pre-treatment of grape skins with water reduced the content of sugar and ensured the production of an extract richer in antioxidant compounds and less sticky. The main active components contained in the extract were quercetin, catechin isomers, ε and α viniferin and myricetin. The ability of the extract to protect fibroblasts, which are considered the most representative cells of the dermis, from oxidative stress was improved by its loading into liposomes, glycerosomes and montanov-glycerosomes. The results achieved suggest that the studied formulations may be added to conventional topical formulations like cream and ointment, to obtain safe and natural cosmeceutical products capable of effectively protecting the skin from oxidative damage. Turning the main winery process residues into components of marketable products fosters the sustainability, and economic and environmental resilience of the winery sector, consistently with the principles of environmentally sound waste management and circular economy. Therefore, the findings of the present study contribute to the perspectives of full integration of winery by-products into the production cycle that originated them through an end-of-waste procedure.

CRediT authorship contribution Statement

Matteo Perra: Investigation, Formal analysis, Data curation, Writing – original draft. **Jesus Lozano-Sanchez**: Methodology, Validation, Writing – review & editing. **Francisco-Javier Leyva-Jimenez**: Investigation, Formal analysis. **Antonio Segura-Carretero:** Methodology, Investigation. **Jose Luis Pedraz:** Investigation. **Gianluigi Bacchetta:** Resources, Writing - review & editing. **Aldo Muntoni:** Data curation, Writing – original draft. **Giorgia De Gioannis:** Data curation, Writing – original draft. **Maria Letizia Manca**: Investigation, Data curation, Writing – original draft. **Maria Manconi:** Supervision, Project administration, Writing – review & editing.

Conflict of interest statement

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.

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