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Antioxidant and neuroprotective effects of nutriosomes and grape pomace phytochemicals in a cell model of oxidative stress and mouse model of Parkinson disease

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The aim of the present study was to evaluate the effectiveness of a Cannonau pomace extract loaded in nutriosomes in intestinal oxidative stress and a subacute model of Parkinson disease and compare the results with those previously obtained with a Nasco pomace extract loaded in the same nutriosomes. In this study, Cannonau pomace was extracted using ultrasound-assisted maceration to obtain a polyphenol-rich extract. The extract at two different concentrations (5 and 10 mg/mL) was loaded in the same nutriosomes previously used to deliver Nasco pomace extract were loaded in nutriosomes. The main physico-chemical and technological characteristics of the obtained vesicles, along with their stability over time were measured. The in vitro biocompatibility and ability of nutriosomes to protect intestinal epithelial cells (Caco-2) from oxidative damages, were evaluated. Finally, the effectiveness of repeated administration of Cannonau extract in dispersion or loaded in nutriosomes against the neurotoxic effects in mice was evaluated. The resulting vesicles had uniform distribution, controlled the release of payloads, were biocompatible, like nutriosomes loading Nasco pomace extract, protected the intestinal cells against damages induced by hydrogen peroxide, in a better extent than the Nasco nutriosomes. However, no significant neuroprotective effects were observed in in vivo experiments performed using a subacute model of Parkinson disease, unlike previous results obtained with Nasco pomace extract loaded nutriosomes. This discrepancy is possibly due to the absence of key polyphenols like procyanidin B2 in the Cannonau extract, which are instead present in the Nasco extract. The findings suggest that while nutriosomes enhance the bioavailability and efficacy of grape pomace extracts in vitro, their therapeutic potential in vivo may depend on the specific phenolic profile of the extract used. Further research is needed to optimize formulations and explore synergistic combinations for improved neuroprotective outcomes.

Keywords Phospholipid vesicles, Neurodegeneration, Antioxidant activity, Neuroprotection, Nanotechnology, Caco-2 cells, Parkinson disease

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Grape pomace, the main by-product of the winemaking process, is rich in beneficial compounds, especially polyphenols, flavonoids, and other antioxidants[1], that, over the last decades, have gained attention at scientific level[2]. Their sustainable extraction not only aligns with the eco-friendly guidelines of the European Union but also allows to obtain valuable compounds with therapeutic, nutritional, and health-promoting potential due to their antioxidant, anti-aging, anti-inflammatory, cardioprotective and neuroprotective effects, as evidenced by various in vitro and in vivo studies[3–6]. Unfortunately, often the translation of these in vitro findings to in vivo settings has been inconsistent, probably because the complex dynamics of biological systems that notably reduce the bioavailability and biodistribution of bioactive molecules, especially in the brain, particularly after oral administration[7–9]. To overcome these challenges, the delivery of these natural chemicals in ad-hoc formulated nanocarriers is deemed to be an effective approach to augment their bioavailability to the brain and minimize required doses[10,11]. Among different nanocarriers, phospholipid nanovesicles stand out due to their versatility, biodegradability, and biocompatibility[12]. However, the journey of optimising the loading of natural molecules within these phospholipid vesicles is complicated and depends on the physico-chemical characteristics of the payload, the desired concentration and the route of administration[13,14]. This knowledge is crucial for *ad-hoc* tailoring formulations to achieve consistent and optimized therapeutic or nutritional benefits. Over the years, phospholipid vesicles have been modified for the desired administration routes and payloads. Nutriosomes, for example, have been introduced to the oral administration of natural antioxidants[15,16]. Nutriosomes are obtained by combining Nutriose®, a water-soluble branched maltodextrin obtained from maize, with phospholipids. They have been widely used in the past years to efficiently deliver several molecules through oral administration, thanks to their peculiar characteristics such as: ability to improve payload bioavailability, targeted delivery of payloads to the intestinal tract, versatility and own probiotic effect[17,18]. In particular, they were effectively employed to enhance the systemic bioavailability of curcumin and quercetin as adjuncts in malaria treatment[16]. In another study, nutriosomes loaded with curcumin were further modified with eudragit, a polyanionic co-polymer that improved the enteric stability of the vesicles, before being orally administered to malaria-infected mice[15]. Obtained results highlighted their ability to significantly increase the survival of animals with respect to curcumin given as a dispersion, probably due to enhanced bioavailability of payload and its antioxidant effect at the intestinal level, which may boost the antimalarial activity at the systemic level[15]. Other studies also proved the ability of nutriosomes to improve intestinal and systemic delivery of grape pomace extracts[1,19,20]. In the most promising, the oral administration of nutriosomes loaded with grape pomace extract from white Nasco cultivar significantly contrasted the dopaminergic neurodegeneration and neuroinflammation in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson disease. Importantly, the oral administration of the same extract in dispersion was found to be ineffective, advocating the superior capacity of nutriosomes, as optimal delivery system[20,21]. Clearly, nanocarriers enhanced the local availability of the whole phytocomplex containing different polyphenols, which variability across different grape cultivars and extraction methods may affect the final in vivo activity[22–24]. For example, in their study Narita et al., evaluated the potential neuroprotective effects of two grape seed extracts from a white cultivar, Koshu, and a red one, Muscat Bailey A[24]. They demonstrated that the extract from white but not that from the red cultivar exerted neuroprotective effects. Comparing the phenolic content of the two extracts they found notable differences, particularly in the amount of small molecular weight polyphenols and procyanidin oligomers, such as procyanidin B1 and procyanidin B2[24].

Considering these findings, the aim of the current study was to understand if another grape pomace extract has the same chemical composition of Nasco extract and, when loaded in nutriosomes, can exert the same anti-neurodegenerative and neuroinflammation effect. In order to this, the new extract was obtained from red Cannonau cultivar, which is cultivated in the same area of Nasco. The extraction was performed with the same method used for Nasco extract and the former was loaded in the same nutriosomes, and its efficacy was evaluated using the same models[17,18]. The novelty of the study lies on deeper analyse and compare the composition of two grape pomace extracts and their in vivo effects at neurological level to select the possible responsible molecules, and if they are effective alone or in combination with other chemicals of phytocomplex. The main physico-chemical (size, zeta potential, polydispersity index) and technological characteristics (entrapment efficiency, payload release) of the obtained Cannonau nutriosomes, along with their stability over time were measured. Moreover, their biocompatibility and ability to protect intestinal epithelial cells (Caco-2) from damages caused by hydrogen peroxide-induced oxidative stress, were evaluated. The effectiveness of repeated administration of Cannonau extract in dispersion or loaded in nutriosomes against the neurotoxic effects in mice was evaluated.

Materials and methods

Materials

Grape pomaces from Cannonau cultivar were provided by a local winery (Cantine Argiolas), harvested in Sardinia in September–October 2020. Samples were dried and stored under vacuum at − 20 °C until use. Lipoid S75 (S75) was purchased from Lipoid (Ludwigshafen, Germany). Nutriose®FM06 was kindly supplied by Roquette (Lestrem cedex, France). Ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteau, gallic acid, trolox and all other reagents of analytical grade were purchased from Sigma-Aldrich (Milan, Italy). Reagents and plastics for cell culture were purchased from Life Technologies Europe (Monza, Italy). MPTP was purchased from MedChemExpress (HY-15608) and was dissolved in distilled water.

Extraction process

The extraction was performed as previously described[20]. Briefly, grape pomaces were grinded to obtain a fine powder with small particles and high specific surface. The resulting powder was kept under a vacuum, at room temperature, in the dark until the extraction process was performed. Briefly, aliquots of 10 g of ground grape pomaces were suspended in 300 mL of a mixture of ethanol and water (70:30 v/v). The suspension was left under constant stirring at 40 °C in the dark for 2 h, the temperature was monitored and maintained below

40 °C throughout the entire process. Further, to facilitate dissolution of chemicals, two ultrasound cycles of 10 min each were employed, one at the beginning and the other after 1 h from the beginning of the extraction process. The resulting suspension was then centrifuged (15 min, 4000 rpm) to remove the coarse fractions. The ethanol was eliminated from the resulting solution under vacuum using a rotavapor (Rotavapor RII, BÜCHI Labortechnik AG, Flawil, Switzerland). Finally, the dispersion of the extract was freeze-dried, and the obtained powder was stored under vacuum in the dark until its use[19].

DPPH, FRAP and Folin–Ciocalteu assays

The ability of the extracts to scavenge free radicals was measured according to the DPPH assay. The Cannonau pomace extract was dissolved (at different concentrations) in ethanol and 20 µL of the ethanolic solutions were added to 1980 µL of DPPH methanolic solution (80 µg/mL). After 30 min of incubation in darkness at room temperature, the absorbance was read against a blank at 517 nm. The antioxidant activity was calculated according to the following equation (Eq. 1):

$$Antioxydantactivity\% = \frac{(A_{blank} - A_{sample})}{A_{blank}} \times 100 \quad (1)$$

where A_{blank} indicates the absorbance of the control (DPPH solution), while A_{sample} is the absorbance of the extract. To quantify the antioxidant activity, a calibration curve was built using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as reference and expressed as mg of Trolox equivalent/g of dry extract[25]. All the experiments were performed in triplicate.

The FRAP assay was used to measure the ability of antioxidant molecules to reduce a ferric-tripyridyltriazine complex into its coloured ferrous form. The FRAP reagent containing 2.5 mL of a 10 mM TPTZ (2,4,6- tripyridy-s-triazine, Sigma) solution in 40 mM chloridric acid, 2.5 mL of 20 mM of ferric chloride and 25 mL of 0.3 mol/L acetate buffer, at pH 3.6, was prepared freshly and warmed at 37 °C. Aliquots of extract sample were mixed with 0.2 mL of distilled water and 1.8 mL of FRAP reagent and incubated at 37 °C for 10 min. The absorbance of reaction mixture was spectrophotometrically measured at 593 nm. Ferrous sulphate (1 mM) was used as the standard solution. The final results are expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM ferrous sulphate. Dilution was applied as necessary if the measured FRAP value exceeded the linear range of the standard curve[26].

The amount of total soluble phenolics was determined according to the Folin-Ciocalteu method[27]. The Cannonau pomace extract was diluted progressively with distilled water, and subsequently, 100 µL of each diluted solution was pipetted in triplicate into a 96-well plate. 50 µL of Folin-Ciocalteu reagent diluted fivefold with water and 50 µL of 10% (w/v) sodium carbonate solution were added to each well, and the plate was incubated for 5 min at room temperature. Colour development at 650 nm was then determined using a microplate reader (Synergy 4, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). Gallic acid was used as reference to prepare a calibration curve using progressive dilutions, results are expressed as gallic acid equivalents/g of dry extract (µmol/g).

Liquid chromatography-quadruple time-of-flight mass spectroscopy analysis of the Cannonau pomaces extract

The composition of the Cannonau pomace extract was analysed with a liquid chromatography-quadruple time-of-flight mass spectrometry to determine the presence and concentration of phenolic compounds. Prior to analysis, calibration curves were build using analytical pure standards of the target compounds. Specifically, concentrations of catechin, quercetin, and gallic acid were measured based on calibration curves. An Agilent 6560 LC/Q-TOF–MS (Agilent Technologies, Palo Alto, CA, United States) equipped with a jetstream electrospray ionization interface was employed. After injection of 8 µL of the methanolic solution of the dried extract, chromatographic separation was carried out using a mobile phase consisting of water (solvent A) and methanol with 0.1 M formic acid (solvent B) to enhance analyte ionization and peak resolution. A Kinetex Evo column (5 µm, C18, 100 Å; Kinetex, Torrance, CA, United States) and a solvent gradient at 0.4 ml/min: 0–15 min from 0 to 100%(B); 15–19 min 100%(B); 19–21 min from 100 to 0% (B); 21–24 min 0%(B) were used. The parameters of the mass spectroscopy were the following: nebulizer (20 psi), drying gas (N2) flow (5 L/min), and drying gas temp (325 °C). The mass spectrometer was used in negative ionization mode with a scanning range from m/z 50 to1700 using the MassHunter analysis workstation software (version 10.0, Agilent technologies).

Preparation and characterisation of nutriosomes

Nutriosomes were prepared as previously reported[20]. Briefly, Phospholipid S75 (120 mg/mL), Cannonau pomace extract (5 or 10 mg/mL), olive oil (100 mg/mL), and Nutriose®FM06 (400 mg/mL) were weighed in a glass vial and hydrated with a mixture of propylene glycol and water (1:1). The dispersions were sonicated (10 + 15 cycles, 5 s on and 2 s off, 13 µm of probe amplitude), using a Soniprep 150 ultrasonic disintegrator (MSE Crowley, London, UK), to obtain homogeneous systems with small particles. To avoid the overheating of the samples, cooling between each sonication has been performed. Vesicles without extract (empty nutriosomes) were also prepared and used as reference. The average diameter, polydispersity index, and zeta potential of empty (control) and extract loaded nutriosomes were measured by means of light scattering technique, using a Zetasizer Ultra (Malvern Instruments, Worcestershire, UK), immediately after their preparation. A stability study has been performed as well, monitoring the main physico-chemical properties (mean diameter, polydispersity index and zeta potential) of grape extract loaded nutriosomes stored at room temperature and in the refrigerator (25 and 4 °C) for 4 months.

The suspensions were purified from the unloaded phytochemicals, dialysing the samples (1 mL) (Spectra/Por® membranes: 12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, Netherlands) against water (1 L) for 2 h, refreshing the water each hour. The antioxidant activity of the vesicles (see paragraph 2.3) was measured before and after dialysis, and the entrapment efficiency was calculated as the percentage of the antioxidant activity of dialyzed versus non-dialysed freshly prepared samples (Eq. 2):

$$EntrapmentEfficiency\,(\%) = \frac{AntioxidantActivity_{dyalised}}{AAntioxidantActivity_{non-dyalised}} \times 100 \quad (2)$$

All the experiments were performed in triplicate.

Release of phytochemicals from nutriosomes

The samples (2 mL) were transferred into dialysis tubes and were dialysed against saline phosphate buffer (25 mL) at pH 7.4, maintaining a temperature of 37 °C with constant stirring (100 rpm) for 72 h[28]. At scheduled time-points, the receptor phase was removed and replaced with an equal volume of pre-thermostated saline phosphate buffer (pH 7.4). The released phytochemicals were quantified by measuring the antioxidant activity of the release solution by the DPPH colorimetric assay and the concentrations were expressed as Trolox equivalents according to the calibration curve obtained using different concentration of Trolox. The amount of phytochemicals released was expressed as a function of the initial antioxidant activity. The value observed at the beginning of the experiment (where antioxidant activity = 0) has been considered as the null release of phytochemicals. The release was mathematically quantified and expressed as percentage of phytochemicals released (quantified measuring the antioxidant activity) as a function of the time.

Biocompatibility of nutriosomes against Caco-2 cells

Human intestinal epithelial cells (Caco-2) were grown as monolayer in 75 cm^2 flasks, incubated at 37 °C with 100% humidity and 5% of carbon dioxide. Dulbecco's Modified Eagle Medium (DMEM) with high glucose, containing L-glutamine, supplemented with 10% foetal bovine serum, penicillin, streptomycin and fungizone, was used as growth medium. The cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well. After 24 h of incubation, the cells were treated for 48 h with Cannonau pomace extract in dispersion (propylene glycol and water mixture) or loaded in nutriosomes and with empty vesicles as well, to evaluate the effect of vesicles themself. Samples were diluted to reach different concentrations of grape extract (10, 1, 0.1, 0.01 µg/mL from 10-cannonau-nutriosomes and 5, 0.5, 0.05, 0.005 µg/mL from 5-cannonau-nutriosomes) in the cell medium. After 48 h, MTT [3(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] (100 µl, 0.5 mg/mL final concentration) was added to each well and the cells were incubated again for 3 h[20]. The formed formazan crystals were dissolved with dimethyl sulfoxide, and the absorbance was measured at 570 nm using a microplate reader (Synergy 4 Reader, BioTek Instruments, AHSI S.p.A, Bernareggio, Italy). The experiments were repeated at least three times, each time in triplicate. Results are reported as percentage of cell viability in comparison with untreated control cells (100% viability).

Evaluation of the protective effect of nutriosomes against cell damage caused by oxidative stress

The in vitro protective effect of the extract loaded nutriosomes against cell damages caused by oxidative stress induced using hydrogen peroxide was evaluated. The cells were seeded into 96-well plates at a density of 7.5×10^3 cells/well. After 24 h of incubation, cells were treated with hydrogen peroxide (1:40,000) and simultaneously with the samples properly diluted to reach 10, 1, 5, 0.5 µg/mL of grape extract. The cells stressed with hydrogen peroxide only, were used as negative control, while untreated cells (100% of viability) were used as positive control. After 4 h of incubation, the cells were washed with PBS, and their viability was determined by the MTT assay (see paragraph 2.7). The experiments were repeated at least three times, and each sample was analysed in triplicate. The results are reported as a percentage of cell viability and are normalized to untreated Caco-2 cells (100%).

In vivo experiments

Animals

Twenty-seven 16–19 weeks old male C57BL/6J mice (Charles River, Calco, Italy) weighing 25 ± 2 g at the beginning of the experiments were used. Mice were housed in a group of 4 per cage under constant temperature and a 12-h light/dark cycle. Standard laboratory chow and tap water were available ad libitum. Animal experiments were approved by the Organism for Animal Welfare (OPBA) of the University of Cagliari and performed in accordance with the ARRIVE recommendations (https://arriveguidelines.org/), the guidelines for animal experimentation of the EU directives (2010/63/EU; L.276; 22/09/2010)[29], and with policies issued by OPBA of the University of Cagliari. Experiments were designed to minimise animal discomfort and to reduce the number of animals used.

Experimental plan

Before treatment, mice underwent one week of handling by experimenters (two daily sessions, 5 min each) to reduce animal distress. Twenty-seven mice were randomly allocated into five experimental groups based on the respective treatments (Fig. 1): (1) saline (SAL) + vehicle, n = 6; (2) Empty nutriosomes + MPTP (20 mg/kg): n = 6; (3) Cannonau suspension (100 mg/kg) + MPTP (20 mg/kg), n = 6; (4) Cannonau nutriosomes (50 mg/kg) + MPTP (20 mg/kg), n = 4; (5) Cannonau nutriosomes (100 mg/kg) + MPTP (20 mg/kg), n = 5. The samples (10 mL/kg of body weight) were administered intragastrically via gavage (18-gauge). To produce the damaging

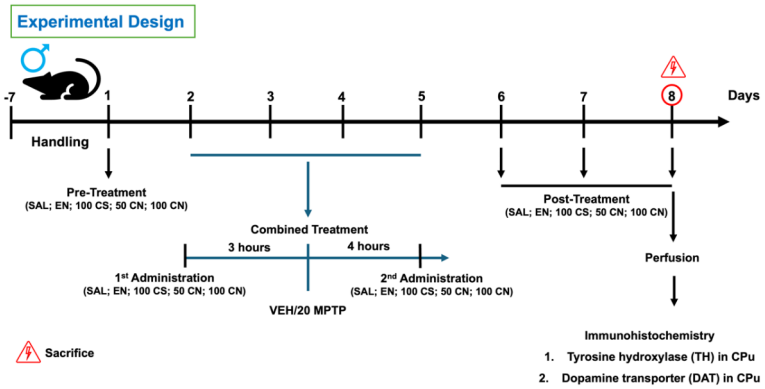

Fig. 1. Illustration of the experimental design reporting the animal groups damaged by intraperitoneal administration of vehicle or MPTP at day 2, 3, 4, 5 and treated by intragastrical gavage with saline (SAL) or empty nutriosomes (EN) or Cannonau suspension (CS) or Cannonau nutriosomes (CN) at day 1, 2, 3, 4, 5, 6, 7, 8 Abbreviations: CPu, Caudate-Putamen nucleus; EN, empty nutriosomes;, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP; VEH, distilled water.

effects, MPTP in water solution (20 mg/kg/day) or distilled water (positive control) were given intraperitoneally for four consecutive days, from day 2 to day 5. On day 1 (pre-treatment), mice intragastrically received the foreseen samples. From day 2 to day 5 (combined treatment), mice received a daily intraperitoneal injection of either MPTP or distilled water and 3 h prior and 4 h later of the injection an intragastrical administration of foreseen samples. The dosage, route, and administration schedule of Cannonau extract in suspension or nutriosomes were determined according to a prior investigation and on the base of in vitro results obtained in the present study[20].

Immunohistochemistry

Tissue preparation

On day 8, thirty minutes after the last intragastric administration of saline or empty nutriosomes or Cannonau suspension or Cannonau nutriosomes, mice were deeply anaesthetized with an intraperitoneal injection of sodium thiopental (Pentothal Sodium, 1 g/50 ml) and sacrificed by transcardial perfusion with ice-cold saline (NaCl, 0.9% w/v) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Subsequently, their brains were isolated, post-fixed in 4% PFA for 2 h, and preserved in PBS 1X (PBS) at 4 °C. The next day, brains were coronally cut on a vibratome (VT1000S, Leica Biosystems) to obtain Sects. (50 µm thick) suited for immunohistochemical analyses. For each mouse, three coronal sections containing the caudate-putamen were obtained according to the following stereotaxic coordinates: 1.34 to 0.74 mm relative to bregma, according to the mouse brain atlas[30].

Tyrosine hydroxylase immunohistochemistry

Free-floating caudate-putamen coronal mouse sections were rinsed three times in PBS 1X and incubated with 1% of hydrogen peroxide (30% v/v, Merck, H1009) in PBS at room temperature (10 min), to block endogenous peroxidase activity. Then, sections were blocked and permeabilized with 5% normal goat serum and 0.1% Triton X-100 at room temperature (20 min) and later incubated with primary antibody directed towards tyrosine hydroxylase (polyclonal rabbit anti-tyrosine hydroxylase, 1:1,000, AB152, Millipore Corporation, MA, United States) at room temperature (2 nights). Thereafter, sections were incubated with biotinylated secondary antibody (Goat anti-rabbit; 1:500, BA-1000 Vector, Peterborough, United Kingdom), then the avidin–peroxidase protocol (ABC, Vector, Peterborough, United Kingdom, PK-4010) was applied for visualization, using 3,3′-diaminobenzidine (Merck, D5637) as a chromogen. Afterward, sections were mounted onto super-frost glass slides, dehydrated, and coverslipped using Eukitt® mounting medium.

Dopamine transporter immunofluorescence

For the immunofluorescence evaluation of dopamine transporter in Caudate-putamen, free-floating sections were rinsed in PB 0.1 M, blocked, and permeabilized in 3% normal goat serum and 0.3% Triton X-100 in PB 0.1 M at room temperature (3 h), followed by incubation in the same solution with the primary antibody (monoclonal rat anti-DAT; 1:1,000, MAB369, Millipore, CA, United States) at 4 °C (2-nights). Then, sections were rinsed three times in PB 0.1 M and incubated with the biotinylated secondary antibody (biotinylated goat anti-rat; 1:200) at room temperature (2 h), followed by incubation with AlexaFlour® 488-labeled streptavidin (1:500, 016-540-084, Jackson ImmunoResearch Europe, Newmarket, United Kingdom) at room temperature (1 h). Afterward, sections were rinsed in PB 0.1 M and mounted onto super-frost glass slides using Mowiol® mounting medium. Omission of either the primary or secondary antibodies served as negative control and yielded no labelling (data not shown).

Yield (%)	FRAP (mM Fe^{2+}/g dry extract)	DPPH (mg Trolox/g dry extract)	TPC (mg GAE/g dry extract)
6.45 ± 1	12 ± 2	306 ± 10	184 ± 7

Table 1. Yield of grape pomace extraction, ferric reducing power (FRAP), free radical scavenging activity (DPPH), and total phenolic content (TPC) of obtained extract. Mean values ± standard deviations were reported.

Polyphenols	Amount present in Cannonau grape pomace extract (mg/kg)	Amount present in Nasco grape pomace extract (mg/kg)
Gallic acid	830 ± 24	182.3 ± 9.52
Procyanidin B2	N.D	4626 ± 26.84
(+)-Catechin	185 ± 10	1375 ± 66.26
(−)-Epicatechin	3.1 ± 0.9	799 ± 38.84
Quercetin	687 ± 21	1087 ± 4.33

Table 2. Liquid chromatography-quadruple time-of-flight mass spectrometry quantitative analysis of polyphenols present in pomace extract from Cannonau and Nasco. The last values were already published[20]. Mean values ± standard deviation are reported (n = 3).

Image acquisition and analysis of dopamine transporter- and tyrosine hydroxylase-positive fibres

Images of a single wavelength were digitalised and captured in RGB by using an epifluorescence microscope (Axio Scope A1, Zeiss, Germany) connected to a digital camera (1.4 MPixels, Infinity 3–1, Lumenera, Canada) as previously described[31]. Coronal sections of the dorsolateral and dorsomedial portions of the Caudate-putamen were captured at ×10 magnification for the analysis of TH immunoreactivity, or ×20 magnification for the analysis of dopamine transporter immunoreactivity. The ImageJ software (National Institutes of Health, United States) was used to quantify the densities of dopamine transporter- and tyrosine hydroxylase-positive fibres in Caudate-putamen. For density quantification, images were first converted to 8-bit, background subtracted, and then the densities of immunoreactive fibres were determined by measuring the mean grey density in fixed regions representing the dorsal Caudate-putamen. Analyses were always performed in a blinded manner in the three sections. No significant differences in the density of dopamine transporter- and tyrosine hydroxylase-positive fibres were found among the three sections of a given area in the same mouse. Thus, values from different levels were averaged, normalised with respect to the group that received distilled water and saline, and expressed as a percentage.

Data analysis and statistics

Statistical analyses were carried out with Prism 8.0.1 software (GraphPad, USA), and data were analysed by one-way (factor: treatment) analysis of variance (ANOVA) followed by either Tukey or Newman–Keuls post-hoc test, when applicable. Results are expressed as mean ± standard deviation and were considered statistically significant if $p < 0.05$.

Results

Extraction of phytocomplex

To extract polar and low polar phytochemicals, Cannonau grape pomace was left to macerate with the improvement of ultrasound in a mixture of ethanol and water (70:30) for 2 h. According to previous studies, the extraction yield was not particularly high, as it was 6.45 ± 1% (Table 1)[25,32]. As first screening, the antioxidant power of the extract was measured using three unspecific tests: total phenolic content, ferric reducing ability and free radical scavenging activity (Table 1).

The antioxidant activity measured by FRAP assay was 12 ± 2 mM Fe^{2+}/g dry extract and that measured by DPPH was 306 ± 10 mg Trolox/g dry extract. The Total Phenolic Content of Cannonau pomace extract, measured according to Folin-Ciocalteu colorimetric assay, was 184 ± 7 mg of gallic acid equivalents/g of dry extract. The value was similar to those of the Cannonau pomace extract (185 ± 10 mg of gallic acid equivalents/g of dry extract) previously measured by Perra et al. and was higher than that of pomace extract from four Portugal varieties (~ 100 mg of gallic acid equivalents/g of dry extract)[32,33].

Analytical characterisation of extract

Through Liquid chromatography-quadruple time-of-flight mass spectrometry analysis the concentrations of gallic acid (840 mg/kg), catechin (185 mg/kg), epicatechin (3.1 mg/kg) and quercetin (687 mg/kg), were quantified comparing their retention time against control pure standards (Table 2). Their amounts were expressed as mg/kg of dried Cannonau pomace extract. The amount of procyanidin B2 in the extract was below the limit of detection.

Preparation and characterisation of nutriosomes

All formulations were obtained by direct dispersion of solid components in water phase and sonication avoiding the use of organic solvents. Propylene glycol was used in the hydrating solution to obtain the same formulation already used with Nasco extract and compare the results. In this previous study, it was selected based on its potential biological relevance as penetration enhancer. Two different concentrations, 5 or 10 mg/mL of Cannonau pomace extract were loaded, obtaining 5-nutriosomes and 10-nutriosomes, 10 mg/mL was the higher concentration that could be stably loaded. Indeed, when higher amounts of extract were added the vesicles were very large and the formation of a precipitate was quickly evident. To better understand the effect of chemicals contained in the extract on the assembling of phospholipid bilayer, empty nutriosomes were prepared and used as reference.

The mean diameter, polydispersity index, zeta potential and entrapment efficiency of the obtained empty and Cannonau loaded nutriosomes were measured (Table 3). Empty nutriosomes were sized ~ 243 nm and the loading of the extract caused a decrease of the mean diameter of 5-nutriosomes (~ 157 nm), and 10-nutriosomes (~ 198 nm, $p < 0.05$ among the three values). The polydispersity index was reduced as well, as that the value of empty vesicles was 0.27, while that of 5-nutriosomes was 0.12 and that of 10-nutriosomes was 0.18. This indicates that the extract components played an important role in the vesicle assembling due to their intercalation inside the bilayer. The zeta potential was always highly negative regardless of the formulation tested (≈-48 mV, $p > 0.05$). 5-nutriosomes and 10-nutriosomes were both able to entrap a high amount of extract, as the entrapment efficiency of fists was ~ 77% and that of formers ~ 85%.

Stability of the formulations on storage

Stability of nutriosomes over time was evaluated storing them at 25 °C in the dark for 4 months and measuring, at scheduled times, the size, polydispersity index and zeta potential (Fig. 2). The storage has been also performed at 4 °C as well, to evaluate the effect of the temperature on their stability. The parameters measured at 4 °C (data not shown) were statistically equal to those at 25° C, confirming the suitability of storage at room temperature, simplifying stoking and distribution. Empty vesicles were not included in stability studies as they have previously demonstrated to be highly unstable over time confirming the key role played by the payloads[20].

After two months of storage, the mean diameter of 5-nutriosomes was constant while after four months, it increased up to ~ 186 nm ($p < 0.05$ versus the values at zero and two months). The mean diameter of 10-nutriosomes increased already at two months of storage and a further increase was evident at four months, up to ~ 256 nm ($p < 0.05$ versus the value at zero months). Polydispersity index of 5-nutriosomes remained almost unchanged, while that of 10-nutriosomes significantly increased up to ~ 0.32 ($p < 0.05$ versus the values at shorter time). The zeta potential was always strongly negative (~ − 44 mV), without significative differences among the different formulations ($p > 0.05$). These results suggest that the quantity of extract loaded influence the stability of formulations over time, as the mean diameter and polydispersity index of 5-nutriosomes were less affected by the time of storage than those of 10-nutriosomes ($p < 0.05$). Previous studies proved that nutriosomes stability over time can be improved by freeze-drying, since Nutriose acts as cryoprotectant, avoiding the break of vesicles during the process and allows the immediate and simple rehydration[16,34].

Release of phytochemicals from nutriosomes

Phytochemicals released from Cannonau nutriosomes over time was measured using a dialysis membrane and compared with the amount released from the corresponding Cannonau pomace extract in suspension, which allowed to evaluate the dialysing capacity of the used conditions (Fig. 3). The percentage of phytochemicals released was calculated as the antioxidant activity in the release medium, at scheduled times, in comparison with the initial antioxidant activity of the formulation, according to previous studies[28].

Using the extract in dispersion, the amount of phytochemicals released was ~ 22% at 2 h, ~ 78% ($p < 0.05$ versus the values obtained from nutriosomes) at 4 h and ~ 100% at 48 h, suggesting a faster release of them in comparison with nutriosomes (Fig. 3). Indeed, at 72 h, only ~ 60% of phytochemicals was released from the vesicles irrespective of the used formulation ($p > 0.05$ between the values obtained from the two formulations at each time point). Results confirmed that nutriosomes were capable of delaying the release of phytochemicals in the media.

Evaluation of the biocompatibility of vesicles

The biocompatibility of the Cannonau pomace extract in dispersion or loaded in nutriosomes was evaluated using Caco-2 cells, considered to be the most representative cells of the intestinal epithelium[19].

	MD (nm)	PI	ZP (mV)		E (%)
Empty nutriosomes	$^{*}243 \pm 12$	0.27	-55 ± 8		–
5-nutriosomes	$^{§}157 \pm 13$	0.12	-51 ± 3		$^{♪}77 \pm 2$
10-nutriosomes	$^{\#}198 \pm 6$	0.18	-41 ± 9		$^{\infty}85 \pm 4$

Table 3. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP) and entrapment efficiency (E) of empty and Cannonau extract loaded nutriosomes. Mean values ± standard deviations are reported (n = 6). For each group (MD, ZP and E) the same symbol (*, §, #,°,♪,∞) indicates values that are not statistically different ($p > 0.05$ among values with same symbol and $p < 0.05$ versus values with different symbol).

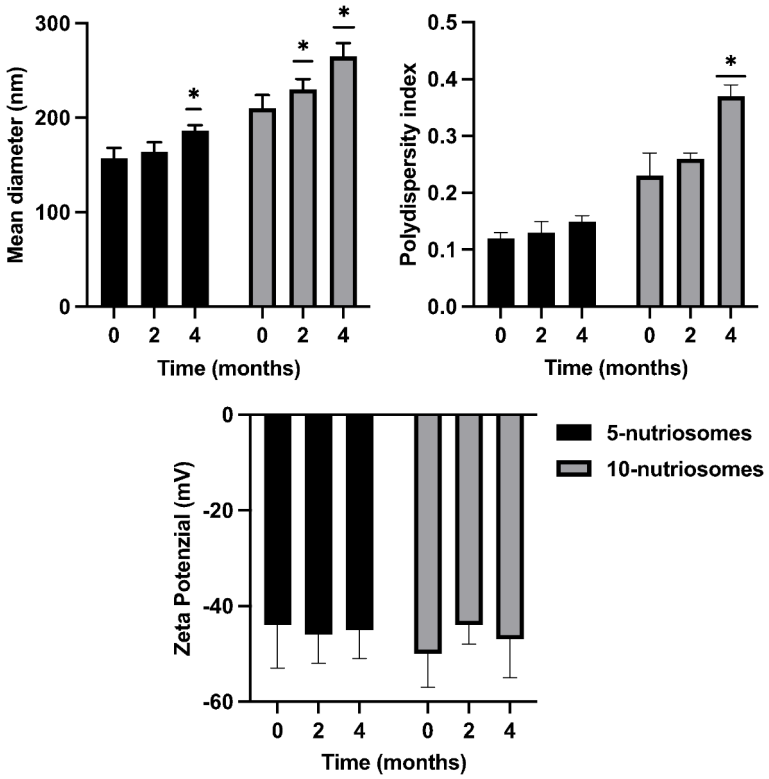

Fig. 2. Mean diameter (nm), polydispersity index and zeta potential (mV) of Cannonau pomace extract loaded nutriosomes stored at 25 ± 1 °C for 4 months. Mean values (bars) ± standard deviations (error bars) are reported (n ≥ 3). Symbol * indicates values statistically different from that of the same formulation at different times, ($p < 0.05$).

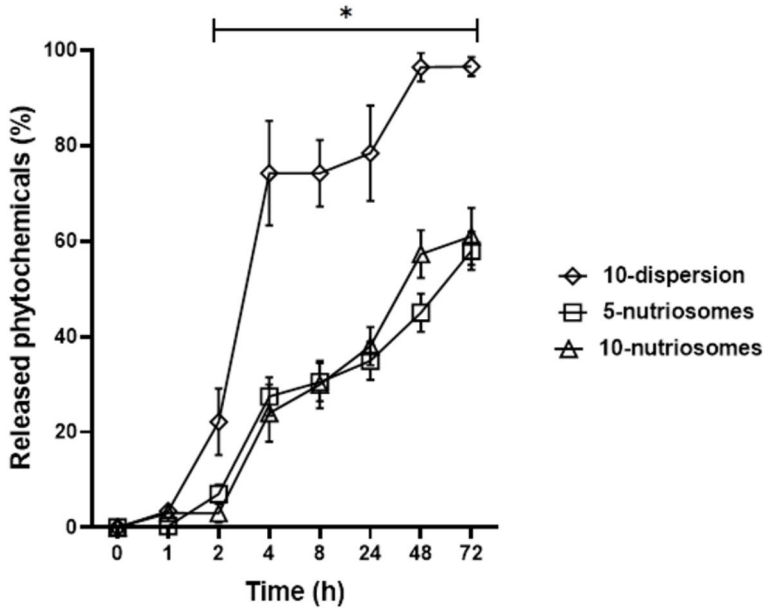

Fig. 3. Amount (%) of the Cannonau pomace extract phytochemicals released from nutriosomes or suspension as a function of time. Mean values (bars) ± standard deviations (error bars) are reported (n ≥ 3). Symbol * indicates values statistically different from that obtained from nutriosomes ($p < 0.05$).

The cells were incubated for 48 h with different dilutions of samples (Fig. 4). Empty vesicles were highly biocompatible confirming the safety of their components against Caco2 cells. When treated with the extract in dispersion, their viability remained ~ 100% ($p > 0.05$ versus the control) irrespective of the concentration tested, indicating its high biocompatibility. When the extract was loaded in nutriosomes, the viability significantly increased up to ~ 120% ($p < 0.05$ vs. the control) using the lowest dilution of 5-nutriosomes and up to ~ 110% ($p < 0.05$ versus the control) using the lowest and highest dilutions of 10-nutriosomes, suggesting the safety of these formulations.

Evaluation of the protective effect of the formulations against cell damage caused by oxidative stress

The ability of the Cannonau pomace extract to inhibit the cell death caused by oxidative stress induced with hydrogen peroxide was evaluated in Caco-2 cells (Fig. 5).

The viability of cells treated with hydrogen peroxide was significantly reduced up to ~ 65% ($p < 0.05$ versus the value of untreated cells) (Fig. 5). The simultaneous treatment with the empty vesicles and the extract in dispersion did not effectively reduce the cell death caused by hydrogen peroxide as the viability was ~ 75% ($p > 0.05$ versus the value of stressed and untreated cells). The loading of 5 mg/mL of extract into nutriosomes protected the cells against oxidative damage as the viability was ~ 100% and ~ 90% for the lowest and the highest dilution, respectively ($p < 0.05$ versus the value of stressed and untreated cells). When cells were treated with 10 mg/mL of extract loaded in nutriosomes, at the lowest dilution, the viability was ~ 90% ($p > 0.05$ versus the value of untreated cells) while using the highest dilution the viability was slightly lower ~ 89% but still statistically different from the viability of stressed and untreated healthy cells ($p < 0.05$). According to these results, Cannonau pomace extract was able to protect CaCo-2 against hydrogen peroxide-induced oxidative stress. A higher effect was noticed when the extract was loaded into nutriosomes.

Immunoreactivity of the tyrosine hydroxylase in the caudate-putamen following MPTP and Cannonau nutriosomes treatment

To investigate the neuroprotective effect of Cannonau nutriosomes in the brain, the protein levels of the enzyme tyrosine hydroxylase, the rate-limiting enzyme involved in the biosynthesis of dopamine in the caudate-putamen of mice neuronal-damaged with MPTP (20 mg/kg/day × 4 by intraperitoneal injection), were evaluated. The damaging treatment significantly reduced the density of tyrosine hydroxylase-positive fibres in the dorsal caudate-putamen (~ 25%, Fig. 6), whereas administration of Cannonau nutriosomes (50 mg/kg) was unable to contrast its neurotoxic effects. Indeed, one-way ANOVA of tyrosine hydroxylase immunoreactivity revealed a significant effect of treatment in the caudate-putamen of damaged mice ($F_{4,22} = 47.80$, $p < 0.0001$). Newman–Keuls post hoc test indicated a significant reduction in the mean grey intensity of tyrosine hydroxylase-positive fibres in mice damaged with MPTP and treated with empty nutriosomes, Cannonau suspension (100 mg/kg)/

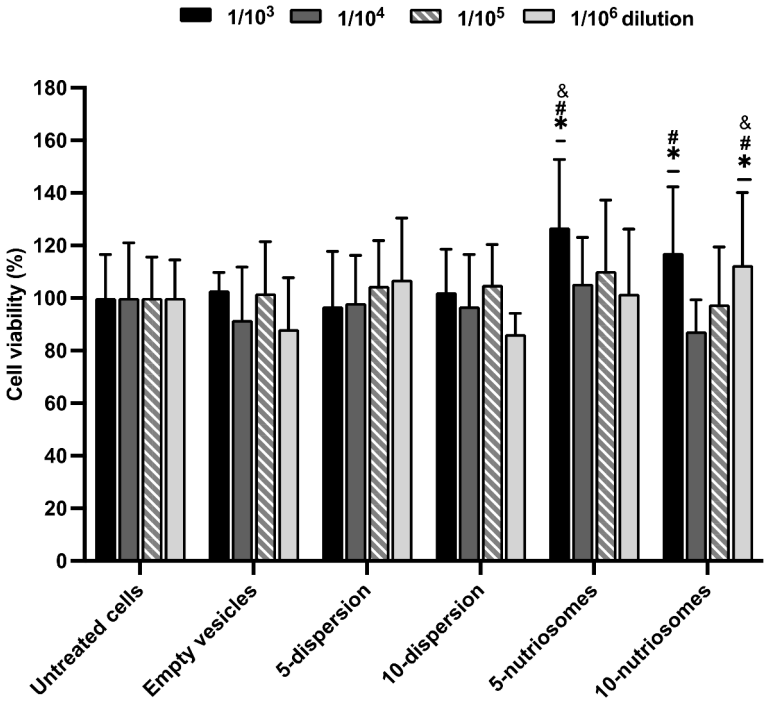

Fig. 4. Viability of Caco-2 cells treated with empty vesicles and with the extract in dispersion or loaded in nutriosomes. Mean values (bars) ± standard deviations are reported, n = 10. Symbol * indicates values statistically different from the control ($p < 0.05$); symbol # indicates values statistically different from the empty vesicles ($p < 0.05$); symbol & indicates values statistically different from the dispersion ($p < 0.05$).

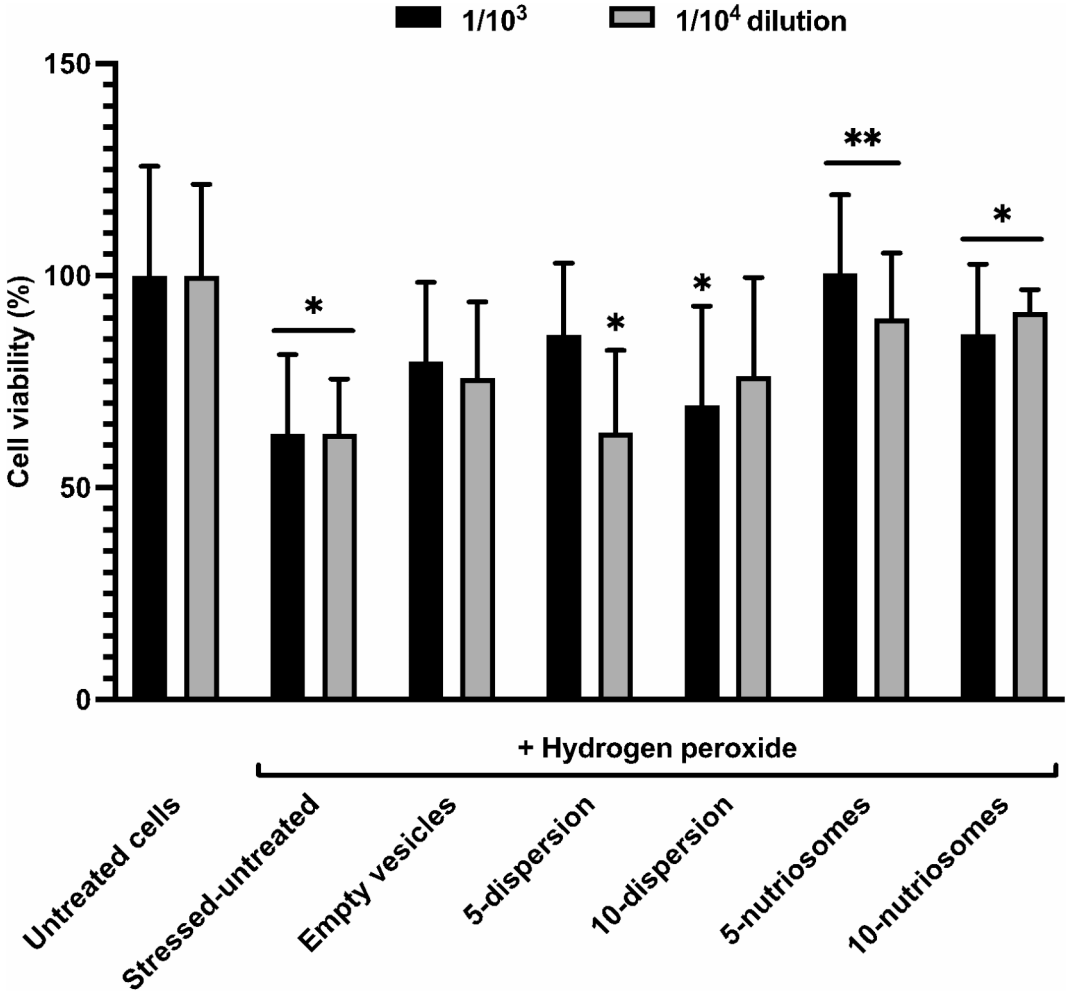

Fig. 5. Viability of Caco-2 cells stressed for 4 h with hydrogen peroxide and untreated or treated with the extract in dispersion or loaded in vesicles. Mean values ± standard deviations (error bars) are reported (n = 10). Symbol (*) indicates values statistically different from the untreated cells ($p < 0.05$). Symbol (**) indicates values statistically different from the stressed and untreated cells ($p < 0.05$).

MPTP or Cannonau nutriosomes (50 or 100 mg/kg)/MPTP compared with that undamaged with MPTP and treated with saline (Fig. 6).

Immunoreactivity of the dopamine transporter in the caudate-putamen (CPu) following MPTP and Cannonau nutriosomes treatment

Next, the effect of Cannonau nutriosomes administration (50 mg/kg) on the expression of the dopamine transporter protein in the caudate-putamen of mice treated with subacute MPTP was assessed. Dopamine transporter is a vital plasma membrane protein that regulates dopamine homeostasis by promoting the reuptake of dopamine from the synaptic cleft into the presynaptic neuron after its release. Findings indicate that subacute MPTP treatment markedly diminished dopamine transporter-positive fibre density in the dorsal Caudate-putamen (~ 60%, Fig. 7). However, Cannonau nutriosomes administration (50 mg/kg) did not mitigate the neurotoxic effects elicited by MPTP.

One-way ANOVA of dopamine transporter immunoreactivity revealed a significant effect of treatment in the caudate-putamen ($F_{4,22} = 85.94$, $p < 0.0001$). Newman–Keuls post hoc test indicated a significant reduction in the mean density of dopamine transporter-positive fibres in mice damaged with MPTP and treated with empty nutriosomes or Cannonau suspension (100 mg/kg) or Cannonau nutriosomes (50 or 100 mg/kg) compared with control group damaged with distilled water and treated with saline (Fig. 7).

Discussion

In previous studies, it was found that the intragastric administration of nutriosomes loading Nasco pomace extract exserts a neuroprotective effect as effectively mitigated the neuroinflammation and neurodegeneration induced in mouse by intraperitoneal injection of MPTP[20,21]. These promising results confirmed the effectiveness of the chemicals contained in this extract and the optimal performances of nutriosomes, as the extract in suspension was not effective. Thus, nutriosomes allowed the phytochemicals contained in the extract to reach

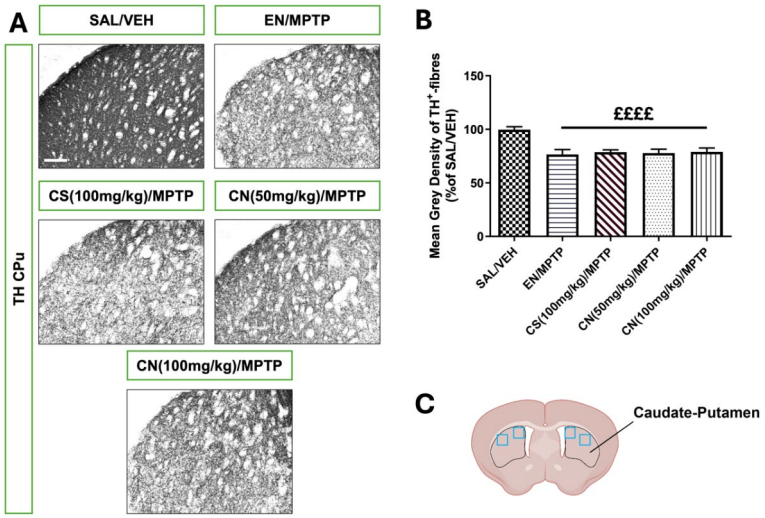

Fig. 6. Effect of Cannonau suspension (CS) (100 mg/kg) and Cannonau nutriosomes (CN) (50 or 100 mg/kg) on the immunoreactivity of tyrosine hydroxylase positive (TH^+)- fibres in the caudate-putamen (CPu) of MPTP-treated mice. (**A**) Representative sections of caudate-putamen immunostained for tyrosine hydroxylase. (**B**) Histogram indicates the mean grey density of tyrosine hydroxylase -immunoreactive (TH^+) fibres in the caudate-putamen. (**C**) Areas of caudate-putamen (blue squares) used for quantitative analysis. Values are expressed as a percentage of the group damaged with distilled water and treated with saline. Symbols within bars indicate the values of individual mice. $p < 0.0001$ vs. control group damaged with distilled water and treated with saline (SAL/VEH). Scale bar = 50 µm.

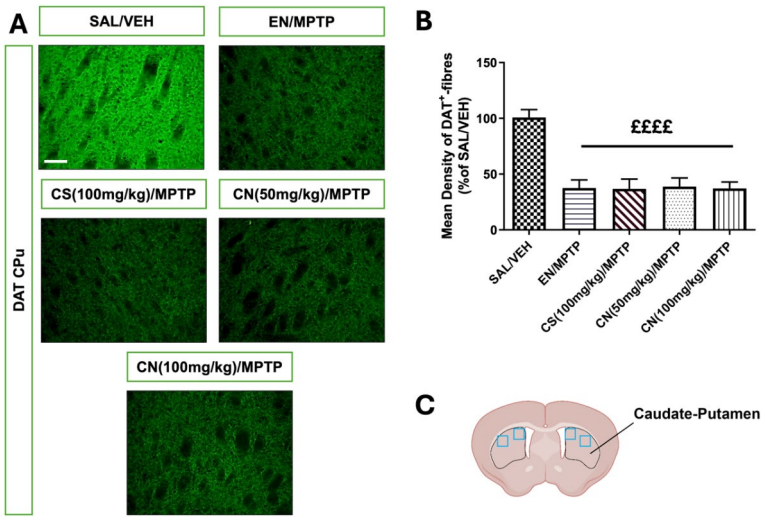

Fig. 7. Effect of Cannonau suspension (CS, 100 mg/kg) and Cannonau nutriosomes (CN, 50 or 100 mg/kg) on the immunoreactivity of dopamine transporter (DAT)-positive fibres in the caudate-putamen of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. (**A**) Representative sections of dopamine transporter immunostained for dopamine transporter. (**B**) Histogram indicates the mean density of dopamine transporter -immunoreactive fibres in the CPu. (**C**) Areas of caudate-putamen (blue squares) used for quantitative analysis. Values are expressed as a percentage of the group damaged with distilled water and treated with saline (SAL/VEH). Symbols within bars indicate the values of individual mice. $p < 0.0001$ vs. group damaged with distilled water and treated with saline. Scale bar = 50 µm.

the brain where they exerted their protective effect. To confirm nutriosomes performance and better understand how the chemical composition of the extract affected the efficacy, in the present study an alternative extract was obtained from the pomace of a different cultivar, Cannonau. This is a Sardinian cultivar like Nasco, but it has a red grape instead of a white one. The same extraction method and conditions previously used with Nasco were applied. If compared to the work of Manca et al., the chosen method allowed the extraction of higher amounts of gallic acid (830 mg/kg versus 35.60 mg/kg), catechin (185 mg/kg versus 88.40 mg/kg) and quercetin (687 mg/kg

versus 31.57 mg/kg) from the same Cannonau pomace, always avoiding the use of toxic solvents and dissipative methodologies, in line with the goal of eco-sustainably valorising agro-industrial residues[35,36].

The Cannonau pomace extract was formulated in the same nutriosomes, improved with propylene glycol, and previously used for the delivery of Nasco pomace extract, at the same concentrations (5 and 10 mg/mL), to compare the results. For the Nasco formulation, propylene glycol was selected due to its well-documented biocompatibility and pharmaceutical-grade safety[37]. Indeed, it is widely used in drug formulations as a co-solvent to enhance the solubility of poorly water-soluble compounds[38]. When added to phospholipid vesicles, it can modulate the fluidity and flexibility of the bilayer membrane or act as penetration enhancer[37].

The loading of Cannonau pomace extract in nutriosomes, improved with propylene glycol, allowed a significant decrease of the mean diameter of vesicles, likely due to the intercalation of phytochemicals within the bilayer and its reassembling. Results are in agreement with previous ones found with Nasco extract, as its incorporation (5 mg/ml) induced a slight, but significant, decrease of the vesicle size from ~ 188 nm (empty nutriosomes) to ~ 141 nm[20].

When tested in vitro, Cannonau nutriosomes were biocompatible, as previously found for those loading Nasco extract, confirming that the carriers were not toxic and the components of both extracts as well. Cannonau extract loaded nutriosomes were able to protect Caco-2 cells against cell death induced by hydrogen peroxide, irrespective of the concentration (5 or 10 mg/mL) and dilution ($1/10^3$ or $1/10^4$) used. Also in this case, the effectiveness was linked to the delivery performances of nutriosomes, as the extract in suspension was not able to protect the cells, probably because the carriers were able to increase the cellular uptake of the antioxidant polyphenols[39–41]. An improved cellular uptake using phospholipid vesicles enriched with Nutriose was also observed by Karim et al.[39]. They found that, if compared to liposomes, these vesicles allowed a higher cellular uptake of delphinidin-3-O-sambubioside, probably related to particle charge and the higher surface hydrophilicity of Nutriose. However, the carrier alone is not enough to ensure the biological activity of delivered molecules and in this case the chemical composition of the extract played a key role. Indeed, the in vitro protective effect of nutriosomes loading Cannonau pomace extract was significantly higher than that of the same nutriosomes loading Nasco pomace extract[20]. This should be related to the higher concentration of gallic acid in the Cannonau pomace extract (830 mg/kg), if compared to that of Nasco, (182.3 mg/kg), as this phenolic molecule is well known for its antioxidant and anti-inflammatory effects at intestinal level[42,43]. Based on the different composition of the two extracts, also their in vivo effect in the brain was different. Specifically, their efficacy was tested in the subacute model of Parkinson induced in mice with MPTP, which cause a Parkinsonian-like phenotype due to the progressive and bilateral degeneration of dopaminergic neurons forming the nigro-striatal pathway[44,45]. The putative neuroprotective activity of formulations was evaluated in the Caudate-putamen of MPTP-treated mice measuring the immunoreactivity of tyrosine hydroxylase and dopamine transporter proteins, two established markers used to assess the synthesis capacity of dopamine and the functionality of dopaminergic terminals. In previous studies, a significant reduction of tyrosine hydroxylase- and density of dopamine transporter-positive fibre was observed in MPTP-treated mice and it was mitigated in animals treated with Nasco pomace extract nutriosomes[20,46,47]. Remarkably, Cannonau pomace extract, in suspension or loaded in nutriosomes, (50 or 100 mg/kg) did not alleviate the loss of tyrosine hydroxylase and dopamine transporter-positive fibres in the Caudate-putamen[20,21]. This lack of neuroprotective effect in vivo might be related to the specific phenolic composition of the Cannonau extract, which differs significantly from the Nasco extract in key components such as procyanidin B2. In fact, the last was particularly rich in procyanidins, in particular procyanidin B2 (4626 mg/kg), (+)-catechin (1375 mg/kg) and (−)-epicatechin (799 mg/kg), known for their neuroprotective effects[48,49]. Chen et al. found that procyanidins obtained from grape seeds may be useful in the prevention of Parkinson disease by activating the Nrf2/ARE pathway and its downstream detoxification and antioxidant enzymes[48]. Unfortunately, the amount of procyanidins contained in Cannonau pomace extract was low, in particular procyanidin B2 fell below the detection limit of the LC-QTOF-MS analysis. This finding aligns with prior investigations that disclosed significant diversity in the phenolic composition of grape pomace extracts across various grape varieties[24,50,51]. Indeed, de la Cerda-Carrasco et al. reported a notably high concentration of procyanidin B2 exclusively in pomaces derived from white grape but not from red grape[50]. An alternative explanation could involve differences in the brain bioavailability and metabolism of the phytochemicals present in Cannonau and Nasco extracts. In this regard, future analyses will be necessary to measure and compare the brain concentrations of the phytochemicals of both extracts to gain a deeper understanding of the differing neuroprotective effects observed in MPTP-treated mice. Indeed, at this stage, it is not possible rule out the possibility that repeated administration of higher concentrations of Cannonau loaded nutriosomes or longer treatment durations may be required to have clear neuroprotective effects.

Nonetheless, based on previous results, it is possible to state that phenolic molecules contained in the Nasco pomace are able to exert a neuroprotective effect but only when loaded in nutriosomes. This effect can be related to their direct action in the brain, or an indirect action through the gut-brain axis, probably modulating the gut microbiota[52]. In fact, it was demonstrated that phenols and nutriosomes as well, when assumed with diet, were able to improve the health of the intestinal microbiota, especially neutralising oxidative stressors and mitigating their damages[53]. The health microbiota can also produce therapeutically relevant compounds with improved permeability at blood–brain barrier level, establishing a gut-brain axis beneficial for the neurodegenerative disorders[52,54]. Analysing the effect of Cannonau extract loaded in nutriosomes, which were more effective at intestinal level than Nasco but ineffective at brain level, it is possible to hypothesize that probably the gut-brain axis may not be implicated in the neuroprotective effect of nutriosomes loaded grape pomace extracts. Therefore, the neuroprotective effect of Nasco pomace extract loaded nutriosomes seem to be presumably due to an increase in the bioavailability of phytochemicals, especially procyanidins, contained in the extract[20,21].

Conclusions

The present study highlights the vast potential of valorising agricultural by-products such as grape pomace through their conversion in health-promoting products, aligning with the principles of a circular economy to address environmental concerns effectively. It confirmed that utilizing an eco-friendly extraction method, it is possible to successfully isolate a blend of potent antioxidant polyphenols, having different compositions and activities depending to the used by-products. The bioavailability and efficacy of these molecules can be improved by their loading in specific nanocarriers, tailored for oral administration and called nutriosomes. Due to their optimal delivery performances and versatility, these vesicles improved the bioavailability of payloads both at intestinal and systemic level, allowing a different effect based on the chemicals contained in the extract. Notably, the absence of key antioxidant polyphenols, such as procyanidin B2 in the Cannonau pomace extract, and in general in the red grape pomace, reduced their effectiveness on counteracting neurodegeneration compared to the extract of white Nasco pomace, that was effective. Differently, Cannonau pomace extract loaded nutriosomes were more effective as nutraceutical to counteract the local oxidative stress. Looking forward, the path is paved for further explorations into optimizing formulations that harness the full spectrum of phytochemicals within different grape varieties. Future studies should focus on determine the specific delivery mechanisms through which nutriosomes increase the bioavailability of the loaded phytochemicals. Additionally, exploring alternative encapsulation techniques or synergistic combinations with other bioactive compounds could enhance the neuroprotective outcomes.

This research not only opens new avenues for the use of grape pomace in health-related applications but also sets a precedent for the sustainable utilization of agricultural residues, providing a blueprint for future studies aimed at converting waste into valuable therapeutic resources.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Declarations

Competing interests
The authors declare no competing interests.

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