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Therapeutic potential of plant-derived extracellular vesicles as nanocarriers for exogenous miRNAs

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ABSTRACT

Cell-to-cell communication strategies include extracellular vesicles (EVs) in plants and animals. The bioactive molecules in a diet rich in vegetables and fruits are associated with disease-preventive effects. Plant-derived EVs (PDEVs) are biogenetically and morphologically comparable to mammalian EVs and transport bioactive molecules, including miRNAs. However, the biological functions of PDEVs are not fully understood, and standard isolation protocols are lacking. Here, PDEVs were isolated from four foods with a combination of ultracentrifugation and size exclusion chromatography, and evaluated as vehicles for enhanced transport of synthetic miRNAs. In addition, the role of food-derived EVs as carriers of dietary (poly)phenols and other secondary metabolites was investigated. EVs from broccoli, pomegranate, apple, and orange were efficiently isolated and

Abbreviations: miRNAs, microRNAs; EVs, extracellular vesicles; PBS, phosphate-buffered saline; NTA, nanoparticle tracking analysis; TEM, transmission electron microscopy; RNA-seq, small-RNA sequencing; RT-qPCR, reverse transcription – quantitative real-time PCR; cDNA, complementary DNA; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum. MTT, thiazolyl blue tetrazolium bromide; DMSO, dimethyl sulfoxide; SEC, size exclusion chromatography; UC, ultracentrifugation; ESI, electrospray ionization; MS, mass spectrometry.

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characterized. In all four sources, 4 miRNA families were present in tissues and EVs. miRNAs present in broccoli and fruit-derived EVs showed a reduced RNase degradation and were ferried inside exposed cells.

EVs transfected with a combination of ath-miR159a, ath-miR162a-3p, ath-miR166b-3p, and ath-miR396b-5p showed toxic effects on human cells, as did natural broccoli EVs alone. PDEVs transport trace amounts of phytochemicals, including flavonoids, anthocyanidins, phenolic acids, or glucosinolates. Thus, PDEVs can act as nanocarriers for functional miRNAs that could be used in RNA-based therapy.

1. Introduction

Cell communication is essential for organism development, and both plant and animal cells employ strategies to facilitate communication. Extracellular vesicles (EVs) are heterogenous non-viable lipid bilayer particles naturally released from cells [1,2], which include (but are not limited to) exosomes, microvesicles, and apoptotic bodies. EVs can transport bioactive molecules, including nucleic acids, such as microRNAs (miRNAs), proteins, lipids, and other metabolites that function as cellular signals and play an important role in intercellular communication [1,3]. Plant cells secrete vesicles that are biogenically and morphologically comparable to mammalian-derived EVs [4,5]. Several terms are used in the literature to describe nano and micro-sized vesicles (50–1000 nm) isolated from plants, including plant-derived extracellular vesicles (PDEVs). Various functions have been attributed to PDEVs, including unconventional protein secretion, RNA transport, and defense against pathogens [6–8]. PDEVs have also been found to transfer small non-coding RNAs (sRNAs) between cells and to be abundant in proteins with antipathogenic characteristics [6,9]. By virtue of their natural origin, and their physicochemical and biophysical features, PDEVs could represent a new class of biocompatible therapeutics [4]. Indeed, PDEVs can be internalized by mammalian cells via different mechanisms, which include clathrin- and caveolae-mediated endocytosis, membrane fusion, or interaction with cellular proteins, where they modulate cellular processes [10,11]. Characterization methods have shown that plant EVs have similar structures to mammalian EVs [5], which may facilitate their uptake, tissue distribution, and ability to produce biological effects in mammalian cells [12]. Moreover, a distinctive feature of vesicle-facilitated intercellular communication is related to the enhanced resistance of bioactive molecules to biodegradability and metabolism [13]. For instance, encapsulated RNA rests protected from RNases and other circulating molecules and can reach distant targets (as hormones do) in a functional state, even when administered orally [14–21].

Epidemiological evidence suggests that adherence to diets rich in vegetables and fruits has a preventive effect against the risk of developing non-communicable chronic diseases (NCDs), such as cardiovascular disease and cancer. This effect has been traditionally associated with the bioactive molecules contained in these foods, including several classes of phytochemicals such as polyphenols, carotenoids, glucosinolates, vitamins, and many other plant secondary metabolites, as well as nucleic acids (i.e., RNAs). Nevertheless, the quantity of these molecules reaching host tissues may not be sufficient to exert bioactive effects [22,23]. Indeed, it is possible to encapsulate bioactive molecules in PDEVs to enhance their biological activity. In this context, strategies are currently being developed for enhancing the levels of particular miRNAs (sRNAs that regulate essential biological processes in plants and animals) inside food-derived EVs, which include mammalian milk [24] and edible-plants [4,20].

Previous studies have shown that certain phytochemicals can be naturally transported within PDEVs. For example, 6-gingerol and 6-shogaol, the active constituents of ginger, are present within ginger-derived EVs [16], and naringin, a major flavonoid found in grapefruits, as well as its functional component naringenin, were present in grapefruit-derived PDEVs [14]. Moreover, glucosinolates, the active constituents of broccoli, including sulforaphane, erucin, iberin, or indole-3-carbinol, were present in broccoli-derived EVs [25]. However, not all secondary metabolites produced in plants are loaded into PDEVs, such as naringenin and vitamin C, since other active constituents of orange juice were absent within orange juice-derived EVs [26].

Despite the growing interest, research on the potential therapeutic use of PDEVs is still in its early phases. Two clear examples of the current gaps in knowledge include the inexistence of standardized protocols for PDEVs isolation and a clear understanding of the actual biological functions of PDEVs [4].

Given the aforementioned, we isolated four food-derived EVs, by combining ultracentrifugation and size-exclusion chromatography (SEC), and we evaluated them as vehicles for enhanced transportation of synthetic miRNAs. Transcriptomic analysis of a human intestinal cell line (Caco-2) was carried out to appraise the biological effects upon exposure to PDEV-loaded miRNAs. Finally, we assessed the endogenous transport of dietary polyphenols and other secondary metabolites (e.g., glucosinolates in broccoli) by food-derived EVs.

2. Material and methods

2.1. Isolation and purification of extracellular vesicles (EVs)

2.1.1. Sample collection

Broccoli, pomegranates, apples, and oranges were purchased from several local greengrocers and supermarkets (Madrid, Spain). These 4 vegetables were selected because they are consumed all over the world. They also contain different polyphenols and phytochemicals. A citrus (i.e., orange), for its exclusivity in flavanones, apple for its content in phloretin/phloridzin, pomegranate for mono and diglucosylated anthocyanins and broccoli for glucosinolates, as well as other polyphenols. Thus covering a broad spectrum of bioactive molecules. Broccoli (*Brassica oleracea L.* var. *Italica)* flower heads were weighed (1 kg), washed with milli-Q water and, after excess water was removed, samples were homogenized at maximum velocity, for 2 min, in milli-Q water using a 1:1.5 wt to volume ratio (w/v). Pomegranate (*Punica granatum L.*) seeds (1 kg) were separated from the peel (exocarp and mesocarp) and homogenized at maximum velocity for 1 min to obtain the juice. Apples (*Malus domestica*, `Royal gala) were washed with milli-Q water, sliced in little portions (the peel was kept, but seeds were removed), and weighed (1.2 kg). Samples were homogenized at maximum velocity, for 2 min, in a 2:1 (w/v) of milli-Q water. Oranges (*Citrus sinensis*) were split in half and squeezed until 1 L of juice was obtained.

2.1.2. Isolation and purification of EVs

2.1.2.1. Ultracentrifugation. To remove plant fibers, large particles, and cellular debris, homogenized samples were sequentially centrifuged (4°C) at 5,000 x g, 10,000 x g, and 25,000 x g for 30 min, and at 35,000 x g for 60 min, in 250 mL containers (Beckman Coulter), using an Avanti Centrifuge J-26XPI (Beckman Coulter) with a JLA 16.250 rotor. Then, to precipitate extracellular vesicles, supernatants were ultracentrifuged at 135000 x g, for 105 min at 4°C, in 25 mL bottles (Beckman Coulter) using an OPTIMA L-90 K Ultracentrifuge (Beckman Coulter) with a 50.2 Ti rotor. Supernatants were discarded, and EV-enriched pellets were dissolved in 700 μL of sterile PBS (pH 7.4) to obtain homogeneous suspensions. Samples were subsequently filtered (0.45 μm, Millex®-GP, Merck Millipore, Burlington, MA, USA) to eliminate large-size particles and stored at − 80°C until used.

2.1.2.2. Size exclusion chromatography (SEC). SEC was performed after the first ultracentrifugation step (UC + SEC) to purify EVs from co-sedimented contaminants further, as previously described [20]. Briefly, thirty mL SEC columns were stacked with 20 mL of Sepharose CL-2B (CL2B300, Sigma-Aldrich) for a final matrix of 1.5 cm (diameter) x 12 cm (height). Columns were preserved with 0.02% azide in PBS at 4ºC to prevent contamination. Before use, columns were equilibrated with filtered (0.22 μm) PBS (pH 7.4). Filtered extracellular vesicles (700 μL) were loaded onto the columns. Elution was performed by gravity using filtered PBS (pH 7.4), and 40 sequential fractions of 500 μL were collected.

2.2. EV detection and characterization

2.2.1. Protein determination by the BCA assay

Protein concentrations were determined using a BCA assay kit (Thermo Scientific, Waltham) according to the manufacturer's instructions. For orange juice, due to color interference with the BCA quantification method, protein levels were assessed by the Lowry method [27], using Folin's phenol reagent.

2.2.2. Nanoparticle Tracking Analysis

Particle size distribution and concentration of extracellular vesicles isolated from broccoli were measured by nanoparticle tracking analysis (NTA) with a LM10 nanoparticle characterization system (NanoSight, Malvern). Three replicates of 60 s capture videos were recorded using a 638 nm laser. Analyses were performed using a NanoSight NTA 3.1. program. EV samples were appropriately diluted (1:100–1:1000) in PBS. Particle size distribution and concentration were analyzed at 23–27ºC. NTA analysis was performed on thawed samples. Zeta potential analyses of EVs were determined using the ZetaView Twin (Particle Metrix, Ammersee). Samples were diluted in pure water at room temperature and analyzed using pulse mode.

2.2.3. Transmission Electron Microscopy

Extracellular vesicles were visualized using transmission electron microscopy (TEM) with negative staining. Samples were placed on copper grids (200 mesh) for 3 min and stained with uranyl acetate for 1 min. Samples were examined (100 kV) on a JEOL JEM 1010 located at the Spanish National Centre for Electron Microscopy (ICTS, Madrid, Spain).

2.2.4. Western Blot

Determination of Evs' distinctive proteins was performed by WB to confirm the isolation procedure was successful. Twenty μL of each SEC recovered fraction, or 50 μg/protein of each sample, were used for protein detection. Protein samples were lysed in reducing conditions and separated on 10% sodium dodecyl polyacrylamide/bisacrylamide gels. Then, proteins were transferred onto nitrocellulose membranes (0.22 μm Millipore) and blocked with 5% skimmed milk (Bio-Rad, Hercules, CA, USA) for 30 min at room temperature. Membranes were incubated with appropriate primary antibodies: anti-PEN1 (CSB-PA875527XA01DOA, Cusabio), anti-BiP (AS09 481, Agrisera), anti-HSP70 (AS08 371, Agrisera), anti-PDR8 (AS09 471, Agrisera), or anti-TET8 (PHY1490S, PhytoAB) at 4 °C (overnight). After incubation, membranes were exposed to secondary anti-rabbit or anti-mouse antibodies conjugated with either Alexa FluorTM 680 or IRDye® 800, for 45 min, at room temperature. Blots were washed three times with TBST buffer after each incubation step and visualized using an Odyssey® infrared imaging system (LI-COR, Lincoln, NE, USA). Image Studio Lite 5.2.5 software was used for image processing.

For Western Blot controls, extracellular vesicles were isolated from apoplast wash fluid (AWF) from *Arabidopsis thaliana* rosettes. AWF was isolated by the infiltration method as previously described [7,28]. Briefly, whole rosettes were vacuum infiltrated with vesicle isolation buffer (20 mM MES, 2 mM CaCl2, and 0.1 M NaCl, pH 6.0 and without β-mercaptoethanol) using a syringe, as previously detailed [28]. Infiltrated plants were placed inside a 50 mL-syringe and centrifuged in 50-mL conical tubes at 1000 g for 10 min at 4ºC. The resulting AWF was ultracentrifuged at 135,000 x g for 105 min at 4ºC, as described above, resuspended in PBS, and filtered through a 0.45-μm membrane.

2.2.5. Phytochemical analysis of EVs

2.2.5.1. Identification of phenolic compounds via HPLC-DAD-ESI/MS^n. The phenolic compounds identification was performed in an Agilent HPLC 1100 series model equipped with a photodiode array detector (model G1315B), a mass detector in series (Agilent Technologies, Waldbronn, Germany), a binary pump (model G1312A), a degasser (model G1322A) and an autosampler (model G1313A), based on the method described by Migues et al. (2018) [29]. The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface and controlled by LC/MS software (Esquire Control Ver. 6.1. Build No. 534.1., Bruker Daltoniks GmbH, Bremen, Germany). A Nucleosil® 100–5 C18 column (25.0 cm × 0.46 cm; 5 μm particle size waters; Macherey-Nagel, Düren, Germany) was used, and the mobile phase was composed of two solvents: the eluent A consisted of water/formic acid (99:1, *v/v*), and the eluent B of acetonitrile. The solvent system started with 8% of B and reached 15% of B at 25 min, 22% at 55 min, and 40% at 60 min, with a wash-out period of 5 min and return to initial conditions afterwards. The mass spectra were acquired with a scan range from *m/z* 100–1200, and the MS parameters were set as follows: the capillary temperature was 350ºC, the capillary voltage was set at 4 kV, the nebulizer pressure was 65.0 psi, and the nitrogen flow rate was 11 L/min. The flow rate was 0.8 mL/min during the whole run, all gradients were linear, and the injection volume was 20 μL. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. For anthocyanins, the mass spectrometry data were acquired in the positive ionization mode, while for non-colored phenolics, the acquisition was done in a negative ionization mode. The MS^n was conducted in the automatic mode on the more abundant fragment ion in MS$^{(n-1)}$. The HPLC system was controlled by ChemStation for LC 3D Systems software Rev. B.01.03-SR2 (204) (Agilent Technologies Spain S. L., Madrid, Spain). The phenolic compounds were tentatively identified based on their elution order, retention times, and ultraviolet-visible and mass spectra features as compared to authentic standards analyzed under the same conditions and data available in the literature [29,30].

2.2.5.2. Identification of isothiocyanates (ITCs) by UHPLC-QqQ-MS/MS. Twenty microliters of each sample were acquired in a UPLC/MS/MS (UPLC-1290 Series and a 6460 QqQ-MS/MS; Agilent Technologies, Waldbronn, Germany). The analytes concentration was calculated using standard curves prepared freshly each day.

The GR and SFN were resolved chromatographically and identified by UHPLC-QqQ-MS/MS. Chromatographic separation was conducted on a ZORBAX Eclipse Plus C-18 Rapid Resolution HD (2.1 *x* 50 mm, 1.8 μm) (Agilent Technologies, Waldbronn, Germany). The column temperatures were held at 10ºC (left and right). The Multiple Reaction Monitoring (MRM) dynamic mode was performed in the positive mode, assigning ± 0.650 min as δ interval time for each retention time and preferential transitions of the corresponding analytes. Dwell time was 30 ms for all MRM transitions. The mobile phases employed were solvent A: ammonium acetate, 13 mM (pH 4 with acetic acid), and solvent B: acetonitrile/acetic acid (99.9:0.1, *v/v*). The flow rate was 0.3 mL/min using the linear gradient scheme (t; %B): (0.0; 12), (0.2; 20), (1.0; 52), (2.5; 95), and (2.5; 12). The optimal ESI conditions for maximal detection of the analytes were: gas temperature, 225ºC; sheath gas temperature, 350ºC; capillary voltage, 3500 V; Nozzle voltage, 1250 V; sheath gas flow, 12%; gas flow, 10; nebulizer, 40. The acquisition time was 2.5 min for each sample, with a post-run of 1.5 min for the column

equilibration. The MS parameter fragmentor (ion optics capillary exit voltage) and collision energy were optimized for each compound to generate the most-abundant product ions for the MRM mode. Data were acquired using the MassHunter software version B.04.00 (Agilent, Waldbronn, Germany). The concentrations of GR and SFN were calculated from the area ratio of the ion peaks of the compounds to those of the corresponding standards.

2.3. miRNA profiling

2.3.1. RNA isolation

Total RNA from samples selected for small RNA sequencing (RNA-seq) was isolated using miRNeasy® Mini kits (Qiagen), according to the manufacturer's instructions. Total RNA for real-time PCR (qPCR) analysis was isolated with the chloroform/phenol method [31]. Before RNA isolation, 1 pmol of cel-miR-39 (Qiagen) was added to samples for spike-in normalization.

2.3.2. Small RNA sequencing and data analysis

Total RNA from broccoli/fruit tissues or broccoli/fruit-purified EVs was isolated following the standard QIAzol protocol (Qiagen). RNA quality and integrity were evaluated using a 4200 TAPE Station or a 2100 Bioanalyzer (Agilent Technologies). A high degree of RNA integrity (RIN quality score $\geq$ 8) was obtained in tissue samples. EV samples showed a scant amount (or absence) of 18 S and 28 S ribosomal RNA, yet many $<$ 25 nucleotides (nt) fragments were present, which could indicate successful miRNA extraction.

Library preparation and sequencing were conducted at the "Fundación Parque Científico de Madrid" (Madrid, Spain). Briefly, total RNA from EVs and tissues was used as input for library preparation, using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (New England BioLabs) and following the manufacturer's recommendations. Libraries were validated and quantified using a 2100 Bioanalyzer (Agilent), and an equimolecular pool was prepared. Small RNA regions ($<$ 200 nt) were purified by polyacrylamide gel electrophoresis and titrated by qPCR using the Kapa-SYBR FAST qPCR kit for LightCycler480 and a reference standard for quantification. The library pool was denatured and seeded on a NextSeq v2.5 flowcell (Illumina), where clusters were formed and sequenced using a NextSeq 500 High Output kit v2.5 (Illumina) in a 1 $\times$ 75 single-read sequencing run on a NextSeq 500 sequencer (Illumina).

The resulting sequence reads were subjected to stringent filters, including removing low-quality reads, repeat sequences, and adaptor sequences, to generate cleaner data. Then, a bioinformatics pipeline (MIRPIPE) for miRNA discovery and profiling was applied as previously described [32]. In brief, filtered reads were aligned against known plant mature miRNAs in miRBase21.0 (November 2016; http://www.mirbase.org/index.shtml) using a maximum of one mismatch criterion. The identified reads were considered as conserved miRNAs, and the number of reads per miRNA was normalized.

2.4. cDNA and RT-qPCR

cDNA synthesis was performed with 500 ng of RNA isolated from cultured cells or 10 μL of total RNA extracted from EVs using the miScript II RT kit (Qiagen) according to the manufacturer's instructions. The miScript SYBR Green PCR kit (Qiagen) and specific primers (Isogen LifeSciences, Netherlands) or LNA-specific oligos (Qiagen) were used to conduct qPCR using a 384-well plate format in a 7900HT Fast Real-time PCR system (Applied Biosystems). Relative expression of miRNAs was determined by normalizing data using cel-miR-39–3p as spike-in or RNU6B as reference. MiRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method [33].

2.5. Selection of candidate miRNAs

Four miRNAs were selected based on the results from two independent small RNA-seq analyses of RNA isolated from either total tissue or EVs. Before further analyses, miRNAs with $<$ 10 reads were discarded. miRNAs were grouped into families, and the most representative miRNAs of each family, presenting identical sequences to miRNAs identified in *Arabidopsis thaliana*, were selected as candidates.

2.6. miRNA loading into broccoli and fruit-derived EVs

Single-stranded miRIDIAN ath-miR159a, ath-miR162a-3p, ath-miR166b-3p, and ath-miR396b-5p mimics, presenting methylation at the 3´-OH end (synthesized by Dharmacon®), were loaded into EVs isolated from broccoli/fruits. A solution containing 25 μM of each of these 4 miRNAs (total concentration = 100 μM) was diluted in siRNA 1 $\times$ Buffer (Thermo Fisher) and incubated with Lipofectamine 2000 reagent (Invitrogen, Thermo Scientific) for 15 min at room temperature, according to the manufacturers' guidelines. Filtered EVs were then incubated with the resulting mixture for 30 min at room temperature and kept at 4° C to minimize lipofectamine action until further purification. EVs transfected with an equal concentration of non-targeting control mimic sequences (Dharmacon) were used as controls. Afterwards, samples were loaded onto the SEC column following the protocol described in the corresponding section.

2.7. RNase A treatment

To assess the protective role of EVs and also to confirm miRNA incorporation in EVs, transfected EVs or free miRNAs were incubated at 37ºC for 30 min, with or without 10 μg/mL of RNase A (Ribonuclease A R6513, Sigma-Aldrich), or a combination of 10 μg/mL RNase A and 1% of TRITON 100-X (Sigma-Aldrich). Total RNA was subsequently purified using QIAzol Lysis Reagent (Qiagen) following the chloroform/phenol method.

2.8. In vitro exposure to EVs

2.8.1. Cell culture maintenance

Human colorectal adenocarcinoma cells (Caco-2) were obtained from the American Type Culture Collection (ATCC) and used between passages 27 and 38. Cells were cultured in DMEM with high glucose (Lonza), supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine (Gibco, Thermo Scientific), and 1% antibiotics (100 mg/mL penicillin and streptomycin) (Gibco). Cells were cultured at 37ºC in a 5% CO_2 humidified incubator. The culture medium was replaced every two days. Cell lines were seeded in 24-well plates (Corning Costar®, Sigma-Aldrich) at an initial density of 0.5 $\times$ 10^5 cells and maintained for approximately 24 h or 48 h until 70% of confluency was reached.

2.8.2. Cell viability, cell cycle distribution, and apoptosis assays

For cell viability analysis, 35,000 cells/well were seeded on 48-well plates. After 24 h, cells were treated with different concentrations of EVs for 24 h. Then, MTT (M2128, Sigma-Aldrich) was added to each well at a final concentration of 0.5 mg/mL for 2 h. Next, MTT was removed, DMSO was added to dissolve the MTT-formazan crystals, and absorbance was measured at 570 nm. Data are shown as the mean of 3 independent experiments performed in triplicates. Cell cycle distribution and apoptosis were analyzed by flow cytometry. For flow cytometry assays, 200,000 cells were seeded in 6-well plates. Cells were treated with different concentrations of EVs (0 (control), 0.1, 1% y 5%) for 24 h and collected using trypsinization. For cell cycle analysis, cells were fixed with 70% ethanol and stored at − 20ºC overnight. The next day, cells were dyed with a solution containing propidium iodide and RNAse. For apoptosis analysis, cells were stained with annexin V and propidium iodide. Results are shown as the mean of three independent experiments.

2.8.3. Cellular uptake of miRNAs transported by fruit-derived EVs

Caco-2 cells were exposed up to 5% of natural (i.e., not artificially loaded) broccoli EVs or broccoli EVs loaded with selected miRNAs for 2 or 24 h. Then, supernatants were discarded, cells were washed twice with PBS, collected in QIAzol Lysis Reagent (Qiagen), and stored at − 80ºC until RNA extraction. Five hundred ng of total RNA were used for RT-qPCR. Unexposed cells (basal culture media) were used as controls. Each experiment was performed in triplicate.

2.9. Transcriptome analysis of Caco-2 cells exposed to exogenous miRNA-loaded and natural EVs isolated from broccoli/fruits

RNA-seq was performed after Caco-2 cells were exposed to 5% of natural or exogenous miRNA-loaded EVs, isolated from broccoli, apple, orange, and pomegranate, to explore their potential impact on gene regulation. PBS-exposed cells were used as controls. Differentially expressed genes (DEG) were identified using the DESeq2 (version 1.36.0) R package, which uses a negative binomial distribution to perform the analysis. Multiple testing correction was performed using false discovery rate (FDR) estimation, and genes displaying FDR values higher than 0.05 were excluded from further analysis. The remaining list of genes was further filtered to include only genes presenting fold changes greater than 1.5 (upregulated) and lower than − 1.5 (down-regulated). Venn diagrams were assembled (using the ggplot R package, version 3.4.2) to identify common DEG among the 4 plant-derived foods. DEG Enrichment analysis was performed using the gene ontology (GO) resource (*http://geneontology.org/*), which connects to the analysis tool from the PANTHER Classification System [34,35].

2.10. Statistical analysis

Statistical differences between groups were evaluated using one-way

A

	Food	Concentration[a] (particles/mL)	Mean[a] (nm)	Mode[a] (nm)	Protein (mg/mL)
EVs (UC)	Apple	$1.29E^{+12} \pm 4.33E^{+09}$	221 ± 7.2	159 ± 8.1	7.6
	Broccoli	$4.28E^{+11} \pm 7.42E^{+10}$	213 ± 15	170 ± 48	14
	Orange	$2.72E^{+12} \pm 5.50E^{+11}$	191 ± 10	153 ± 5.1	2.8
	Pomegranate	$8.90E^{+11} \pm 4.56E^{+10}$	268 ± 14	201 ± 14	3.5
EVs (UC+SEC)	Apple	$1.79E^{+11} \pm 1.84E^{+10}$	253 ± 13	217 ± 33	0.16
	Broccoli	$2.45E^{+10} \pm 3.45E^{+09}$	241 ± 6.8	174 ± 19	0.59
	Orange	$3.30E^{+08} \pm 1.96E^{+07}$	167 ± 5.7	137 ± 6.2	0.05
	Pomegranate	$1.25E^{+11} \pm 4.28E^{+09}$	233 ± 2.6	177 ± 11	0.13

[a]Mean ± SD

B

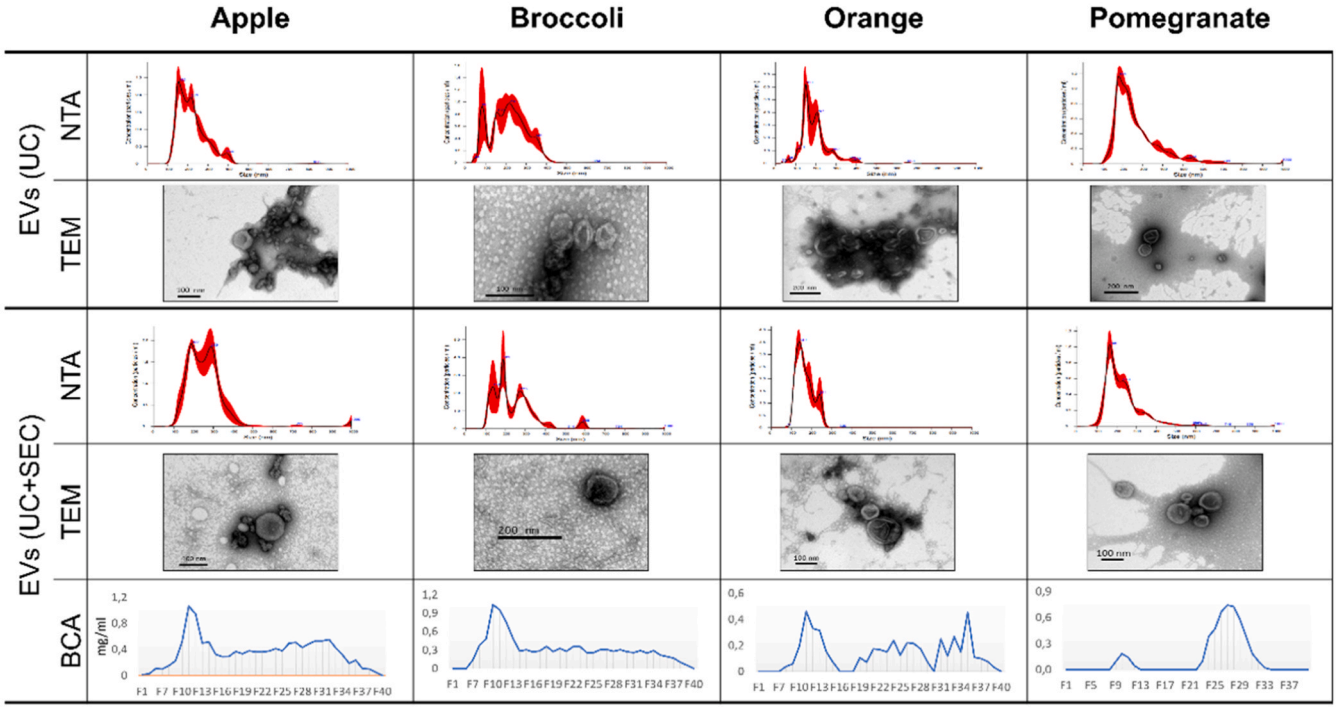

Fig. 1. : Characterization of broccoli/fruit-isolated EVs. **A)** Particle concentration, mean, mode size, and protein yield of EVs isolated with one cycle of ultracentrifugation (UC) or EVs isolated with UC combined with SEC (UC + SEC). **B)** Size distribution profile (NTA) and morphology (TEM) of EVs isolated by UC and UC + SEC. Protein elution profile (BCA) of EVs isolated by UC + SEC.

analysis of variance (ANOVA), comparing the mean of each treatment with that of controls, followed by Dunnett's or Bonferroni's post hoc tests. Data were expressed as means ± SD unless stated otherwise. Statistically significant differences were considered at * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$, **** $P \leq 0.0001$. All analyses were performed using GraphPad Prism V.9.

3. Results

3.1. Fruit-derived EVs isolation and characterization

Four fruits/vegetables (i.e., apple, orange, pomegranate, and broccoli) were selected to evaluate the potential therapeutic properties of isolated EVs. Differential ultracentrifugation (UC) was used as the starting method for isolating EVs. After the initial UC step, the particle size distribution of EVs ranged from 50 to 500 nm, with a mean size of 213 ± 15 nm for broccoli, 268 ± 14 for pomegranate, 221 ± 7.2 nm for apple, and 191 ± 9.9 nm for orange (Fig. 1, A and B). The presence and morphology of food-derived EVs isolated by UC were confirmed by transmission electron microscopy (TEM) (Fig. 1B). To further purify EVs, SEC was performed after UC (UC+SEC). Protein analysis of the 40 fractions collected revealed that most EVs were enriched in fractions F10-F14, while protein contaminants were present in fractions F16-F40 (Fig. 1B). The profile of protein contaminants was heterogeneous between foods. For instance, broccoli presented higher protein levels in EV fractions, while orange samples had higher protein levels in the contaminant fractions. As expected, contaminant protein concentration considerably decreased after SEC purification (Fig. 1A). The NTA profile of the fractions presenting the highest protein concentration (F10–14) showed that EVs ranged from 70 to 300 nm with a mean size of 241 ± 6.8 nm for broccoli, 233 ± 2.6 for pomegranate, 253 ± 13 nm for apple, and 167 ± 5.7 nm for orange (Fig. 1, A and B).

Furthermore, both protein and particle concentrations decreased after SEC. As performed for UC-isolated EVs, TEM was used to assess the size and shape of UC+SEC EVs and confirm they were surrounded by a plasmatic membrane (Fig. 1B). Characterization by Western blot confirmed the EVs isolation was accomplished successfully (Fig. 2). Broccoli EVs reacted for markers present in either *Arabidopsis* AWF or EVs, including TET8, PEN1, BIP, and HSP70, while pomegranate reacted against BIP and HSP70 and orange against HSP70. The z-potential of EVs was also evaluated and ranged from − 23.59 ± 0.93 mV for broccoli, − 26.33 ± 1.48 mV for pomegranate, − 21.26 ± 1.06 mV for orange, and − 19.65 ± 0.30 mV for apple.

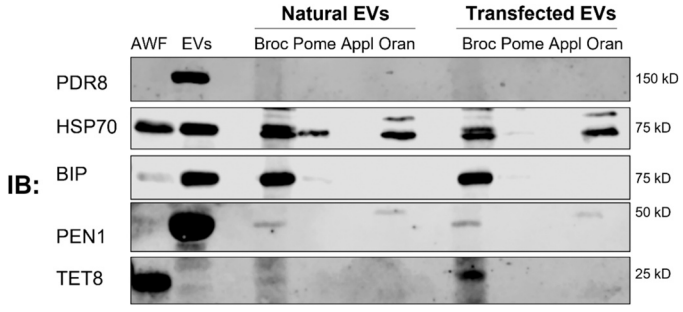

Fig. 2. : EV-associated proteins analyzed in *Arabidopsis* apoplast wash fluid (AWF), *Arabidopsis* extracellular vesicles (EVs), and natural and transfected food-derived EVs. Equal volumes of food-derived EVs were loaded. Samples were electrophoresed on 10% SDS−PAGE gels and analyzed by WB using anti-PDR8, anti-HSP70, anti-BiP, anti-PEN1, and anti-TET8 antibodies. Representative image of two independent experiments. Broc, broccoli; Pome, pomegranate; Appl, apple; Oran, orange.

3.2. Small RNA profiling of broccoli/fruit-derived EVs

The expression profile of miRNAs in the whole fruit, broccoli inflorescences, and UC + SEC-purified EVs was analyzed by small RNA sequencing. After normalization, miRNAs < 10 read counts were discarded for subsequent analyses. miRNAs were then grouped into families, and the percentage represented by each family in the total number of miRNAs was calculated (Supplementary Fig. S1). From each food plant, the 10 most representative miRNA families were identified (Fig. 3A). Interestingly, EVs were enriched in 10 miRNA families, encompassing more than 80% of miRNAs identified by RNAseq. Four miRNA families were common to total tissue and isolated EVs (miR159, miR162, miR166, and miR396) for all 4 foods studied here (Fig. 3B). Finally, 4 miRNAs (i.e., ath-miR159a, ath-miR162a-3p ath-miR166b-3p, and ath-miR396–5p) belonging to the 4 common miRNA families identified above were selected for subsequent analyses based on abundancy (according to read counts) and identical sequence to those of *Arabidopsis thaliana* (*ath*) (Table 1).

3.3. Endogenous miRNAs are transported in broccoli/fruit-derived EVs

The levels of the 4 selected miRNAs were measured by RT-qPCR to verify their endogenous transport within natural (unloaded) broccoli/fruit-derived EVs, isolated using UC and further purified by SEC. In general, miRNAs were enriched in fractions F10-F14 (Fig. 4).

3.4. Exogenous miRNAs can be loaded into broccoli/fruit-derived EVs

Broccoli/fruit-derived EVs were loaded (via lipofection) with the 4 previously selected miRNA candidates to assess if they could be loaded with exogenous miRNAs. As anticipated, the levels of miRNAs transfected into EVs were at least 20-fold higher than those found in natural (non-transfected) EVs (Fig. 5), indicating that loading exogenous miRNAs into EVs was effective. Moreover, unintended alterations in EVs' size, shape, or protein profile, potentially resulting from the transfection process, were assessed. The protein profiles and the sizes and shapes of transfected EVs were similar to natural EVs (Fig. 5). Western blot analysis also showed no major changes in protein markers after loading with the 4-above mentioned miRNAs (Fig. 2). Zeta potential analyses showed no significant changes in PDEVs for broccoli (−21.23 ± 1.45 mV), pomegranate (−26.06 ± 3.39 mV), or orange (−20.31 ± 0.60 mV), while apple (−27.11 ± 1.01 mV) slightly changed their z-potential after miRNA loading.

3.5. Fruit-derived EVs protect RNAs from RNase degradation

Degradation of miRNAs after treatment with RNase A and (or) Triton X-100 was compared among 1) free (naked) miRNA, 2) native EVs, and 3) miRNAs loaded into EVs. The survival percentage of each miRNA was calculated by comparing each sample type with its corresponding control, i.e., 1) untreated free miRNAs, 2) untreated natural EVs, or 3) untreated loaded EVs. As expected, free miRNAs were extensively degraded in the presence of RNase A, whereas RNA cargos were protected from RNase A degradation (Fig. 6). Treatment with Triton X-100 (which decomposes vesicle structure, exposing its cargo) combined with RNase A resulted in substantial degradation in free and encapsulated miRNAs, suggesting that miRNAs were loaded inside the vesicles. In general, Triton X-100 treatment alone reduced surviving miRNAs but was not as extensive as that observed when RNAse A was applied simultaneously.

3.6. Broccoli/fruit-derived EVs restrain proliferation in vitro

3.6.1. Unloaded EVs

Caco-2 cells were exposed to up to 5% of foodstuff-derived EVs isolated by UC or UC + SEC for 24 h (Fig. 7). Broccoli-derived UC-isolated

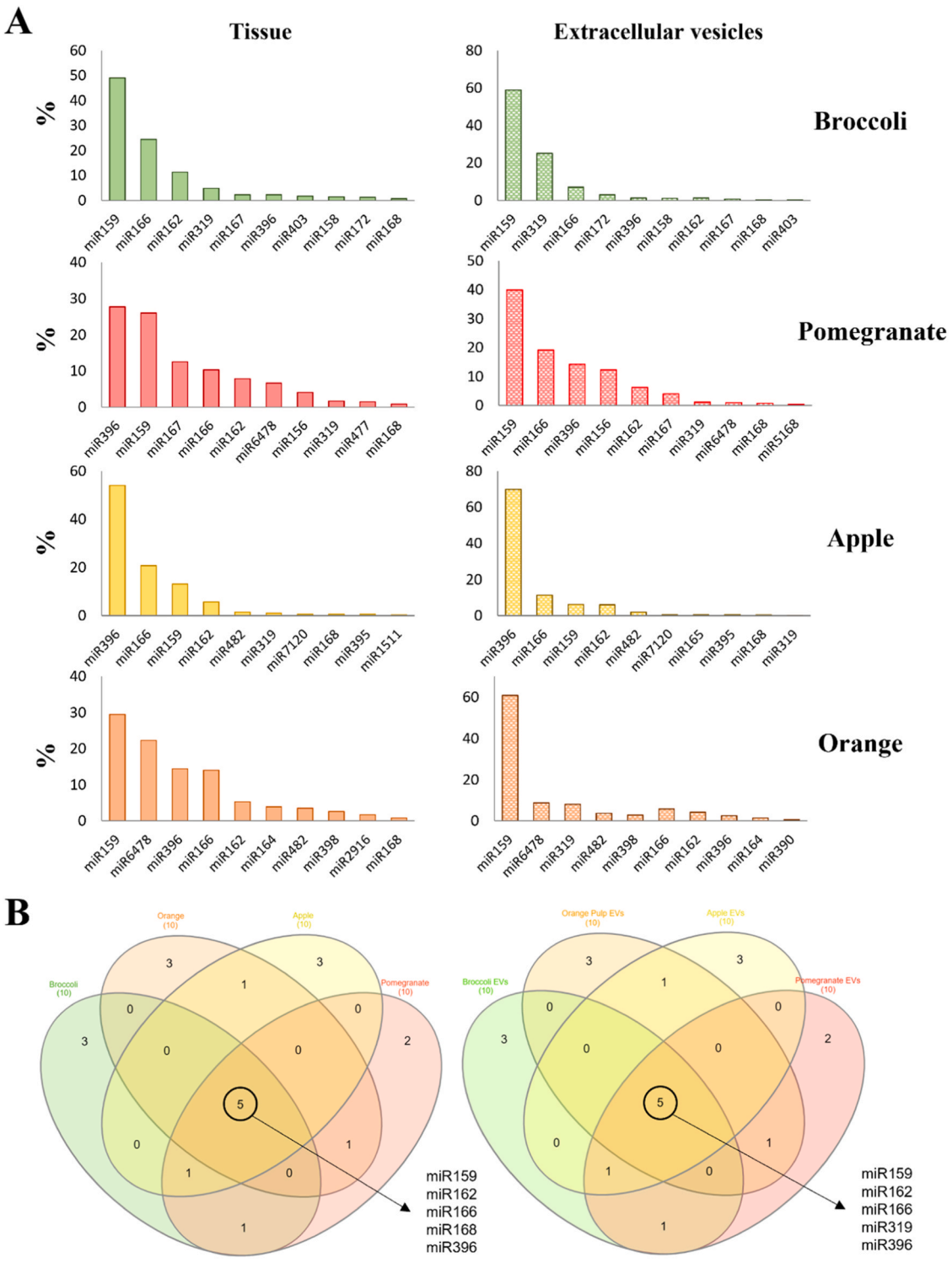

Fig. 3. : A) Selection of the 10 most representative miRNA families found for each foodstuff. "%" represent the relative amount of miRNA family present in each food. B) Identification of common miRNAs between the 4 selected foodstuffs, both in total tissue (left) and in isolated EVs (right).

Table 1
Name, sequence, and MIMAT of methylated miRNAs selected for further analyses.

Name	Sequence	MIMAT
ath-miR159a	UUUGGAUUGAAGGGAGCUCUmA	0000177
ath-miR162a-3p	UCGAUAAACCUCUGCAUCCAmG	0000182
ath-miR166b-3p	UCGGACCAGGCUUCAUUCCCmC	0000190
ath-miR396b-5p	UUCCACAGCUUUCUUGAACUmU	0000945

EVs reduced cell viability by 50%, and UC + SEC-isolated EVs by approximately 75%. However, no significant differences were found between UC and UC + SEC regarding cell viability (Fig. 7). As in the case of broccoli, exposure to pomegranate and apple UC-isolated EVs reduced survival percentages to around 60%, yet the effect caused by UC+SEC-purified EVs was marginal (Fig. 7). Both UC and UC + SEC-isolated broccoli EVs resulted in around 50% of cell survival when a different cell line (HepG2) was tested (Supplementary Fig. S2). HepG2 cell viability

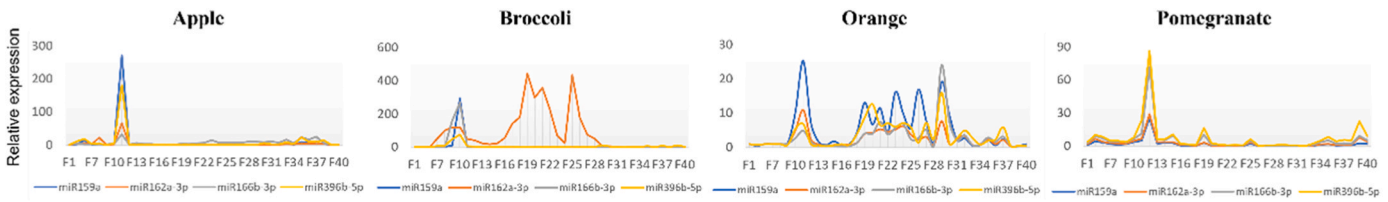

Fig. 4. : Elution profile of candidate miRNAs naturally transported (endogenous) in EVs isolated by UC + SEC.

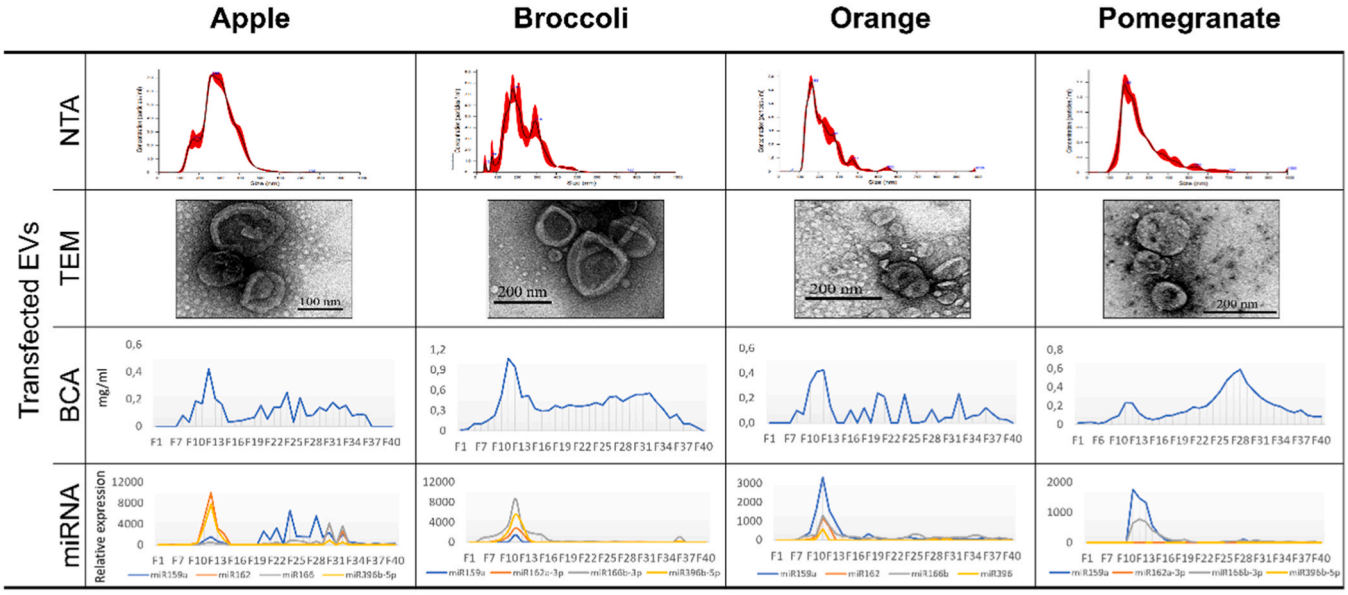

Fig. 5. : Size distribution profile (NTA) and morphology (TEM) of EVs isolated by UC + SEC and transfected with exogenous miRNAs. Elution profiles of protein (BCA) and selected miRNAs of EVs isolated by UC + SEC and transfected with exogenous miRNAs.

was not affected by both apple-isolated EV types, whereas pomegranate EVs reduced cell viability by around 25% (Supplementary Fig. S2). Neither UC nor UC + SEC isolated orange EVs significantly affected cell viability in both cell lines.

3.6.1.1. Apoptosis and cell cycle distribution analyses of broccoli EVs. As broccoli EVs showed the most consistent effect in cell viability for both cell lines tested, apoptosis (Annexin V staining) and cell cycle distribution analyses were performed using broccoli-isolated EVs. Exposure to 5% of broccoli EVs led to around 50% of apoptosis induction (30% and 20% of early and late apoptosis, respectively) in Caco-2 cells (Fig. 8A), which were mainly found at the sub-G1 stage (Fig. 8B).

On the other hand, results confirmed that, compared to broccoli UC-isolated EVs, exposure to UC+SEC-isolated EVs produced a moderate apoptotic effect in Caco-2 cells (Fig. 9).

3.6.2. miRNA-loaded EVs

To check if miRNA-loaded EVs also affected the viability of Caco-2 cells, these were exposed to increasing concentrations of loaded or unloaded EVs isolated from broccoli, apple, orange, or pomegranate and purified by SEC. In all cases, at the lowest tested concentration (1%), miRNA-loaded EVs significantly reduced cell viability compared to unloaded EVs (Fig. 10). However, this effect was not dose-dependent at the concentrations tested. Noteworthy, in the case of broccoli, unloaded (natural) EVs, per se, significantly affected cell viability in a dose-dependent manner.

3.7. Exogenous miRNAs transported within broccoli/fruit-derived EVs are taken up by Caco-2 cells

Caco-2 cells were exposed to 5% of natural broccoli EVs or loaded with selected miRNAs for 24 h (Fig. 11). As expected, cells did not take up free miRNA. As natural broccoli EVs contain endogenous miRNAs, it would be expected that some cellular uptake would be seen, as it did. Nevertheless, cells exposed to ath-miR159a, ath-miR162a-3p, ath-miR166b-3p, and ath-miR396b-loaded EVs showed uptake levels, at least 10-fold higher than natural EVs.

3.8. Transcriptome analysis of Caco-2 cells exposed to miRNA-loaded and unloaded EVs isolated from broccoli/fruits shows a shared gene regulation impact

Since broccoli/fruit-derived miRNA-loaded EVs seemed to exert a more drastic effect on Caco-2 cells, at least regarding cell viability, RNA-seq analysis was performed to explore if the underlying impact on gene regulation could help explain this finding. Thus, Caco-2 cells were exposed to 5% of miRNA-loaded or unloaded EVs isolated from broccoli, apple, orange, and pomegranate. PBS-exposed cells were used as controls. Among the list of differently expressed genes found for each condition, only a few were common to the four foodstuffs for natural EVs vs. control and transfected vs. control (Figs. 12A and 12B, respectively).

For each foodstuff, differently expressed genes between control and miRNA-loaded and unloaded EVs were analyzed using gene ontology (GO) term enrichment using PANTHER gene list analysis tools. Regarding broccoli, the analysis revealed that miRNA-loaded and unloaded EVs had a similar gene regulation impact, affecting biological processes such as cellular response to xenobiotic (GO:0071466) and

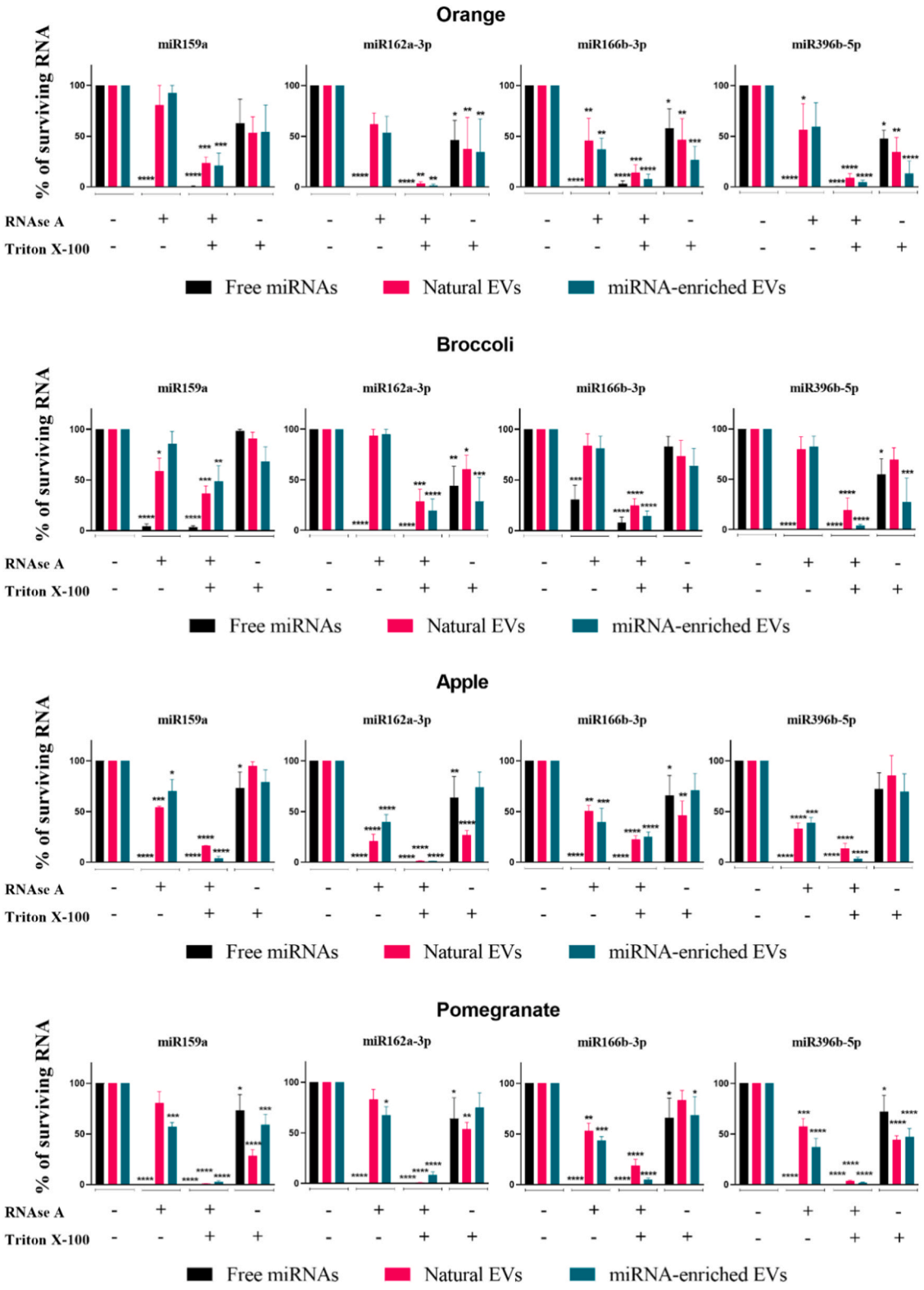

Fig. 6. : RNase A protection assay. Survival percentage for free miRNA, natural EVs and transfected EVs (miRNA-enriched EVs) after treatment with RNase A, Triton X-100 or both, compared to untreated samples (controls, considered as 100%). Values represent means ± SEM; n = 3 independent experiments. * ** * $P \leq 0.0001$; * ** $P \leq 0.001$; * * $P \leq 0.01$; * $P \leq 0.05$.

lipid (GO:0071396) stimuli and the corresponding metabolic processes (GO:0006805 and GO:0006629, respectively). The regulation of signal transduction (GO:0009966), the regulation of the MAPK cascade (GO:0043408), and the regulation of the endothelial cell apoptotic process (GO:2000351) were also identified. On the other hand, differently expressed genes were not found when comparing unloaded and miRNA-loaded EVs, suggesting that the impact on gene expression of the selected miRNAs loaded into broccoli-derived EVs was scarce, if any.

In the case of orange, the GO analysis revealed that miRNA-loaded

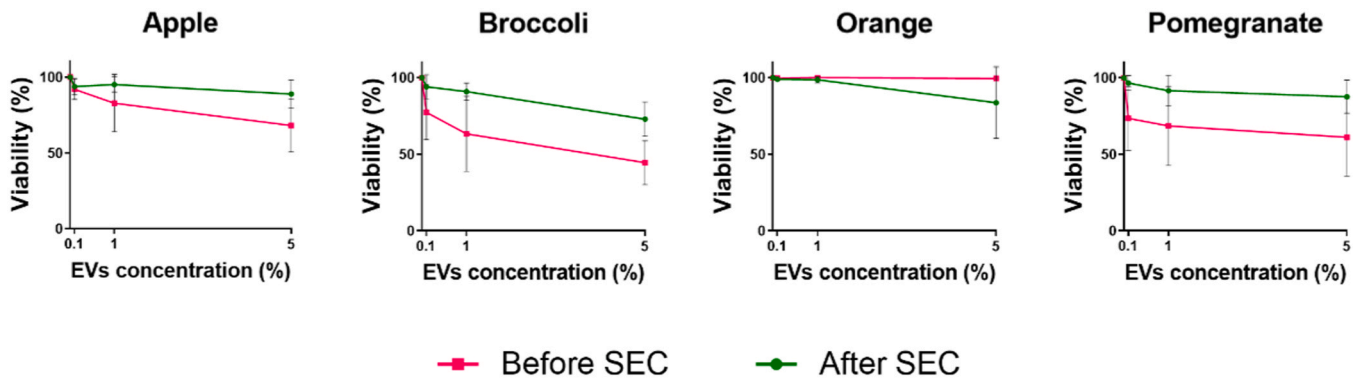

Fig. 7. : Survival percentages of Caco-2 cells exposed to UC-isolated EVs before or after SEC purification for 24 h. Values are means ± SEM (n = 3).

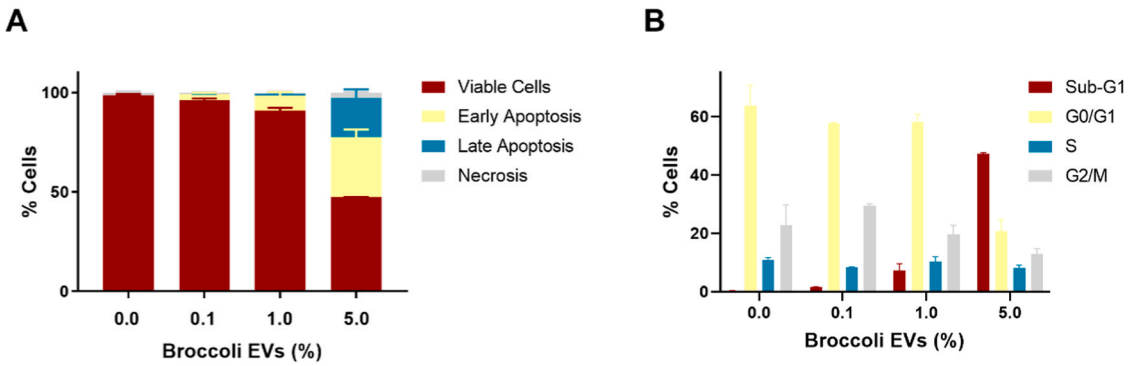

Fig. 8. : Apoptosis (**A**) and cell cycle (**B**) distribution analyses of Caco-2 cells exposed to different concentrations of broccoli-derived EVs isolated by ultracentrifugation.

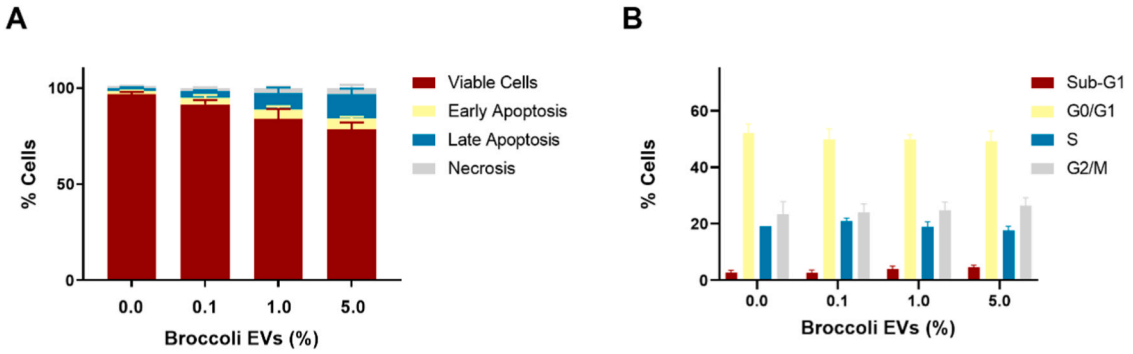

Fig. 9. : Apoptosis (**A**) and cell cycle (**B**) distribution analyses of Caco-2 cells exposed to different concentrations of broccoli-derived EVs isolated by ultracentrifugation and further purified by SEC.

and unloaded EVs had a similar gene regulation impact affecting biological processes related to ATP synthesis (GO:0015986, GO:0042776) and ATP biosynthetic (GO:0006754) and metabolic (GO:0046034) processes, as well as oxidative phosphorylation (GO:0006119) and nucleoside triphosphate biosynthetic processes (GO:0009142), among others. As in the case of broccoli, differently expressed genes were not found when comparing unloaded and miRNA-loaded EVs, further suggesting that the impact on gene expression of the selected miRNAs loaded into orange-derived EVs was also very limited, if any. Concerning pomegranate EVs, the GO analysis revealed that the differently expressed genes found between unloaded EVs and controls were associated with humoral immune response (GO:0006959) and response to chemokine (GO:1990868). On the other hand, no statistically significant results were found for the very few differently expressed genes found between miRNA-loaded EVs and controls nor between loaded and unloaded EVs. Finally, although differently expressed genes were found between miRNA-loaded and unloaded apple-isolated EVs and controls, the GO analysis did not bring back statistically significant associations with biological processes or pathways.

3.9. Broccoli/fruit-derived EVs transport residual amounts of dietary (poly)phenols

As seen in the previous section, miRNA-loaded EVs and unloaded EVs caused an analogous impact on gene regulation, suggesting that the selected miRNAs had minor effects on the observed transcriptomic alterations, at least at the concentration tested. The (poly)phenolic cargo of broccoli/fruit-isolated EVs was assessed in three fractions (i.e., juice, supernatant, and EVs) to explore if other bioactive molecules (such as (poly)phenols) naturally present in unloaded EVs could be associated

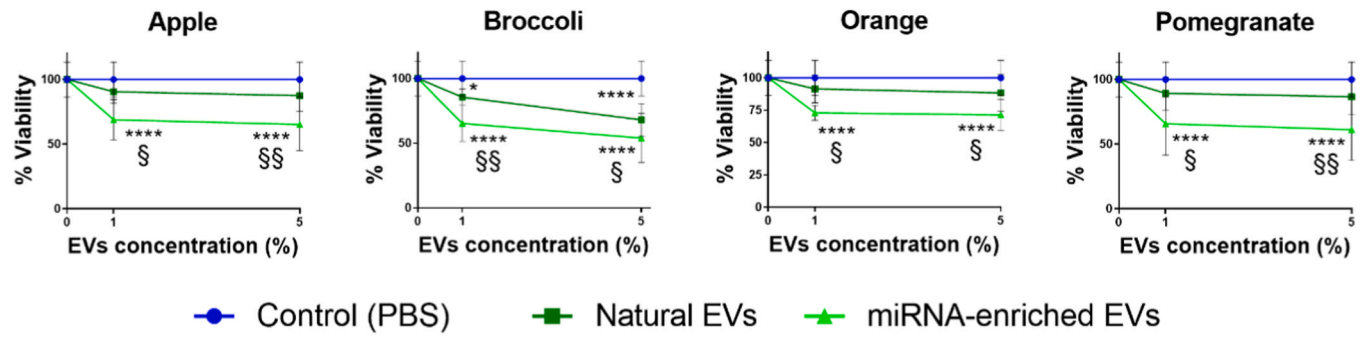

Fig. 10. : Survival percentages of Caco-2 cells exposed to EVs, either unloaded (natural) or loaded with selected miRNAs, for 24 h. Values are means ± SEM (n = 3). miRNA-enriched EVs vs. control: ****P ≤ 0.0001; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; miRNA-enriched vs natural EVs: $^{§§§§}P \leq 0.0001$; $^{§§§}P \leq 0.001$; $^{§§}P \leq 0.01$; $^{§}P \leq 0.05$.

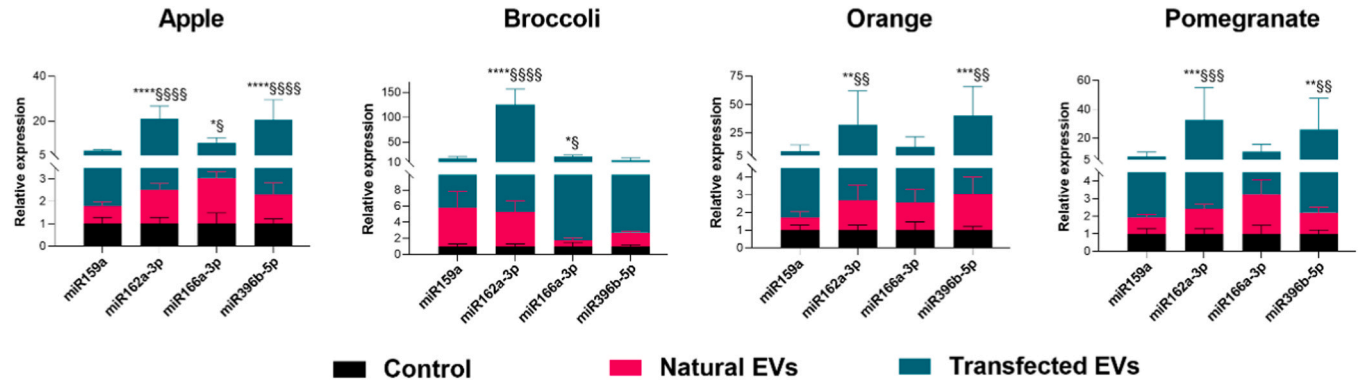

Fig. 11. : Relative levels of selected miRNAs found in Caco-2 cells exposed to 5% of broccoli EVs, either unloaded (natural) or loaded (transfected) with selected miRNAs, for 24 h. Non-treated cells were used as controls. Values are means ± SEM; n ≥ 3 independent experiments. Transfected EVs vs. control: **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; Transfected vs natural EVs: $^{§§§§}P \leq 0.0001$; $^{§§§}P \leq 0.001$; $^{§§}P \leq 0.01$; $^{§}P \leq 0.05$.

with their biological actions. Phytochemical analyses revealed that the large majority of detected (poly)phenols were present in juice and supernatant, whereas most (poly)phenols were not detected or present in trace amounts in EVs (Table 2 and Supplementary Figs. S3-S6), which suggests (poly)phenols are likely minor actors in the actions of the EVs derived from these plant foods. For instance, broccoli sulforaphane (SFN) and indole-3-carbinol (I3C), two well-known isothiocyanates (ITCs) with antiproliferative properties in colon cancer cells were present in the juice and supernatant, while only residual amounts were detected in the EVs (Fig. 13).

4. Discussion

PDEVs are biogenically and morphologically comparable to mammalian-derived EVs and have attracted much attention as potentially scalable and sustainable alternatives to the latter. In vitro and in vivo findings have demonstrated that PDEVs possess intrinsic therapeutic activities and can also be used as efficient and biocompatible nanocarriers of bioactive molecules. However, there are still several challenges to address before basic EV research can be translated into clinical applications. Currently, major hurdles include the lack of standardized isolation and bioactive molecule-loading protocols, as well as solid methods for the characterization of EVs from various plant sources. In this sense, results consistency regarding the downstream applications of EVs will depend on developing more efficient, specific, reliable, and reproducible EV extraction and purification methods. Indeed, the scientific community calls for rigor and standardization in plant EVs research [36]. In this context, the International Society of Extracellular Vesicles (ISEV) has recently updated the guidelines for minimal information for studies of extracellular vesicles (MISEV) [37], which have been taken into consideration here. However, in contrast to what happens with mammalian EVs, there are no specific guidelines for research on PDEVs.

The growing research interest in mammalian-derived EVs has led to the promotion of numerous EVs isolation methods, such as ultracentrifugation, ultrafiltration, size-exclusion chromatography (SEC), precipitation, and immunoaffinity capture [38–40]. The idea is to take advantage of the physical properties of EVs, such as size, density, charge, solubility, and surface-exposed proteins, to isolate these vesicles from other biological contaminants [41]. Both differential and (or) gradient ultracentrifugation have been widely reported in PDEVs research [14, 16,25]. Here, we used ultracentrifugation as the first step to isolate and concentrate broccoli/fruit-derived EVs. As mentioned, ultracentrifugation has been widely used to isolate EVs, but this procedure has important shortcomings, such as co-sedimentation of non-EV-associated proteins and protein aggregates. As the ISEV highly advises the use of more than one purification method to obtain mammalian EVs [37,42], it seems reasonable to consider these recommendations regarding the isolation and characterization of PDEVs. SEC has been applied to EV isolation from various sources [4,43] and was tested here as a complementary method for further purification of broccoli/fruit-derived EVs. EV characterization results, including size, shape, concentration, and protein yield, indicated that combining these purification methods led to successfully isolating EVs from broccoli, pomegranate, apple, and orange. Previous studies have shown that these vegetables/fruits are valid sources of EVs. For example, methods such as ultracentrifugation have been used to isolate apple EVs [44,45], a combination of tangential flow filtration and SEC was applied to recover pomegranate EVs [43], and, in the case of orange EVs, ultracentrifugation alone [15,18], ultracentrifugation followed by SEC [26,46], and ultrafiltration followed by

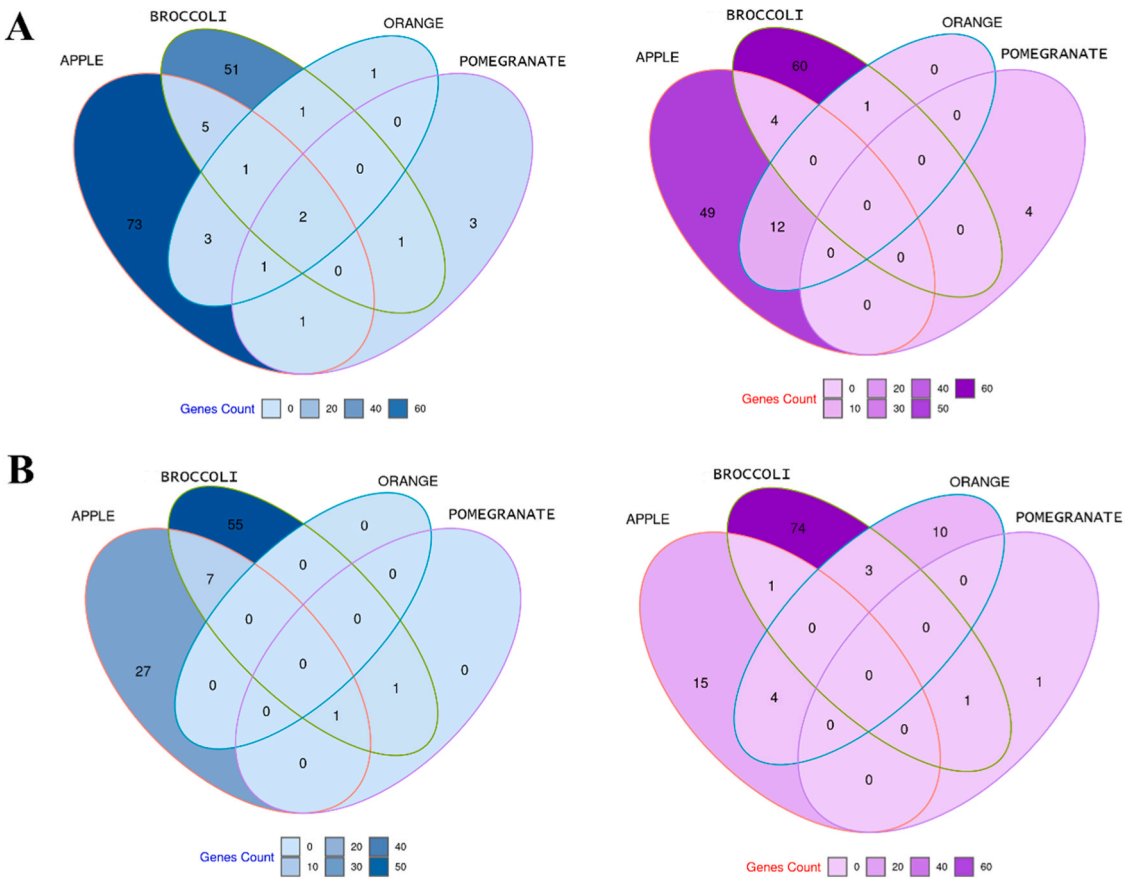

Fig. 12. : A) Common differently expressed genes between natural EVs and controls. Upregulated (left) and downregulated (right). **B**) Common differently expressed genes between miRNA-transfected EVs and controls. Upregulated (left) and downregulated (right).

Table 2
Phytochemical analysis of phenolic compounds in EVs isolated from broccoli and selected fruits.

Source	Compound	M-/M+ (*m/z*)	Rt (min)[a]	Concentration (μmol/mL)[b]		
				Juice	Supernatant	EVs
Orange	*p*-Coumarate	M- 355	9.4	0.0256 ± 0.012	0.0323 ± 0.003	N.D.
	Ferulate	M- 385	9.7	0.0548 ± 0.003	0.0732 ± 0.029	N.D.
	Feruloyl-glc	M- 355	12.3	0.1239 ± 0.088	0.0715 ± 0.018	N.D.
	Sinapoyl-glc	M- 385	12.5	0.0495 ± 0.006	0.0442 ± 0.013	N.D.
	Vicenin 2/Apigenin 6–8-di-C-glc	M- 593	13.5	0.6663 ± 0.471	0.6868 ± 0.035	N.D.
	3-FeruloylQA	M- 367	23.1	0.0422 ± 0.025	0.0523 ± 0.011	N.D.
	Hesperidin	M- 609	26.7	1.324 ± 0.687	1.600 ± 0.288	0,0942 ± 0067
	Dehydrotrimer FA	M- 575	30.5	0.0245 ± 0.003	0.0245 ± 0.003	N.D.
Broccoli	*p*-coumaroylQA	M- 325	7.4	Traces	Traces	N.D.
	3-FeruloylQA	M- 367	11.1	Traces	Traces	N.D.
	K-3-(sinap)diglc-7glc	M- 977	12.2	0.0406 ± 0.002	0.0405 ± 0.002	N.D.
	K-3diglc	M- 609	17.2	0.0421 ± 0.039	0.0434 ± 0.039	N.D.
	K-3glc	M- 447	26.6	0.0409 ± 0.002	0.0406 ± 0.002	N.D.
Pomegranate	3-FeruloylQA	M- 367	12.4	0.0468 ± 0.001	0.0045 ± 0.006	N.D.
	6'-β-hexopyranosyloeoside	M- 551	14	0.0299 ± 0.002	0.0015 ± 0.002	N.D.
	6'-β-hexopyranosyloeoside	M- 551	14.8	0.0121 ± 0.000	Traces	N.D.
	Caffeic acid hex-deriv.	M- 533	25.9	0.0107 ± 0.002	0.0021 ± 0.003	N.D.
	Dp-3,5diglc	M+ 627	4.9	0.0288 ± 0.019	0.058 ± 0.006	N.D.
	Cy-3,5diglc	M+ 611	6.8	0.2102 ± 0.158	0.2802 ± 0.063	0,0026 ± 0004
	Pg-3,5diglc	M+ 595	8.6	0.0054 ± 0.000	0.0129 ± 0.012	N.D.
	Cy-3glc	M+ 449	11.3	0.0829 ± 0.032	0.0819 ± 0.087	0.0096 ± 0.0008
	Pg-3glc	M+ 433	13.6	Traces	Traces	N.D.
Apple	Q-glc	M- 463	22.3	0.039 ± 0.000	0.0392 ± 0.001	N.D.
	Q-pent	M- 433	25.9	0.0388 ± 0.000	0.0389 ± 0.000	N.D.
	Q-rhamnoside	M- 447	26.6	0.0393 ± 0.001	0.0394 ± 0.001	N.D.

[a] Retention time;
[b] Mean±SD; N.D., not detected. glc= glucoside, QA= quinic acid, FA= ferulic acid, diglc= diglucoside, K= Kaempferol, Q= quercetin, hex= hexose, deriv.= derivative, Dp =Delphinidin, Cy= cyanidin, Pg= pelargonidin, pent= pentoside, rham= rhamnoside

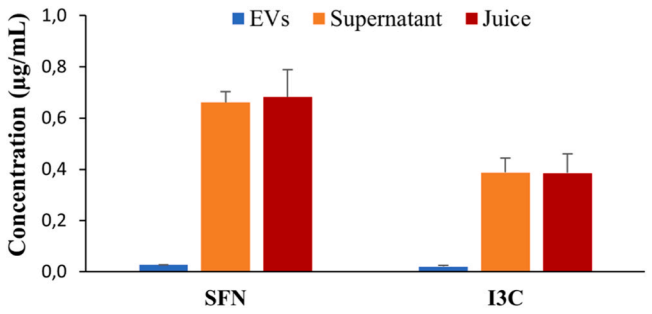

Fig. 13. : SFN and I3C present in different broccoli fractions, determined by UHPLC 3Q-MSMS.

ultracentrifugation [47], have all been used. The values obtained here for the z-potential, a parameter used for assessing EVs condition, were generally similar to other studies [25] [26]. Even though SEC is one of the most efficient techniques for exosome purification because of its ability to remove contaminants and preserve the integrity of exosomes, its use for large-scale separations requires careful handling. For example, any separation into fractions requires further characterization of each fraction and elution of the sample, generating - as a by-product - the mobile phase. Moreover, the discontinuity of the process for EV separation may limit the use of this technology for large-scale productions.

Although the general principles of MISEV guidelines seem to be applicable to the field of PDEV research, recommendations regarding specific protein markers for mammalian EVs cannot be applied to plant EVs [36]. In this context, we analyzed, by Western Blot, proteins previously described to be present in extracellular vesicles (including apoplastic fluids) and found that PEN1, TET8, or HSP70 could be valid candidates as protein biomarkers of broccoli EVs, and HSP70 also for pomegranate and orange EVs. HSP70 has been previously found in several EV proteomic studies performed with *Arabidopsis*, *Nicotiana*, olive, or sunflower [36]. One possible explanation of why antibodies work better for broccoli EVs than the selected fruits might be that broccoli belongs to the *Brassicaceae* family, as does *A. thaliana,* which is used to generate most plant antibodies. Other common proteins found in plant EVs, including PDR8/ABCG36, only reacted to *A. thaliana* EVs and not the food-derived EVs tested here.

Following small RNA sequencing analysis in whole tissue and SEC-purified EVs, the most abundant common miRNA families (based on read counts) found in the four plant foods, i.e., miR159, miR162, miR166, and miR396, were selected for further analysis. These miRNAs were confirmed to be naturally (endogenously) transported within purified broccoli/fruit EVs. As endogenous miRNAs may not be present in sufficient amounts to exert biological effects, we evaluated whether broccoli, pomegranate, apple, and orange EVs could be enriched with exogenous miRNAs with potential bioactivity and act as protective nanocarriers. Loading of the four above-mentioned miRNAs (using lipofection) did not cause major changes in the purified EVs' size, concentration, or shape. As expected, exogenous miRNA levels were several fold-times higher than endogenous miRNAs. In addition, RNase protection assays confirmed that exogenous miRNAs were predominantly located within EVs. Successful examples of the incorporation of bioactive molecules within food-derived extracellular vesicles or membrane vesicles include astaxanthin [48] and sulforaphane [25] for broccoli EVs and mRNA vaccines and dexamethasone for orange EVs [15,18,49]. Here, we show that extracellular miRNAs with potential therapeutic purposes can also be efficiently loaded into PDEVs.

miR159 is one of the most abundant miRNAs present in the four PDEVs studied here, especially in broccoli EVs. Since plant miR159 was previously shown to affect the proliferation of breast cancer cells [50], it seemed reasonable to test whether EVs could influence the viability of other types of cancer cellular models. We found that most EVs exerted cytotoxic effects when ultracentrifugation was the sole method of isolation; however, when a SEC purification step was added, only broccoli EVs persisted in causing cytotoxic effects, thus, reinforcing the need for standardization of the methodology used in plant-derived EVs research.

Exposure to exogenous miRNA-loaded EVs (miR159a, miR162a, miR166b, and miR396b) had a toxic effect on Caco-2 cells (5% of EV volume was sufficient to reduce their viability), suggesting that these miRNAs might contribute to the cytotoxic effects of PDEVs. In previous studies, broccoli membrane vesicles [25,51] or EVs [52] were found to exert in vitro antiproliferative activity.

RNA-seq analysis was performed to explore if the underlying impact on gene regulation could help explain why broccoli/fruit-derived miRNA-loaded EVs seem to exert a more drastic effect on the viability of Caco-2 cells compared to natural EVs. However, only a very reduced number of differently expressed genes were common to the four foodstuffs analyzed, suggesting that a shared mechanism of action is unlikely. Similarly, for each foodstuff, when DEG found for control vs. miRNA-loaded and control vs. unloaded EVs were analyzed using bioinformatic tools, a shared mechanism of action could not be detected between the different EV sources. Regarding broccoli, GO term enrichment results seem to reinforce the observations that unloaded (natural) broccoli EVs can affect cellular viability per se (Fig. 10), as DEG were, among other processes, associated with the regulation of the MAPK cascade, which plays a key part in translating extracellular inducements to a widespread diversity of cellular responses, including cell growth, migration, proliferation, differentiation, and apoptosis [53]. In this regard, processes associated with regulating endothelial cell apoptosis were also identified here. On the other hand, transcriptomic results do not seem to be associated with apoptosis or other non-apoptotic cell-induced death mechanisms that could help explain the decrease in cellular viability seen in cells exposed to orange-, apple- and pomegranate-isolated EVs transfected with selected miRNAs (Fig. 10). Most surprisingly, DEG could not be identified between unloaded and loaded EVs, preventing further comparison between both types of EVs using bioinformatic tools. Thus, further studies are needed for clarification.

As mentioned above, some of the bioactive properties of broccoli have been associated with miRNAs [50,54]. Still, this vegetable is greatly recognized as a source of health-promoting phytochemicals, including phenolic compounds (flavonoids, chlorogenic and sinapic acid derivatives), nitrogen-sulfur derivatives (i.e., glucosinolates (GSLs), which can be hydrolyzed to generate bioactive isothiocyanates (ITCs)), vitamins (A, C, K, and B6) and minerals (selenium, potassium, and manganese). For instance, the ITCs sulforaphane (SFN) and indole-3-carbinol (I3C) have been shown to exert beneficial health properties [55], particularly in cancer research [56,57]. SFN, erucin, iberin, and I3C have been previously found in broccoli membrane vesicles at low concentrations [25]. Here, SFN and I3C were also detected in SEC-purified EVs, as well as trace amounts of other (poly)phenols. One possible explanation for the differences found regarding the concentration of glucosinolates is that most studies used bulk methods for EVs isolation, including discontinuous sucrose gradient ultracentrifugation [58], while ultracentrifugation plus an additional step of purification (SEC) were used here.

Although some other polyphenols, including naringin or naringenin, were present in grapefruit-derived PDEVs [14], information on the transport of other polyphenols in PDEVs is scarce. Here, we show that other polyphenols are also transported in SEC-purified EVs from fruits, including cyanidin 3-glucoside and cyanidin 3,5-diglucoside in pomegranate EVs or hesperidin in orange EVs. Why and how some (poly) phenols and not others are incorporated within EVs is unknown and deserves further investigation. Moreover, whether these contribute to the reported biological effects has been poorly described [14,59] but warrants further research. In this context, recent data suggest that mammalian (including humans) EVs might transport dietary

polyphenols [60,61], but whether the amount transported is relevant for biological effects to occur is unknown.

While preclinical studies with plant- or fruit-derived EVs have shown promising results, there are still several challenges and knowledge gaps that have to be addressed for the successful translation of PDEVs into clinical applications. For example, in addition to the pitfalls described above, limitations in the large-scale production of biocompatible PDEVs for commercial applications exist. The methods we used, i.e. ultracentrifugation and SEC have limited scalability potential. While different other methods of PDEVs purification have been described [4] none of them alone reaches the desired levels of yield, purity, scalability, sutainability, or throughput. Moreover, for therapeutic applications, the isolation of PDEVs would need to meet current good manufacturing practices, which is rare in pre-clinical studies.

There is also a need to find suitable and reliable markers of PDEVs. One reason for this is the relatively small number of PDEVs proteomics analyses and proper databases. Clearly, additional plant EV proteomics datasets are needed to start establishing a set of reliable markers [36]. The use of reliable markers will also help to detect contaminations from cytosolic or enzyme proteins during the isolation procedure. Indeed, the destructive procedure (different from that of extracellular apoplastic fluid content isolation) could artificially produce nanoparticles/microsomes or release other contaminants [36]. As for other food-derived EVs, e.g. milk-derived EVs, one possible contaminants is proteins. While the protein content of PDEVs appears to differ among species [4], their proportions are typically lower than those of mammalian EVs. Hence, further proteomic analyses will help to uncover protein contaminants during the isolation procedures. In summary, we report here that PDEVs (i.e., broccoli, pomegranate, apple, and orange) have the potential to be versatile nanoplatforms for the delivery of extracellular miRNAs. Our findings suggest that PDEVs increase the biological stability of exogenous miRNAs against RNase degradation and ferry miRNAs toward cellular uptake in vitro, regulating gene expression and exerting biological activity. Broccoli EVs stand out for their effect on cell viability, whereas this effect only seemed to occur for fruit-derived EVs when they were transfected with miR159a, miR162a-3p, miR166b-3p, and miR396b-5p. Results also show that PDEVs transport low levels of phytochemicals, including flavonoids, anthocyanidins, phenolic acids, or glucosinolates. As duly noted, some results will require further investigation, and we stand by the appeal for rigor and standardization in plant EVs research to facilitate solid comparisons between different studies and sources. As the pharma industry searches for scalable, sustainable, cost-effective, stable, and low immunogenic drug delivery nanovesicles, PDEVs might be suitable candidates for future RNA-based therapies.

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CRediT authorship contribution statement

AD and MCLH designed the paper. JTC and AD wrote the paper. MCLH, JTC, LdP-A, AdS-L, LB, CC, DAM, CG-V and ER performed the experiments and/or data collection. LAC conducted RNAseq and bioinformatic analysis. AD, MCLH, CG-V, DAM, JMO, and JCE obtained funding. FV, JCE, ER, DAM, CG-V and AD revised and edited the manuscript. All authors have read and approved this manuscript.

Declaration of Competing Interest

none.

The authors declare no conflict of interest associated with this work.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2023.106999.

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