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Cardoon cell cultures as a biofactory for extracellular vesicles with antisteatotic activity in an in vitro model of non-alcoholic fatty liver disease

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A R T I C L E I N F O

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A B S T R A C T

Food-derived extracellular vesicles (EVs) hold growing interest in their applications across diverse fields, including nutraceuticals and functional foods. Plant-derived EVs present a sustainable, cost-effective alternative to other sources, addressing challenges related to safety and scalability. This research investigates the potential of cardoon (*Cynara cardunculus* L. var. *altilis*) cell suspension cultures (CSCs) as a novel biotechnological platform for EV production. EVs were isolated from cardoon CSC-conditioned media using differential ultracentrifugation and size exclusion chromatography and characterized through nanoparticle tracking analysis, interferometry, and transmission electron microscopy, showing a narrow size distribution and round-shaped morphology. Comparative proteomic and metabolomic analyses of cardoon calli and CSC EVs revealed a significant enrichment of bioactive proteins and secondary metabolites in the EV preparations. These components include key molecules associated with plant defence mechanisms and potent antioxidant and anti-inflammatory activities.

In an in vitro model of non-alcoholic fatty liver disease (NAFLD) using HepG2 cells, cardoon EVs exhibited notable hepatoprotective properties. They reduced reactive oxygen species (ROS) and nitric oxide (NO) levels, enhanced cell viability, and decreased lipid accumulation, demonstrating efficacy comparable to metformin, employed as lipid-lowering drug. Mechanistically, CSC EVs activated the SIRT-1/AMPK signalling pathway, a key regulator of lipid metabolism. Under lipotoxic conditions, EV treatment enhanced AMPK phosphorylation and upregulated SIRT-1 expression. Pharmacological modulation of SIRT-1 further confirmed that CSC EVs regulate this pathway.

These findings highlight the potential of cardoon EVs in tackling oxidative stress and lipid dysregulation, positioning them as a promising natural therapeutic candidate for NAFLD. By advancing the understanding of EVs isolated from plant cell cultures, this study opens avenues for their application in treating metabolic and liver-related disorders, reinforcing the potential role of EVs in nanomedicine, biotechnology, and the development of new functional food supplements.

1. Introduction

Cells have evolved sophisticated mechanisms for intercellular communication, with extracellular vesicles (EVs) being one of the most remarkable. These small, membrane-bound, non-replicating particles, typically ranging from 50 to 200 nm, are naturally secreted by cells and

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serve as vesicles for transferring bioactive molecules, including lipids, proteins, and nucleic acids (such as microRNAs and long non-coding RNAs) (Théry et al., 2018). This form of communication is a highly evolved mechanism for influencing other cells locally and systemically, and fascinatingly, it also facilitates communication between different organisms and across biological kingdoms (inter-kingdom communication) making them potential delivery systems and innovative therapeutic tools in various biomedical applications (Chen et al., 2021; Raposo & Stahl, 2019).

EVs are also an integral and functional part of our diet, naturally occurring in a diverse array of food products. They are found in dairy items like milk (Tong et al., 2023), fermented products (Pérez Martínez et al., 2023), probiotics (Seo et al., 2018; Tong et al., 2021), as well as in plant products, including fruits and juices. Interestingly, plant-derived vesicles (PDVs) have demonstrated a diverse array of health-promoting properties, including antioxidant, antimicrobial, anti-inflammatory, anticancer, and wound-healing effects (Alfieri et al., 2021; Jin et al., 2024; Lian et al., 2022; Stanly et al., 2020). While their use for various aspects of human health is now well established, challenges persist due to non-standardized purification protocols affected by plant genetic diversity, seasonal factors, and environmental conditions, which also result in relatively low yields. Not least, current destructive isolation methods produce heterogeneous mixtures containing genuine EVs, other small vesicles, and non-vesicular contaminants coming from damaged cells and tissues, challenging their characterization (Pinedo et al., 2021). This ambiguity also contributes to the lack of a clear classification of PDVs, pointing to the need for alternative plant-based biotechnology systems that ensure standardized purification while preserving biomolecular cargo and reproducible bioactivity.

A promising solution to these challenges is the use of plant biotechnology platforms, including in vitro plant cell and organ cultures (Ambrosone et al., 2023; Conte et al., 2025). Conditioned media of cell suspension cultures (CSCs) and hairy roots (HRs) offer significant advantages for EVs collection, yielding high quantities of pure EVs free from microbial contamination and pesticides. These systems allow for precise control of growth conditions, optimizing factors like pH, temperature, nutrient availability, and oxygen concentration to enhance the production of EVs with desired characteristics, making them suitable for scalable and reliable applications in human health.

In recent years, our group has successfully purified and thoroughly characterized EVs from the conditioned media of HR cultures of two *Salvia* species, demonstrating their potential as innovative, non-conventional sources for therapeutic applications, particularly in treating cancer and Parkinson's Disease (PD) (Boccia et al., 2022; Vestuto et al., 2024).

Here, we expand current knowledge on the therapeutic potential of plant EVs derived from in vitro systems by using CSCs of cardoon (*Cynara cardunculus* L. var. *altilis*) as a novel source for EVs production. Cardoon is an excellent choice for a healthy diet. It is rich in dietary fibre, which aids digestion, and contains significant amounts of vitamins and minerals, including vitamin C, vitamin K, potassium, magnesium, and calcium. These nutrients contribute to various health benefits, such as supporting the immune system, promoting bone health, and maintaining cardiovascular function. Additionally, cardoon is a source of antioxidants like flavonoids, which help combat oxidative stress and inflammation. Beyond its nutritional profile, cardoon is abundant in bioactive compounds, notably phenolic acids (such as mono- and dicaffeoylquinic acids), inulin, fibre, and sesquiterpene lactones. These constituents have been extensively studied for their health-promoting properties, including antimicrobial, anti-inflammatory, antioxidant, lipid-lowering, cytotoxic, antidiabetic, cardiotonic, and hepatoprotective activities (Mandim et al., 2023; Silva et al., 2022). The synergistic effects of these bioactive compounds make cardoon a valuable component in both traditional diets and modern therapeutic applications. In addition, cardoon callus cultures offer a versatile platform for the large-scale production of high-value compounds from primary and secondary metabolism. Advancements in genetic engineering have further enhanced this platform, enabling the accumulation of higher levels of oleic acid and specialized metabolites by targeting key fatty acid desaturase biosynthetic genes through overexpression and RNA interference (RNAi)(Cappetta et al., 2022; Pirona et al., 2025).

In this work, conditioned media of cardoon CSCs have been employed as source for EV purification. Biophysical analyses successfully confirmed the presence of EVs in the conditioned media of CSCs, providing detailed information on their size and characteristics. Moreover, biomolecular analysis of cardoon EVs revealed an array of proteins and secondary metabolites with interesting biological roles. Finally, the potential therapeutic effects of cardoon-derived EVs in vitro were investigated, focusing on their ability to support liver health and counteract steatosis using human HepG2 hepatocarcinoma cells. This study explored the potential antioxidant properties, effects on cell viability under steatotic conditions, and the capacity to modulate lipid accumulation. The results indicate that cardoon EVs hold significant promise as potential therapeutic agents for addressing non-alcoholic fatty liver disease (NAFLD). Finally, the findings from this research highlight the innovative use of cardoon cell cultures as non-conventional sources for producing EVs with potential hepatoprotective benefits.

2. Materials and methods

2.1. *Cynara cardunculus* cell suspension cultures production

Cardoon (*Cynara cardunculus* L. var. *altilis*) calli, established using leaves from the genotype "Spagnolo" as described by Paolo and collaborators (Paolo et al., 2021), were grown in the dark at 24 ± 1 °C on Gamborg B5 medium (GB5, Duchefa Biochemie, Haarlem, Netherlands, #G0209) supplied with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/l adenine, 0.1 mg/l kinetin, 3% (*w/v*) sucrose, 8% (w/v) agar, adjusted to pH = 5.8. For cell suspension cultures, 1.0 g of friable fresh callus was inoculated in a 250 ml flask containing 100 ml of GB5 liquid medium and incubated on an orbital shaker at 100 rpm in the dark at 24 ± 1 °C. Cells were subcultured every 12 days by transferring 10 ml of cell suspensions into 90 ml of fresh GB5 medium. Living cells were visualized using fluorescein diacetate (FDA, 5 mg/ml). Ten μl of FDA were added to 1 ml of cell suspension and slides were examined using a Leica DM6000B epifluorescence microscope. Images were captured with a DFC365 FX CCD camera and LAS AF software (Leica Microsystems).

2.2. Isolation of EVs from cell suspension cultures

EVs were isolated from conditioned media harvested from ten-day-old subcultured cardoon CSCs corresponding to the exponential phase as reported previously by our group (Paolo et al., 2021). EVs were purified using two widely established methods: differential ultracentrifugation and size exclusion chromatography.

2.2.1. Differential ultracentrifugation (dUC)

The dUC purification protocol involved two initial low-speed centrifugation of the cell culture media previously collected in 50 ml centrifuge tubes. The initial centrifugation was conducted at 500 × *g* for 10 min at 4 °C followed by a second centrifugation step at 2000 × *g* for 20 min at 4 °C. The supernatant was transferred to Beckman polycarbonate tubes, suitable for high-speed centrifugation and centrifuged at 15,000 × *g* for 20 min using a Beckman Type 70 Ti rotor, effectively pelleting larger microvesicles (typically >300–500 nm). The supernatants were then filtered using 0.45 μm nylon membrane filter (Merck Millipore) to eliminate cellular debris and organelles, ensuring cleaner downstream processing. The filtered supernatant was transferred into new polycarbonate tubes (Beckman Coulter, No. 355631) in a Beckman Coulter Optima™ L-90 K and ultracentrifuged at 100,000 × *g* for 3 h at 4 °C with a Type 70 Ti rotor to isolate extracellular vesicles (with expected sizes between 50 and 200 nm), which sedimented to form a pellet at the bottom of the tubes. The pellet was then gently resuspended in 30–60 μl of MilliQ water and stored for up to one month at −80 °C for subsequent biophysical, proteomic, metabolomic and bioactivity analyses.

2.2.2. Size exclusion chromatography (SEC)

The SEC protocol involved a first step of centrifugation at 10,000 × *g* for 10 min at 4 °C. The resulting supernatant was then filtered using 0.45 μm filters to eliminate cell debris. EVs were separated using Izon's qEV Size exclusion chromatography columns (Gen 2 qEV), with elution performed using sterile PBS according to the manufacturer's guidelines. The purified EV preparations were stored up to one month at −80 °C for subsequent biophysical, proteomic, metabolomic and bioactivity analyses. To ensure sterility throughout the process, the SEC purification was performed under a laminar flow hood.

2.3. Characterization of cardoon CSC EVs

2.3.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining

For the preliminary screening of EV preparations, SDS-PAGE was performed. 10 μl of each purified EV sample, containing approximatively 10^{10} particles/ml, was resolved on a 10% SDS-PAGE polyacrylamide gel. The gel was subsequently silver-stained following previously described protocols (Boccia et al., 2022; Vestuto et al., 2024). EV preparations displaying clear and intact protein profiles were selected for subsequent analyses.

2.3.2. Nanoparticle tracking analysis (NTA)

The concentration and hydrodynamic diameters of cardoon EV samples were determined using Nanoparticle Tracking Analysis (NTA) with a NanoSight NS300 instrument (Malvern Instruments, Malvern, UK) following the manufacturer's guidelines. Before analysis, samples were diluted 1:100 with milli-Q water to achieve an approximate count of 20–120 particles per frame optimal for NTA. The NanoSight NS300 instrument, equipped with a green laser and sCMOS camera, was set up with the following parameters: camera level 15, acquisition time of 60 s, and detection threshold 4. Three videos per sample were recorded at room temperature under flow conditions with a syringe infusion rate of 30. The recorded video data were subsequently processed using NanoSight NTA 3.4 software. All measurements were performed in biological triplicates.

2.3.3. Interferometric light microscopy (ILM) by Videodrop (Myriade)

Cardoon EV size and concentration were also measured by interferometric light microscopy using a Videodrop instrument (Myriade, Paris), following the manufacturer's instructions. For each measurement, 6 μl of sample were loaded onto the chip. Data were acquired using Videodrop software (v. 2.5.5.6797), with a minimum of 300 particles tracked per video.

2.3.4. Transmission electron microscopy (TEM)

Before using a carbon film-supported 300 mesh copper grid, a glow discharge was performed for 30 s at 15 mA. Ten μl of EVs (10^9 particles/ml) were fixed with 2.5% glutaraldehyde in PBS and deposited on parafilm. The grid was placed on the drop for 5 min, then washed with distilled water for 1 min. It was subsequently immersed for 1 min in a drop of 3 ml of 2% Methylamine Vanadate (NanoVan) diluted in water, followed by another 1-min wash with distilled water. TEM images were captured using a FEI Tecnai T20 (FEI Europe, Eindhoven, Netherlands) in the Laboratorio de Microscopias Avanzadas at Universidad de Zaragoza (Spain).

2.4. Proteomic and metabolomic profiling of cardoon calli and CSC EVs

2.4.1. Proteomic profiling and bioinformatic analyses

Proteomic profiling of cardoon calli (30 μg) and 50 μl of cardoon EV preparation (10^9 particles/ml) was performed as previously described (Vestuto et al., 2024). Briefly, proteins were separated by 12% SDS-PAGE. The gel bands underwent a trypsin in-gel digestion procedure. Mass spectrometry analysis of the peptide mixtures was performed using Q-Exactive Orbitrap instrument (Thermo Fisher Scientific), coupled with a nanoUltimate3000 UHPLC system (Thermo Fisher Scientific. Peptide separation was achieved on a capillary EASY-Spray PepMap column (0.075mmÅ ~ 50 mm, 2 μm, Thermo Fisher Scientific) using aqueous 0.1% formic acid (A) and CH3CN containing 0.1% formic acid (B) as mobile phases and a linear gradient from 3 to 40% of B in 45 min and a 300 nL·min^{-1} flow rate. Mass spectra were acquired over an m/z range from 375 to 1500. Protein characterization was performed using the Proteome Discoverer software, against a reviewed *Cynara cardunculus* database (SwissProt) with the following parameters: trypsin cleavage; carbamidomethylation of cysteine as a fixed modification and methionine oxidation as a variable modification; a maximum of two missed cleavages; false discovery rate (FDR), calculated by searching the decoy database, 0.05. Proteomic analyses were performed in duplicate on two different vesicle preparations and two cardoon calli extracts. Gene ontology analyses of Biological Process, Molecular Function and Cellular Component categories were performed using the ShinyGo 0.80 analysis tool (http://bioinformatics.sdstate.edu/go80/), applying a False Discovery Rate (FDR) threshold of 0.05 and focusing solely on pathways containing more than two proteins.

To identify conserved plant EV markers, *C. cardunculus* CSC EV proteome was compared with the previously reported apoplastic EV proteome of the plant model species *Arabidopsis thaliana* (Rutter & Innes, 2017).

FASTA sequences of the 113 cardoon EV proteins were retrieved from the UniProt database and aligned with human sequences (genome assembly GRCh38.p14) using the blastp mode of BLAST on Ensembl (https://www.ensembl.org/index.html). A homologous alignment was considered valid if the identity within the region met or exceeded 50%. The complete Vesiclepedia database (downloaded and accessed on 10 January 2025) was imported into FunRich, 3.1.3. The top 100 proteins identified in EVs according to Vesiclepedia, were obtained from ExoCarta (http://exocarta.org/) and compared with the identified human homologs.

2.4.2. Metabolomic profiling

For cardoon calli analysis, the sample was extracted with an 8:2 EtOH/H_20 hydroalcoholic solution (1:20 matrix/solvent ratio) using UAE (Ultrasound-assisted extraction) for 15 min. The obtained extract was rotavapor-dried and then lyophilised. To promote the extraction of specialized metabolites from cardoon CSC EVs the procedure for vesicles disrupts was setup: 3 cycles of freezing in dry ice for 5 min and thawing for 5 min in a bath heated to 40 °C with UAE was performed.

The extracts were analysed on an Orbitrap Q-Exactive Classic Mass Spectrometer (Thermo Fisher Scientific) coupled with an Ultimate300 UHPLC system (Thermo Fisher Scientific). A Luna® C18 150 × 2 mm, 3 μm (100 Å) column (Phenomenex®, Castel Maggiore, Bologna, Italy) was used to optimize the separation. As mobile phase aqueous 0.1% formic acid (A) and CH_3CN containing 0.1% formic acid (B) were used in this method setup: isocratic 5% B for 5 min, followed by a linear gradient to 60% in 30 min. The flow rate is 0.2 ml/min. Mass spectra were acquired over an m/z range from 100 to 2000 in both polarities. For qualitative analysis, the following standards were used: ferulic acid 4 O-hexoside, syringic acid 4 O-hexoside, caffeoyl 4-ferulic acid, ferulic acid 3 O-hexoside, chlorogenic acid, 3,4-dicaffeoylquinic acid, 18-hydroxylinoleic acid and 17-hydroxylinolenic acid were obtained from Sigma Chemicals Company (Milan, Italy).

2.5. Bioactivity and cellular uptake of cardoon CSC EVs

2.5.1. Cell culture, NAFLD model set-up and drug treatment

Human hepatocellular carcinoma cell line HepG2 (HB-8065) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in Minimum Essential Medium (MEM, 4500 mg/ml glucose) supplemented with 10% (*v/v*) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 5% non-essential amino acids. Cells were routinely grown in culture dishes (Corning, Corning, NY) in an environment containing 5% CO_2 at 37 °C and splitted every 2 days. Prior to cell seeding, cell growth and viability were evaluated by phase-contrast microscopy and trypan blue exclusion analysis (Covelli et al., 2023). In each experiment, cells were placed in a fresh medium, treated with EVs (10^9 particles/ml) either alone or in combination with oleic and palmitic acid (OA/PA) (Sigma Aldrich, St. Louis, MO, USA), in a 2:1 ratio (final concentration: OA 500 μM, PA 250 μM in 1% BSA solution), for 24 h to induce non-alcoholic fatty liver disease (NAFLD). For experimental validation, cells were also exposed to metformin (Sigma Aldrich, St. Louis, MO, USA; 1 mM), as a reference lipid-lowering drug, the Sirtuin 1 inhibitor EX527 (Sigma-Aldrich, St. Louis, MO, USA; 10 μM) and the Sirtuin 1 activator SRT2104 (MedChemExpress, Monmouth Junction, NJ, USA; 10 μM). Each treatment and analysis were performed in at least three independent experiments.

2.5.2. MTT assay

Cell viability was established by measuring mitochondrial metabolic activity with 3-[4,5-dimethylthiazol-2,5-diphenyl-2H-tetrazolium bromide (MTT). Briefly, HepG2 (30 × 10^3 cells/well) was plated into 96-well plates, then cardoon EVs were added in co-administration with OA/PA for 24 h. For dose–response MTT experiments, EVs were tested across a concentration range of 10^2 to 10^8 particles/ml. Then, a concentration of 10^8 particles/ml was selected for all subsequent assays.

Afterwards, MTT reagent (0.5 mg/ml) for 1 h was added. Then, 100 μl per well of 0.1 M isopropanol/HCl solution was added to dissolve the formazan crystals. The absorbance was measured at 570 nm, using a microplate reader (Multiskan Go, Thermo Scientific, Waltham, MA, USA). Cell viability was expressed as a percentage relative to the untreated cells cultured in medium with 0.1% DMSO and set to 100%, whereas 10% DMSO was used as positive control and set to 0% of viability. To assess the specificity and bioactivity of cardoon EVs, various controls were employed. As negative controls, cells were treated with heat-inactivated EVs and EV-depleted medium. Heat inactivation of cardoon EV preparations was performed by incubating the EVs at 95 °C for 1 h using a thermoblock as reported previously by our group (Boccia et al., 2022). The supernatant from the final ultracentrifugation step used for EV collection (100,000 ×*g* for 3 h at 4 °C) was used as the EV-depleted medium control. For comparative analyses, EV preparations from other plant species, including *Salvia sclarea* and *Salvia dominica*, were used as additional controls. These EVs were isolated and characterized according to our previous work (Vestuto et al., 2024).

2.5.3. Calcein AM/PI live-dead dual staining

Calcein AM is a cell-permeant dye that can be used to determine cell viability in most eukaryotic cells (Zhao et al., 2022). In live cells, the non-fluorescent calcein AM is converted to a green, fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases, while propidium iodide (PI) is used to stain dead cells (red channel). In this experiment, cells were grown (3 × 10^4 cells/well) in 96-well. The cells were treated with cardoon EVs alone and in the presence of OA/PA for 24 h. Then, the medium was discarded, and the cells were washed with PBS three times. The cells were co-stained with calcein AM (2 μM) and PI (3 μg/ml) (Sigma-Aldrich, St. Louis Missouri, USA). After 20 min, the stained cells were washed, and representative images of live cells were acquired using ZOE Fluorescent Cell Imaging System (Biorad, Hercules, California, USA).

2.5.4. EV fluorescent labelling and cellular uptake

Cardoon EVs were labelled using BODIPY® FL N-(2-aminoethyl) maleimide (Thermo Fisher Scientific, MA, USA), as previously described (Vestuto et al., 2024) with slight modifications. Briefly, conditioned media from *C. cardunculus* cultures were sequentially centrifuged at 500 ×*g* for 10 min, 2000 ×*g* for 20 min, and 15,000 ×*g* for 20 min. The supernatants were then filtered through a 0.45 μm membrane filter, and 10 mM BODIPY dye was added to stain the small EV-enriched fraction. After a 15-min incubation at room temperature, labelled- EVs were collected by ultracentrifugation at 100,000 ×*g*. Fluorescently labelled EVs (10^9 particles/ml) were administered to HepG2 cells (seeding density: 8 × 10^4 cells/ml), which have been pre-seeded in 24-well plates containing 12 mm round cover glasses. After a 24-h incubation, the cells were stained for confocal analysis as previously reported (Conte et al., 2024). Briefly, the cells were washed extensively with PBS containing 0.1% Tween 20, fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked for 1 h with blocking buffer (0.5% BSA in PBS containing 0.1% Tween 20). The cells were then incubated overnight with TRITC-Phalloidin (2 μg/ml), followed by washing with PBS containing 0.1% Tween 20, and stained with DAPI (1 μg/ml, Thermo Fisher Scientific) to visualize the nuclei. Confocal images were captured using a Zeiss LSM 710 microscope.

2.5.5. Reactive oxygen species (ROS) detection

ROS were measured using 10 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma Aldrich, St. Louis, MO, USA) (Miranda et al., 2024). To test the effect of cardoon CSC EVs on ROS neutralization, HepG2 cells were seeded (30 × 10^3 cells/well) in 96-well plates allowing them to adhere for 24 h. Next, cells were incubated with cardoon EVs and OA/PA. After 24 h, the medium was removed, and the cells were washed twice with PBS. A staining solution containing DCFH-DA in serum-free medium without phenol-red was added for 20 min at 37 °C in the dark. The fluorescence signal (excitation/emission 485 nm/535 nm) was read in endpoint mode using a PerkinElmer EnSpire multimode plate reader. Representative live images were acquired using ZOE Fluorescent Cell Imager microscope (Biorad, Hercules, California, USA).

2.5.6. Nitric oxide (NO) detection

NO levels were measured using 5 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM Diacetate, Thermo Fisher Scientific, Waltham, MA, USA) (Chimento et al., 2021). To test the effect of cardoon EVs on NO neutralization, HepG2 cells were seeded (30 × 10^3 cells/well) in 96-well plates allowing them to adhere for 24 h. Next, cells were incubated with cardoon EVs and OA/PA. After 24 h, the medium was removed, and the cells were washed twice with PBS. A staining solution containing DAF-FM in serum-free medium without phenol-red was added for 20 min at 37 °C in the dark. The fluorescence signal (excitation/emission 495 nm/515 nm) was read in end point mode using a PerkinElmer EnSpire multimode plate reader. Representative live images were acquired using ZOE Fluorescent Cell Imager microscope (Biorad, Hercules, California, USA).

2.5.7. Oil red O (ORO) staining

ORO staining was used to assess lipid droplets formation in cells. The HepG2 cells were seeded (40 × 10^3 cells/well) in 24-well plates and cultivated overnight. The cells were treated with cardoon EVs and OA/PA for 24 h. The treated cells were washed twice with PBS and fixed with 10% formaldehyde for 15 min at room temperature. Cells were then incubated with 60% isopropanol for 5 min, washed and subsequently stained with Oil Red O solution for 20 min. Images were acquired using an optical microscope (Axioshop 40, Zeiss. Magnification, 40×). Quantitative analyses were performed by dissolving stained lipid droplets in isopropanol. The absorbance was measured at 510 nm, using a microplate reader (Multiskan Go, Thermo Scientific, Waltham, MA, USA) (Mun et al., 2019).

2.5.8. Western blot analysis

HepG2 cells were seeded in 24-well plates and allowed to adhere for 24 h. Cells were then treated either alone, with OA/PA, OA/PA plus metformin, or OA/PA plus EVs. After 24 h of treatment, cells were washed twice with PBS, detached using a scraper, and pelleted by centrifugation at 18,800 ×*g* for 1 min at 4 °C. Total proteins were extracted using a lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2% NP-40, 1% sodium deoxycholate, and protease/phosphatase inhibitor cocktail) for 30 min on ice. Lysates were then clarified by centrifugation at 18,800 ×*g* for 15 min at 4 °C.

Total protein (30 μg) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes using a Bio-Rad minigel apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked for 1 h at room temperature in PBS containing 0.1% Tween-20 and 10% non-fat dry milk, and subsequently incubated overnight at 4 °C with the appropriate primary antibodies under gentle agitation. β-tubulin was used as a loading control. The following antibodies were employed: mouse monoclonal anti-SIRT1 (Thermo Fisher Scientific, Waltham, MA, USA), rabbit polyclonal anti-phospho-AMPK (Thermo Fisher Scientific), and mouse monoclonal anti-β-tubulin (Santa Cruz Biotechnology, Dallas, TX, USA).

After washing with PBS/0.1% Tween-20, membranes were incubated with the corresponding HRP-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h at room temperature. Protein–antibody complexes were visualized using an enhanced chemiluminescence detection system (ECL kit, Amersham, Germany). Signals were acquired with the LAS 4000 imaging system (GE Healthcare, Chicago, IL, USA), and densitometric analysis was performed using ImageJ software (version 1.47).

2.5.9. Statistical analysis of the bioactivity tests

Data are reported as mean ± SD of results from three independent experiments. Statistical analysis was performed using an analysis of variance test (ANOVA), and multiple comparisons were made with the Bonferroni's test with GraphPad Prism 8.0 software (San Diego, CA, USA). Significance was assumed at $p < 0.05$.

2.6. Data availability

The data supporting this article have been included as part of the

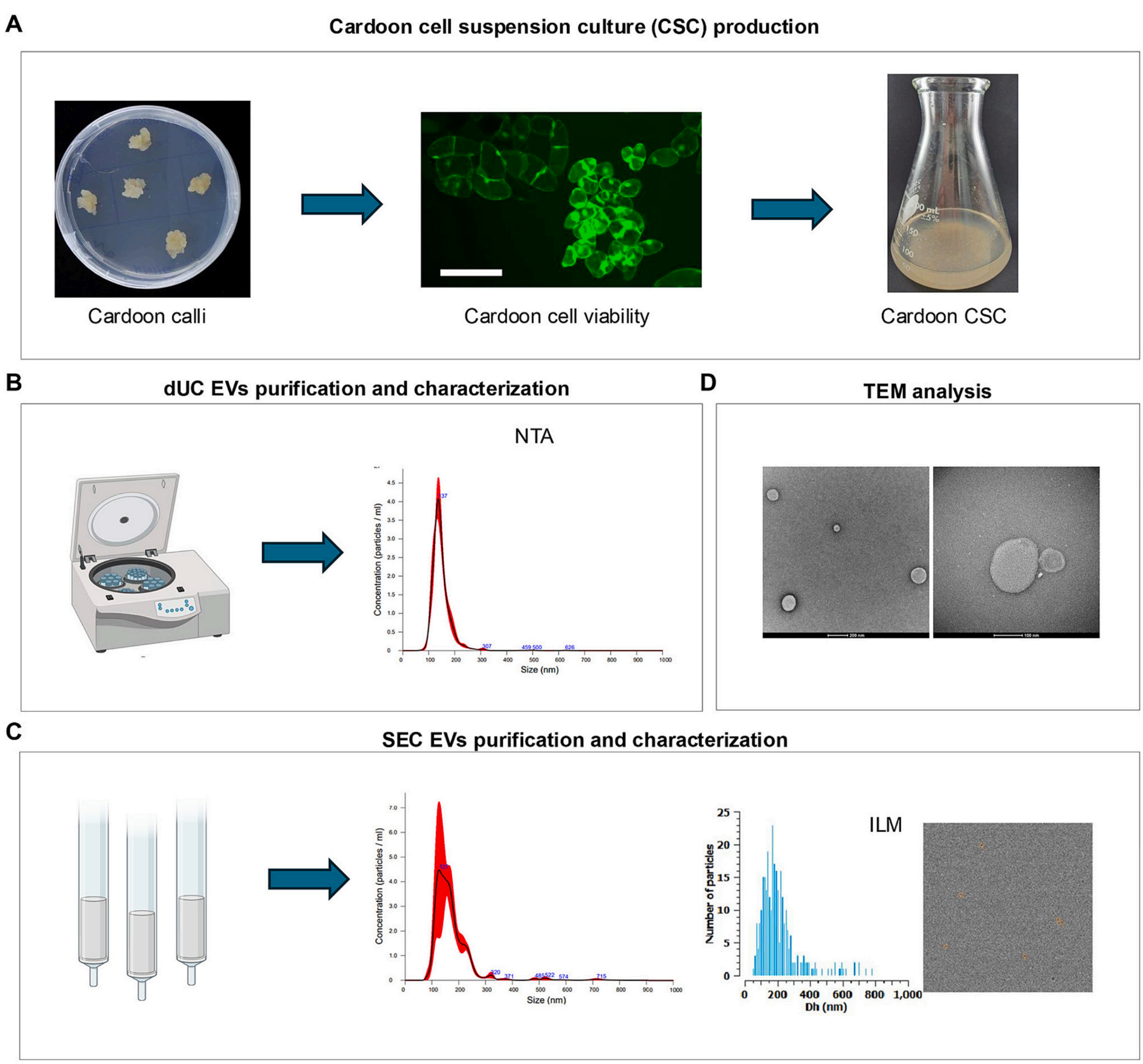

Fig. 1. Isolation and characterization of EVs from cardoon cell culture media. (A) Representative images of cardoon CSCs (scale bar: 100 μm) employed as EV biofactories. (B) NTA measurements illustrate the size distribution of EVs purified from CSCs by dUC; (C) NTA and ILM show the size distribution of EVs purified from CSCs by SEC. (D) TEM image and close-up detail displaying the round-shaped morphology of *C. cardunculus* EVs. Scale bars: 200 nm left image; 100 nm, right image).

Supplementary Information. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD059086.

3. Results

3.1. Isolation and characterization of extracellular vesicles from Cynara cardunculus cell suspension cultures

Live cardoon cells, visualized using fluorescein diacetate staining, were derived from callus cultures and inoculated into liquid media, as depicted in Fig. 1A. The CSC medium carrying the EVs was collected on day 10 which corresponds to the exponential growth phase as previously reported (Paolo et al., 2021). EV isolation was carried out using dUC and SEC, as schematically illustrated in Fig. 1B and C.

To preliminary assess the integrity of the isolated EVs, we performed silver staining, which revealed intact protein bands and well-defined migration patterns (Fig. S1). NTA was employed to determine the size distribution and concentration of dUC-isolated EVs (Fig. 1B). EVs exhibited a concentration of $2.26 \times 10^9 \pm 2.07 \times 10^7$ particles/ml with a size distribution ranging approximately from 100 to 200 nm and an average diameter of 147 nm. Additionally, the size distribution and concentration of EVs isolated by SEC were also determined using NTA and interferometric light microscopy (ILM) further validating these findings (Fig. 1C). Specifically, NTA revealed that SEC-isolated EVs had a concentration of $4.45 \times 10^8 \pm 1.04 \times 10^8$ particles/ml with an average diameter of 172.2 (size range ≈ 90–250 nm). Similarly, ILM analysis provided consistent results, reporting an average EV diameter of 184 nm and a concentration of 6.1×10^8 particles/ml. Transmission electron microscopy (TEM) images of grouped and individual CSC EVs identified them as round-shaped biogenic nanoparticles with diameters ranging from approximately 90 to 200 nm, in line with NTA and ILM analyses (Fig. 1D).

The combined use of two isolation techniques, along with complementary analytical methods such as NTA, ILM and TEM, effectively validated the presence of EVs in the conditioned media of cardoon cell suspension cultures. Furthermore, this integrated approach offered comprehensive insights into the size distribution and key characteristics of cardoon EVs.

3.2. Protein composition of Cynara cardunculus calli and CSC EVs

EVs harbour a diverse array of proteins that play crucial roles in cellular communication and response to environmental stimuli. Thus, beyond their morphological and dimensional characteristics, a detailed proteomic profiling of EV-associated proteins is crucial for confirming their identity and providing insights into their potential functional roles.

In this study, we conducted a gel-based proteomic analysis of both the cardoon callus and EVs, identifying 469 and 113 proteins respectively (see Tables S1 and S2). The protein composition of EVs includes typically EV-associated proteins such as cytoskeletal components (e.g actin, A0A103YD79), nucleic acid binding proteins (A0A124SH24, A0A118K4P0), chaperone proteins like chaperonin Cpn60 (A0A103XLE7) and Heat shock protein 70 (A0A124SG09), and different ribosomal constituents. Notably, we also identified defence-related proteins such as chitin-binding proteins (A0A103YFE0), chitinase (A0A103YJB2), endoglucanases (A0A103XQY5, A0A103XQV0) and various membrane proteins, including an alpha-mannosidase (A0A103YBM4) and the plant exosomal marker tetraspanin 8 (TET-8) (A0A118K766). Several hydrolases involved in cell wall remodelling were also detected, including a pectinesterase (A0A118JTX1), a pectin acetylesterase (A0A103XIX7), and glycoside hydrolases (A0A103P7R6, A0A103YI04, A0A118K002).

To elucidate further the biological significance of EVs, we performed a Gene Ontology (GO) enrichment analysis focusing on Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF). As shown in Fig. 2A, the callus proteome predominantly consists of proteins associated with metabolic activities, particularly those involved in small molecule and organic acid metabolism, as well as protein folding processes. In contrast, CSC EVs exhibited a distinct profile of metabolic processes together with an enrichment of proteins involved in the translation and gene expression categories (Fig. 2B). Regarding "Cellular Component" category, proteins identified in the callus were enriched in terms related to the cytoplasm, mitochondria, and proteasome (Fig. 2A), whereas CSC EVs were primarily associated with the ribosome, ribonucleoprotein complex and extracellular region (Fig. 2B). Notably, the GO terms related to peroxidase activity enrichment indicate an important role of EVs in the oxidative stress responses. Moreover, hydrolase, RNA binding and peptidase activities are all specifically enriched in EV preparations. These findings reveal key proteomic differences between callus and CSC EVs, emphasizing their role in plant signalling, stress responses, defence mechanisms and other important functions including antioxidant properties, which hold potential relevance for agriculture and biomedical applications.

To identify common EV markers in plants, we conducted a detailed comparative analysis of the proteome of cardoon EVs with those of the model plant species *Arabidopsis thaliana*, reported by Rutter and collaborators in 2017 (Rutter & Innes, 2017). This analysis revealed 18 common proteins, including alpha-mannosidase, actin, glucanase, chaperonin Cpn60 and tetraspanin-8 (Table S3). Finally, by searching for homolog proteins included in the EVs public database ExoCarta (http://www.exocarta.org), we found that several proteins detected in cardoon EVs have also a human homolog with a sequence identity >50%, including actin, several ribosomal proteins and chaperonins. Notably, five of these proteins are also listed in the Top 100 most frequently identified EV-associated proteins in Vesiclepedia (Table S4). This indicates that cardoon EVs possess evolutionarily conserved markers shared between the plant and animal kingdoms.

3.3. Metabolomic profiling of Cynara cardunculus calli and CSC EVs

LC-HRMS analysis was performed separately on cardoon cell extracts and EV extracts. For both types of extracts, the negative ionization mode yielded better results compared to the positive mode, particularly for callus extracts. The identity of the detected metabolites was verified by comparison with analytical standards. As shown in Table 1, the analysis revealed that the callus extract is notably rich in polyphenolic compounds. Compounds 5, 7, 8, 9 and 10 show the fragments at *m/z* 191 and *m/z* 161 corresponding to deprotonated quinic acid and dehydroxylated and deprotonated caffeic acid respectively. Other identified polyphenols are ferulic and coumaric acid derivatives, which show fragments at *m/z* 193 (for compounds 3, 5 and 6) and *m/z* 163 (compound **2**), respectively. Notably, the chromatogram also highlights the presence of various polyhydroxylated fatty acids (PHFAs), within the C18 class, exhibiting one or two unsaturations and appearing prominently toward the end of the chromatogram.

Regarding cardoon EVs, the analysis of specialized metabolites revealed a significant reduction in polyphenolic molecules compared to the callus extracts and the presence of several polyunsaturated fatty acids (Table 2). Additionally, a significant presence of triterpenes was identified, including compounds 9, 10, 11, and 12, further confirming the unique composition of EVs and their potential specialized biological functions.

3.4. Bioactivity and cellular uptake of C. cardunculus EVs in human hepatocellular carcinoma cell lines

3.4.1. Cytoprotective effects of CSC EVs against hepatic steatosis

Cardoon contains high levels of bioactive compounds, such as phenolic acids, flavonoids, and sesquiterpene lactones, which have been shown to reduce oxidative stress and inflammation often associated with

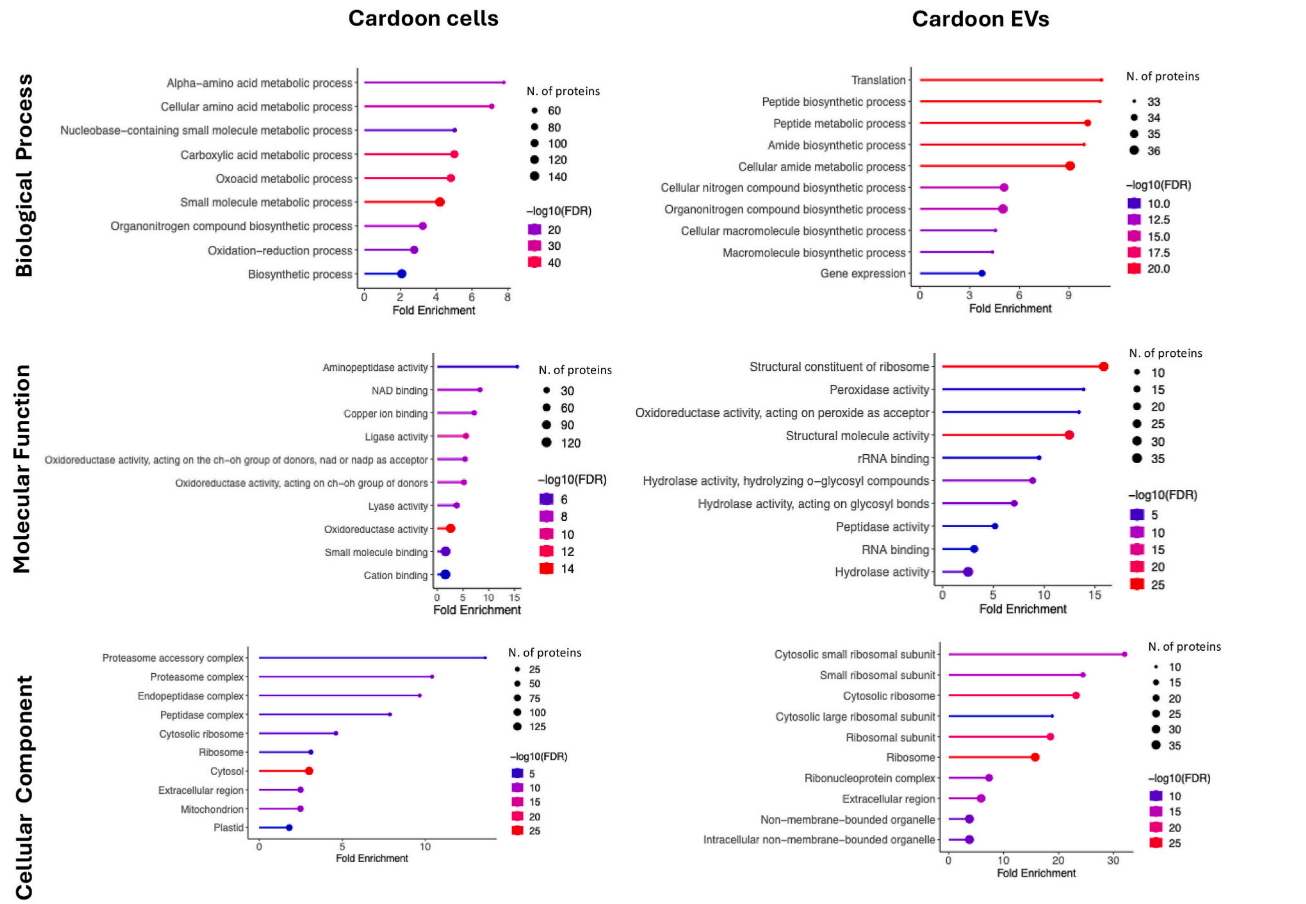

Fig. 2. GO enrichment analyses of the calli and EVs proteomes were performed using the specific analysis tool ShinyGo 0.80 with a False Discovery Rate (FDR) cutoff of 0.05. Proteins identified in calli (A) and EV (B) proteomes were classified in terms of biological process (BP), cellular component (CC) and molecular function (MF) in which they are involved. The number of proteins identified, and the fold enrichment are indicated.

Table 1
Specialized metabolites from *C. cardunculus* calli characterized by LC-MS. (R_t = retention time; MSI = Metabolomics Standards Initiative).

Peak	Compound	Rt	[M-H]$^-$	MS2	MSI level[a]	Error (ppm)
1	quinic acid	1.91	191.0551	173; 163; 147	1	0.52
2	coumarinic acid	2.07	163.0390	147; 119; 91	1	0.53
3	ferulic acid 4 *O*-hexoside	8.74	355.1029	193; 178; 175; 149; 134	1	1.36
4	syringic acid 4 *O*-hexoside	8.91	359.0970	197; 182; 153; 138	1	−0.55
5	Caffeoyl 4-ferulic acid	10.24	355.1029	193; 178; 175; 161; 149; 134	1	0.53
6	ferulic acid 3 *O*-hexoside	12.91	355.1029	193; 178; 175; 149; 134	1	0.53
7	chlorogenic acid	13.41	353.0867	191; 161	1	0.47
8	3,4-dicaffeoylquinic acid	18.45	515.1190	353; 191; 179; 161; 134	1	1.16
9	tricaffeoylquinic acid	22.13	677.1508	515; 353; 191; 179; 134	2	1.18
10	tricaffeoylquinic acid	22.99	677.1508	515; 353; 191; 179; 134	2	1.18
11	trihydroxyoctadecadienoic acid	25.62	327.2167	309; 291	2	−1.22
12	trihydroxyoctadecenoic acid	27.11	329.2320	313; 293	2	2.12
13	dihydroxyoctadecenoic acid	35.78	311.2212	293	2	−1.60
14	dihydroxyoctadecenoic acid	36.54	311.2212	293	2	−1.60
15	dihydroxyoctadecenoic acid	37.21	311.2212	293	2	−1.60
16	dihydroxyoctadecenoic acid	39.39	311.2212	293	2	−1.60
17	18-hydroxylinoleic acid	42.36	295.2274	277; 233	1	−0.67
18	17-hydroxylinolenic acid	43.87	293.2115	275; 231	1	−1.02

[a] Sumner et al., 2007.

liver diseases like non-alcoholic fatty liver disease (NAFLD), hepatitis, and liver fibrosis (Ferro et al., 2022; Gebhardt & Fausel, 1997; Oppedisano et al., 2020). Additionally, cardoon's bioactive have been reported to improve lipid metabolism and protect hepatocytes from lipid accumulation (Ferro et al., 2020; Rondanelli, Giacosa, et al., 2013; Rondanelli, Monteferrario, et al., 2013) making it a promising candidate for treating metabolic liver dysfunctions.

Prior to evaluating the hepatoprotective effects of cardoon CSC EVs, their effective concentrations were determined by MTT assay in HepG2 cells exposed to oleic acid and palmitic acid (OA/PA) to induce steatosis (Fig. S2). The dose–response analysis showed a progressive recovery of cell viability with increasing EV concentrations, with the strongest protective effect observed at 10^8 EVs/ml. This concentration was therefore selected as the working dose for further experiments.

Table 2
Specialized metabolites from cardoon EVs characterized by LC-MS. (R_t = retention time; MSI = Metabolomics Standards Initiative).

Peak	Compound	Rt	[M-H]$^-$	MS2	MSI level[a]	Error (ppm)
1	quinic acid	1.81	191.0551	173; 163; 147	1	0.52
2	trihydroxyoctadecenoic acid	27.11	329.2320	313; 293	2	2.12
3	dihydroxyoctadecenoic acid	35.78	311.2212	293	2	−1.60
4	dihydroxyoctadecenoic acid	36.54	311.2212	293	2	−1.60
5	dihydroxyoctadecenoic acid	37.21	311.2212	293	2	−1.60
6	dihydroxyoctadecenoic acid	39.39	311.2212	293	2	−1.60
7	18-hydroxylinoleic acid	42.36	295.2274	277; 233	1	−0.67
8	17-hydroxylinolenic acid	43.87	293.2115	275; 231	1	−1.02
9	Triterpene	28.95	503.3367	485	2	−0.59
10	Triterpene	32.45	487.3413	465	2	−1.02
11	Triterpene	35.32	487.3413	465	2	−1.02
12	Triterpene	44.38	471.3471	453	2	0.63

Subsequently, the hepatoprotective activity of CSC EVs was assessed in the same in vitro NAFLD model. As shown in Fig. 3, cardoon EVs significantly improved cell viability in this steatosis model.

Particularly, a double cell staining was performed in HepG2 cells using calcein AM and PI. As observed in Fig. 3A, the number of calcein AM-positive control cells is significantly higher than in OA/PA-treated cells. At the same time, the number of PI-positive cells is significantly increased in OA/PA-treated cells, suggesting enhanced cytotoxicity. Cardoon EVs effectively reversed the cytotoxic effects in the steatosis model, restoring cellular conditions to levels comparable to the untreated control and significantly matching the protective effects observed with the positive control, metformin. These observations were further quantitatively validated by MTT assay (Fig. 3B). While OA/PA treatment significantly reduced cell viability (51.89 ± 1.28%, $p < 0.01$ vs. Ctrl), CSC EVs increased viability to 86.26 ± 8.82%, a level comparable to that achieved with metformin ($p < 0.01$ vs. OA/PA). These results indicate that cardoon EVs provide strong hepatoprotective effects against fatty acid-induced cytotoxicity in vitro.

To further evaluate the specific bioactivity of cardoon EVs, appropriate negative controls were employed, including heat-inactivated EVs, EV-depleted medium, and EVs derived from other species such as *Salvia sclarea* and *Salvia dominica*, previously described by our group. Notably, heat-inactivated EVs retained a slight residual bioactivity, likely attributable to thermostable components within their biocargo (Fig. S3 A). In contrast, neither the EV-depleted medium nor EVs isolated from other plant species exhibited any detectable bioactivity (Fig. S3 B). These results emphasize that antisteatotic activity of cardoon-derived EVs is driven by species-specific molecules within their biocargo and is partially attributed to the presence of thermostable components in the cardoon EVs.

3.4.2. Cellular uptake of CSC EVs in HepG2 cells

To assess whether cardoon CSC EVs are actively taken up by liver cells, HepG2 cells were incubated with BODIPY-labelled EVs for 24 h (Fig. 4). Confocal microscopy analysis revealed significant EV uptake in HepG2 cells as indicated by distinct green fluorescence within the cells. The untreated control cells exhibited no detectable fluorescence in the green channel, confirming the specificity of EV uptake. These findings support the effective internalization of *C. cardunculus* EVs by HepG2 cells, suggesting that these vesicles could deliver bioactive compounds directly to liver cells.

3.4.3. In-cell antioxidant activity

Oxidative stress, characterized by elevated production of reactive oxygen species (ROS) and nitric oxide (NO), contributes to liver cell damage and progression of liver diseases. It is well known that exposure of liver cells to OA/PA triggers the production of ROS (Teixeira et al., 2023) and NO (Zhang et al., 2019).

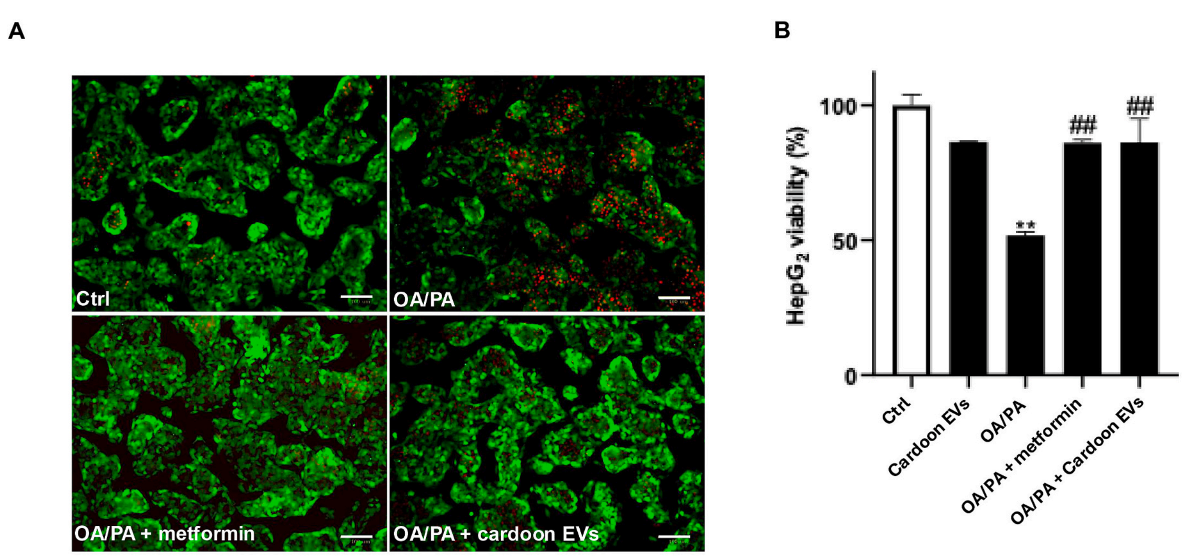

Fig. 3. Hepatoprotective effect of cardoon EVs against OA/PA-induced cytotoxicity. (A) Representative images of calcein/PI double staining after treatments. Live cells emit green fluorescence due to calcein AM staining, indicating intracellular esterase activity, while dead cells fluoresce red as PI intercalates into DNA upon membrane compromise. EV concentration: 10^8 particles/ml. Scale bars: 100 μm. (B) HepG2 viability was examined by the MTT assay. The viability variations were calculated as percentage of viable cells in treated cultures compared to untreated ones. Metformin (1 mM) was used as positive control. Data are showed as the mean ± SD of three different experiments performed in triplicate. ** $p < 0.01$ vs. Ctrl. ## $p < 0.01$ vs. OA/PA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

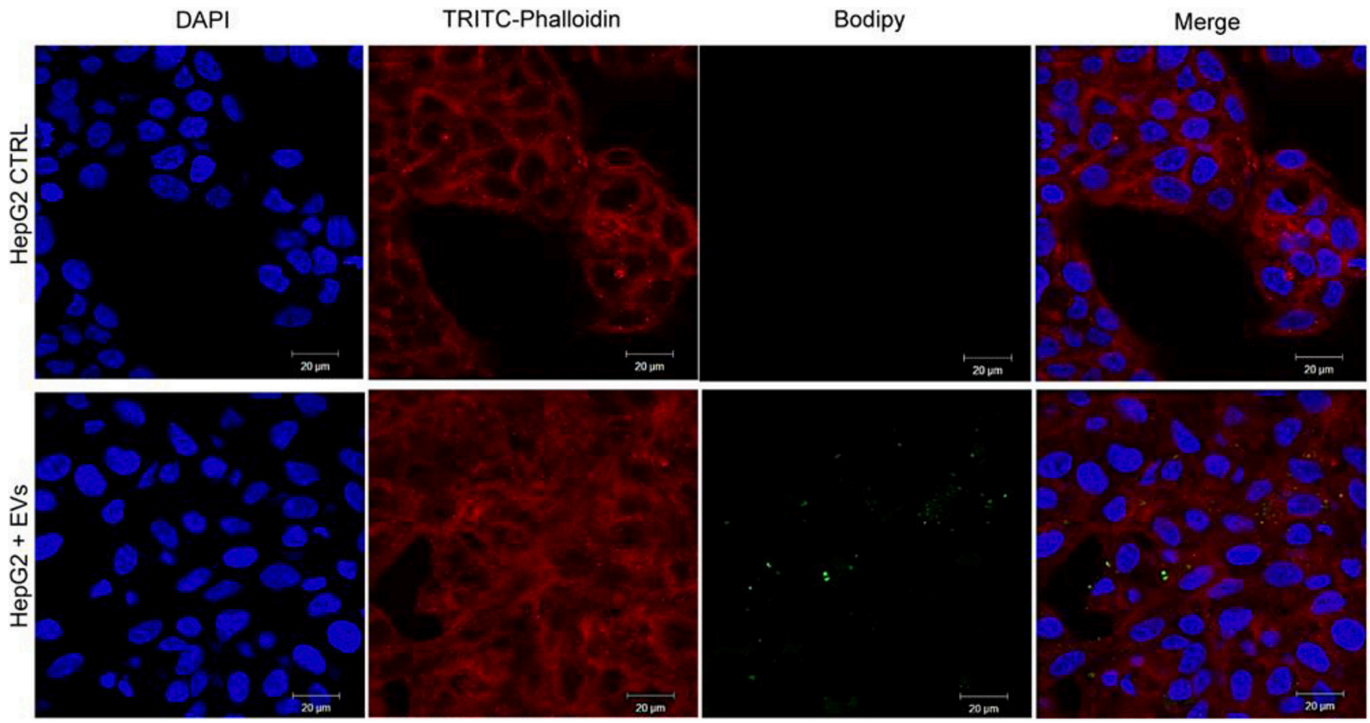

Fig. 4. Uptake of cardoon EVs in HepG2 cells by confocal microscopy. Cells were stained with phalloidin-TRITC (actin, red) and DAPI (nuclei, blue). EVs were labelled by BODIPY (green). Upper panel: HepG2 control cells. Lower panel: BODIPY-labelled EV uptake after 24 h of treatment (10^9/ ml). EV concentration: 10^8 particles/ml. Magnification 63×/1.4 numerical aperture. Scale bars: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We evaluated the antioxidant potential of *C. cardunculus* CSC EVs in HepG2 cells exposed to OA/PA-induced oxidative stress. HepG2 cells treated with OA/PA exhibited significantly increased levels of ROS and NO compared to the control group ($p < 0.001$ vs. Ctrl). In contrast, treatment with cardoon EVs markedly suppressed ROS and NO production ($p < 0.01$ vs. OA/PA), demonstrating the antioxidant effects of cardoon EVs after 24 h of exposure (Fig. 5).

3.4.4. Effect of CSC EVs on the reduction of lipid accumulation in NAFLD model

The presence of hydroxylated fatty acids and triterpenes candidate cardoon EVs as potential therapeutics to target metabolic diseases characterized by inflammation, dyslipidemia, and oxidative stress. NAFLD is associated with excessive lipid accumulation in hepatocytes, which disrupts cellular metabolism, promotes further lipid deposition, and exacerbates liver dysfunction (Ipsen et al., 2018).

Herein, we evaluated the potential of cardoon EVs to reduce lipid accumulation in HepG2 cells treated with OA/PA. Intracellular lipid accumulation was visualized using ORO staining (Cui et al., 2010). As shown in Fig. 6, OA/PA treatment led to a marked increase in lipid accumulation within HepG2 cells compared to the control group, indicating a pronounced induction of NAFLD-like conditions (390.64 ± 16.77% lipid accumulation, $p < 0.001$ vs. Ctrl). Conversely, the addition of CSC EVs significantly reduced lipid accumulation in a dose-dependent manner. Notably, cells treated with EVs exhibited a substantial decrease in lipid droplet formation (242.67 ± 7.25% lipid accumulation, $p < 0.01$ vs. OA/PA), as shown in Fig. 6. These results underscore the lipid-lowering effect of CSC EVs, suggesting their potential as a therapeutic agent to counteract lipid accumulation associated with NAFLD (Fig. 6).

To further elucidate the molecular mechanisms triggered by CSC EVs, we first examined the modulation of the SIRT-1/AMPK axis, a key regulatory pathway in hepatic lipid metabolism. Western blot analysis revealed that, under lipotoxic conditions induced by OA/PA treatment, CSC EVs, significantly enhanced AMPK phosphorylation ($p < 0.001$ vs. Ctrl) reaching levels substantially exceeding those induced by metformin, which served as a positive control. SIRT-1 expression was also markedly upregulated, attaining levels comparable to those observed with metformin ($p < 0.001$ vs. Ctrl) (Fig. 7A, B). Based on these findings, we next explored the functional contribution of SIRT-1 using the selective activator SRT2104 and the inhibitor EX527. Consistent with a SIRT-1-dependent mechanism, SRT2104 potentiated the protective effects of CSC EVs, resulting in increased mitochondrial metabolic activity ($p < 0.05$ vs. OA/PA + Cardoon EVs) and a more pronounced reduction of lipid accumulation ($p < 0.05$ vs. OA/PA + Cardoon EVs). In contrast, EX527 attenuated the beneficial effects of CSC EVs, leading to reduced mitochondrial function ($p < 0.001$ vs. OA/PA + Cardoon EVs) and higher intracellular lipid levels ($p < 0.01$ vs. OA/PA + Cardoon EVs) (Fig. 7C, D). These results show that CSC EVs exert their anti-steatotic effects, at least in part, through the activation of the SIRT-1/P-AMPK pathway, and that modulation of SIRT-1 activity critically influences their metabolic impact.

4. Discussion

Molecular farming using plant cell and tissue cultures (PCTCs) is a widely adopted approach for producing valuable bioactive compounds for pharmaceutical, cosmetic and food sectors (Apone et al., 2020; Gubser et al., 2021; Tschofen et al., 2016). Very recently, the European Food Safety Authority (EFSA) Panel on Nutrition, Novel Foods and Food Allergens approved apple fruit cell culture biomass as a novel food intended as an ingredient for food supplements in adults (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) et al., 2023). This approval represents a significant milestone in the advancement of cellular agriculture, opening new opportunities for its application in the food sector. In this context, CSCs-based biofactories, particularly those derived from edible plants, may represent promising food biotechnological systems for the production of EVs. Indeed, plant cell cultures offer several advantages over mammalian-derived vesicles, including

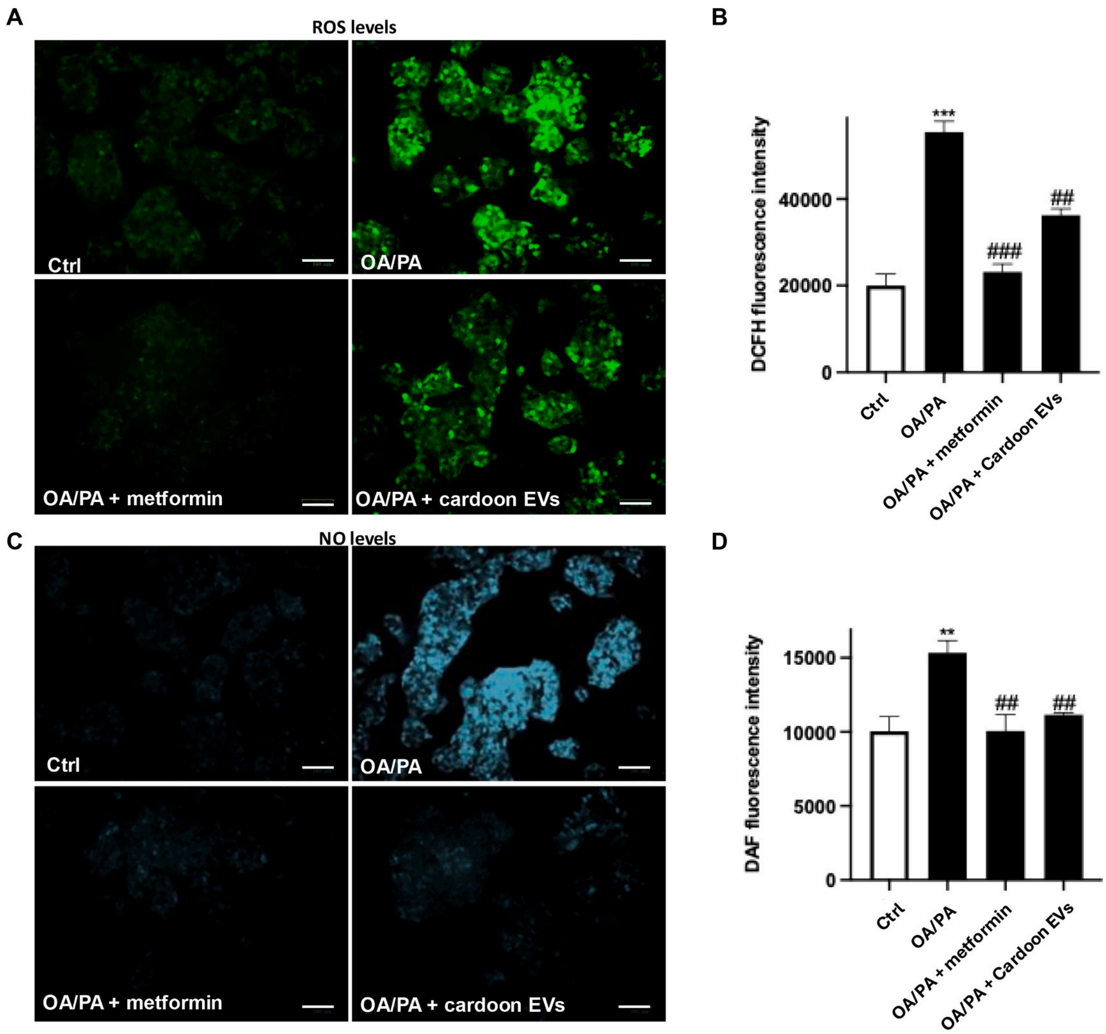

Fig. 5. Effects of cardoon CSC EVs on OA/PA-induced oxidative stress in HepG2 cells. Fluorescence images of cells treated with cardoon EVs (10^8 vesicles) on ROS (**A**) and NO production (**C**), evaluated by DCFH-DA and DAF-FM. Steatosis was induced using OA/PA (in a 2:1 ratio) for 24 h. Quantitative analysis of ROS (**B**) and NO (**D**) production were reported. Data are showed as the mean ± SD of three different experiments performed in triplicate. ** $p < 0.01$ vs. Ctrl, *** $p < 0.001$ vs. Ctrl. ## $p < 0.01$ vs. OA/PA; ### $p < 0.001$ vs. OA/PA. Scale bars in A and C: 100 μm.

scalability, lower production costs, reduced immunogenicity, and elimination of pathogenic contamination risks. Additionally, their ability to ensure consistent yields and minimize batch-to-batch variability makes them a robust platform for EV production. Precise control over culture conditions, such as pH, nutrient levels, and elicitor addition, further allows the enhancement of EV bioactivity and implementation of bioengineering strategies to improve their therapeutic potential (Ambrosone et al., 2023). Moreover, very often, the media of plant in vitro cultures are a waste of the process. Therefore, their recovery for the isolation of EVs could be an excellent circular bioeconomy strategy.

Woith et al. (Woith et al., 2021) initially reported the isolation of EVs from *Craterostigma plantagineum* Hochst. CSCs. Since then, only a limited number of studies have identified EVs from other plant cell cultures, including *Nicotiana tabacum* (tobacco), *Panax ginseng* (Korean ginseng), *Aster yomena* (kalimeris), *Picea abies* (Norway spruce), *Stevia rebaudiana* and *Vaccaria hispanica* and the model species *Arabidopsis thaliana* (Cho et al., 2021; Kankaanpää et al., 2024; Kim et al., 2022; Kırbaş et al., 2025; Kocholatá et al., 2024; Yugay et al., 2023). However, despite these advancements, our understanding of plant EV biodiversity remains highly fragmented. The limited range of studied species represents a significant gap in the knowledge of plant-derived EVs, particularly in terms of their molecular composition, biological functions, and potential applications. This lack of comprehensive research is a missed opportunity, as in vitro cultures could serve as an ideal and underutilized source for systematically exploring the diversity and functional potential of plant EVs. Expanding studies in this area is critical to uncover an extensive collection of plant EVs and their roles in both biological and biotechnological contexts.

Cardoon, a highly versatile plant valued for its applications in bioenergy, food, and pharmaceuticals, presents a novel and sustainable source of EVs (Barracosa et al., 2019). The in vitro cultivation of cardoon is straightforward, and its EVs exhibit size distribution and morphology consistent with those reported for other CSCs. Our proteomic analyses reveal that cardoon EVs are enriched with proteins commonly associated with plant-derived EVs, including actin, heat shock proteins (HSPs), alpha-mannosidase, and the well-established exosomal marker TET-8 (Pinedo et al., 2021). In addition to these conserved components, cardoon EVs exhibit a notable enrichment of antioxidant-related and stress-response proteins, including key players in plant-pathogen interactions such as chitinases, endoglucanases, proteinases, and leucine-rich repeat-containing (LRR) proteins. Collectively, these proteins may synergistically endow EVs with a critical role in mediating defence mechanisms and adapting to environmental stressors, reinforcing findings from previous studies (Cai et al., 2018; De Palma et al., 2020; Regente et al., 2017; Rutter & Innes, 2018). It is worth mentioning that the proteomes of EVs derived from CSCs remain poorly explored so far. A

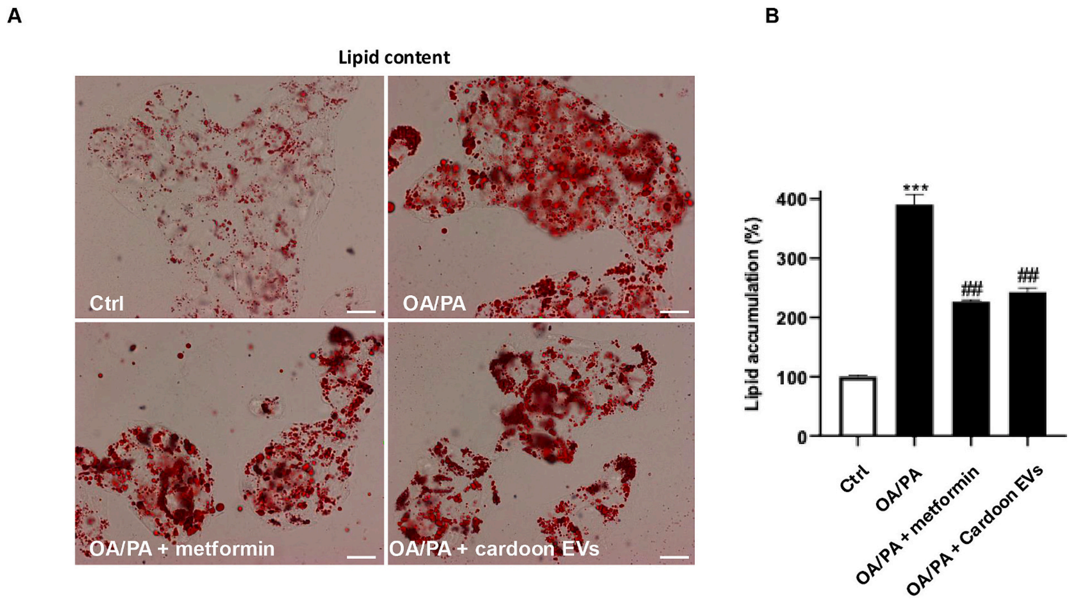

Fig. 6. ***C. cardunculus*** **CSC EVs reduces the level of lipid accumulation.** (**A**) Oil-red O staining of HepG2 cells untreated (control group), treated with OA/PA (in a 2:1 ratio), treated with EVs (10^8 particles/ml) and OA/PA, for 24 h. Metformin (1 mM) was used as a control. Scale bar: 100 μm. (**B**) Quantitative analysis of lipid accumulation was reported. Data are showed as the mean ± SD of three different experiments performed in triplicate. *** $p < 0.001$ vs. Ctrl. ## $p < 0.01$ vs. OA/PA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recent study has shown that Norway spruce EVs are enriched with laccases, peroxidases and several hydrolases, enzymes also abundant in cardoon EVs (Kankaanpää et al., 2024). These proteins are integral to cell wall metabolism, particularly in lignin polymerization and cross-linking of wall components. Interestingly, similar markers have been identified in EVs purified from the apoplastic fluid of other plant species (Ekanayake et al., 2024; Prado et al., 2014; Regente et al., 2017), indicating that these proteins are shared between EVs derived from cell cultures and those isolated directly from plant tissues. This overlap further reinforces the hypothesis that plant EVs, regardless of their origin, play crucial roles also in cell wall remodelling. Additionally, the presence of such proteins may also provide insights into the mechanisms underlying EV release in plants. It is plausible that these enzymes facilitate EV secretion by enzymatically modifying or deforming the plant cell wall, enabling vesicle release into the extracellular space. Further research is needed to validate these hypotheses.

Yugay et al. identified conserved proteins such as heat shock protein and sucrose synthase in the proteome of callus-derived EVs from *Arabidopsis thaliana* (Yugay et al., 2023). These proteins were also detected in cardoon-derived EVs and EVs purified from the apoplastic fluid of different plant species (Rutter & Innes, 2017)·(Chaya et al., 2024)·

Although the overlap includes only a limited number of proteins, it suggests that certain fundamental components are shared between EVs derived from CSCs and those from apoplastic fluid. These shared features appear to be conserved also across EVs from diverse plant species, suggesting potential commonalities in their biogenesis and functional roles. These findings also underline the need to identify universal markers for genuine plant EVs, regardless of whether they are purified from the apoplast or conditioned media. Expanding proteomic datasets and conducting comparative studies of EVs isolated from CSCs and apoplastic fluids will be critical for elucidating their biogenesis, biological functions, and evolutionary conservation, while also paving the way for broader biotechnological applications.

The metabolite profiles of cardoon callus and EVs reveal intriguing similarities and differences. In the callus extracts, phenolic compounds such as quinic acid, chlorogenic acid, dicaffeoylquinic acids, tricaffeoylquinic acids and ferulic acid derivatives are prominent. These findings are consistent with previous metabolomic studies on cardoon, which identified chlorogenic acid, along with other phenolic compounds, as the most abundant metabolites (Barbosa et al., 2024; Docimo et al., 2020; Graziani et al., 2020). These bioactive molecules are widely recognized for their potent antioxidant activities, which contribute significantly to cardoon's established therapeutic potential in skincare applications, anti-inflammatory treatments, and oxidative stress mitigation (Bagdas et al., 2020; Nguyen et al., 2024).

Additionally, calli extracts contain also a variegate repertoire of hydroxy fatty acids (HFAs) and polyhydroxyfatty acids (PHFAs). The EV biocargo is also particularly enriched in HFAs and PHFAs, including trihydroxyoctadecenoic acid, dihydroxyoctadecenoic acids, hydroxy-octadecadienoic acid, and hydroxy-octadecatrienoic acid. These compounds, characterized by their unsaturated C18 backbones and hydroxyl substitutions, stand out as prominent components in cardoon EVs. Lipidomic studies on EVs derived from other plant sources, such as ginseng and edible plants including ginger and grape, have mainly reported phospholipids, sphingolipids, sterols and specialized metabolites (e.g., gingerols and shogaols), but have not described a comparably abundant fraction of C18 HFAs or PHFAs in the vesicle cargo (Bahri et al., 2024; Cho et al., 2021; Wang et al., 2025). To our knowledge, no PHFA-enriched lipid profile has been reported in other plant-derived EVs characterized to date, indicating that cardoon EVs may possess a distinctive lipid signature.

Their presence in EVs is highly significant as these HFAs and PHFAs, are involved in key biological processes such as modulating cellular oxidative stress, regulating inflammatory pathways, limiting atherogenesis and maintaining membrane integrity (Calder, 2010; Eckhardt, 2023; McReynolds et al., 2020; Vangaveti et al., 2010). Overall, their specialized lipid profile suggests that cardoon EVs may be optimized for lipid-based signalling, influencing membrane dynamics and responses to oxidative stress. Moreover, the potential of HFAs to regulate lipid metabolism highlights their relevance in addressing inflammatory and metabolic disorders, where disrupted lipid signalling is a key factor.

Additionally, the presence of terpenes in EVs, not detected in the callus extracts, highlights a unique aspect of the EV metabolome. Terpenes are known for their antimicrobial, anti-inflammatory, and antioxidant properties (Cox-Georgian et al., 2019). Interestingly, the presence of terpenes in EVs suggests their role as specialized carriers for these bioactive compounds, potentially facilitating their delivery and bioavailability to target cells or tissues. Supporting this hypothesis,

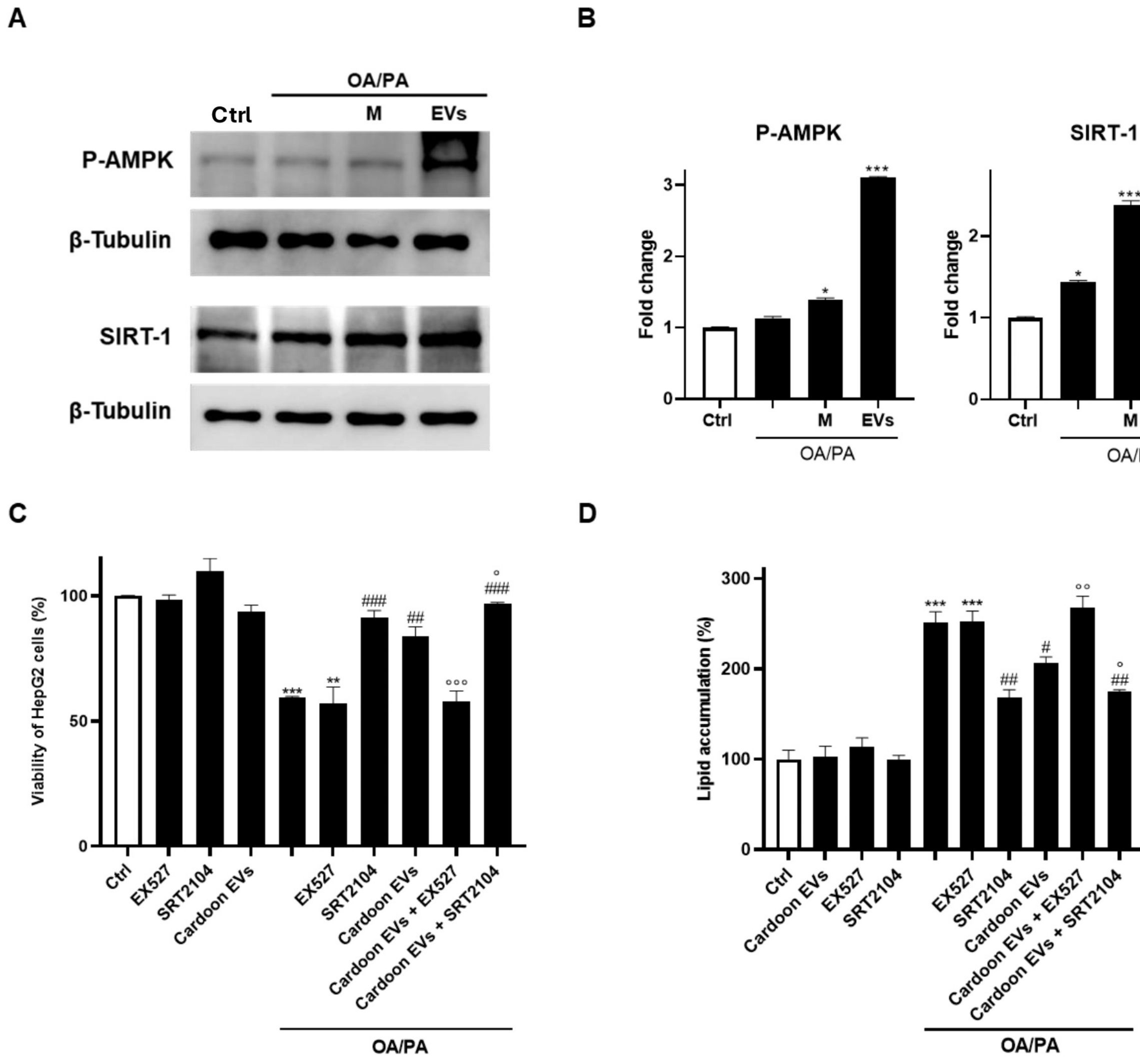

Fig. 7. Anti-steatotic effects exerted by *C. cardunculus* CSC EVs are mediated by the pathway SIRT-1/P-AMPK. (**A**) Western blotting for evaluation of SIRT-1/P-AMPK modulation. (M: metformin; EVs: cardoon EVs). (**B**) Related densitometry of western blotting analyses. (M: metformin; EVs: cardoon EVs). (**C**) Mitochondrial metabolic activity tested by MTT assay using SRT2104 and EX527 as activator and inhibitor of SIRT-1, respectively. (**D**) Quantitative determination of lipid accumulation in HepG2 cells through spectrophotometric analysis using SRT2104 and EX527 as activator and inhibitor of SIRT-1, respectively. Values are shown as mean ± SD ($n = 3$). *, ** and *** denote respectively $p < 0.05$, $p < 0.01$ and $p < 0.001$ vs. Ctrl; #, ## and ### denote respectively $p < 0.05$, $p < 0.01$ and $p < 0.001$ vs. OA/PA; °, °°, °°° denote respectively $p < 0.05$, $p < 0.01$ and $p < 0.001$ vs. OA/PA + Cardoon EVs.

triterpenes were also identified in the EVs derived from *Salvia sclarea* and *Salvia dominica*, suggesting that plant EVs preferentially transport lipophilic molecules over more hydrophilic compounds (Vestuto et al., 2024). Similar observations have been reported (Woith et al., 2021), indicating a potential evolutionary strategy for EVs to selectively carry bioactive lipids or hydrophobic metabolites with specific biological or ecological roles. Whether this selectivity is driven by inherent chemical affinities between lipophilic metabolites and EV membranes or by an active, regulated mechanism of selective metabolite packaging remains unclear. Future studies employing advanced metabolomic profiling and functional analyses will be crucial to uncover the molecular processes that govern the potential selective loading of lipophilic compounds into plant EVs.

In recent years, food-derived EVs have emerged as promising therapeutical tools in nanomedicine, particularly due to their resilience to gastric fluids and the capability to reach the intestine intact, where they may influence the gut microbiota, reinforce the gut barrier, and modulate the gut-liver axis, offering new promising possibilities for digestive system and overall well-being (Zhang et al., 2024).

In this study, we explored in vitro the potential therapeutic applications of cardoon EVs as multifunctional bioactive agents for addressing liver-related metabolic disorders, with a particular focus on non-alcoholic fatty liver disease (NAFLD). NAFLD, a leading cause of liver disease globally, affects nearly 25% of the adult population and is linked to significant adverse health outcomes and elevated mortality rates (Younossi et al., 2016). Confocal microscopy confirmed the efficient internalization of *C. cardunculus* EVs into HepG2 cells, underscoring their suitability as delivery vehicles for bioactive compounds. The observed beneficial effects stem likely from the blend of bioactive molecules within the EVs, including phenolic compounds, hydroxylated fatty acids, and triterpenes. Upon NAFLD induction, cardoon EVs significantly reduced elevated levels of ROS and NO in HepG2 cells, mitigating oxidative damage. This protection might be critical for preventing the progression of liver diseases such as NAFLD. Interestingly, lipid modulation, likely facilitated by HFAs like dihydroxyoctadecenoic acid, enables cardoon EVs to reduce intracellular lipid accumulation in hepatocytes. Remarkably, these effects are linked to activation of the SIRT-1/AMPK pathway, which plays a central role in coordinating energy metabolism and antioxidant defenses in hepatocytes (Hou et al., 2008). The functional relevance of SIRT-1 was supported by pharmacological modulation, as activation enhanced, and inhibition attenuated, the protective effects of cardoon EVs. These findings highlight that CSC EVs not only limit lipid accumulation but also bolster cellular resilience to oxidative damage, suggesting a multifaceted mechanism of action that integrates metabolic regulation and stress response pathways.

Furthermore, these beneficial effects were comparable to those of metformin, a widely used lipid-lowering and insulin-sensitizing agent, underscoring the therapeutic promise of CSC EVs. Additionally, anti-inflammatory properties, likely driven by the synergic effects of triterpenes and HFAs/PHFAs, and quinic acid, suggest that cardoon EVs may help address chronic low-grade inflammation often associated with metabolic disorders. Taken together, these results position cardoon EVs as promising plant-derived nanotherapeutics for metabolic liver diseases. Their ability to target key regulatory pathways such as SIRT-1/AMPK suggests potential applications in managing NAFLD, obesity-related inflammation, and broader cardiometabolic conditions. However, since the present study relies on a single in vitro hepatic cell model, the integration of advanced 3D organoid systems and appropriate animal models will be essential to validate and generalize these findings. Accordingly, future investigations will extend to these more complex experimental systems to comprehensively assess the efficacy, biodistribution, and safety of cardoon EVs. Moreover, a thorough evaluation of cardoon EV formulation stability and storage conditions is required, as plant EVs are known to transport chemically labile bioactive compounds whose integrity and biological activity may be compromised during processing and storage, thereby critically influencing their translational potential.

5. Conclusions

This study shows the in vitro hepatoprotective and antioxidant properties of cardoon-derived EVs and underscores the potential of plant cell cultures as a scalable, reliable, and customizable platform for EV production. By offering a standardized and eco-friendly alternative to animal-derived EVs, plant cell culture platforms could transform the production of EV-based therapeutics. Likewise, although plant cell cultures offer clear scalability advantages in principle, large-scale production, downstream purification, standardization, and cost-effectiveness of EV manufacturing, together with the improvement of isolation techniques through more efficient production and recovery systems, remain key objectives for future EV biotechnological development. Therefore, future studies should focus on optimizing cardoon cell culture conditions to further enhance their yield and bioactivity and investigate their efficacy in in vivo models, potentially advancing these vesicles as viable candidates for clinical applications in liver disease and beyond. Additionally, plant cell cultures can be elicited to boost the production of desired bioactive compounds and targeting peptides, enabling the development of food-derived EV nutritional supplements or precision therapeutics tailored to specific medical needs. Based on our evidence, metabolic engineering of polyhydroxylated fatty acids or triterpenes in cardoon, through both overexpression and knockdown strategies will be a promising approach to clarify the role of these bioactives in EVs and to generate bioengineered EVs with enhanced value for human health.

CRediT authorship contribution statement

Elisa Cappetta: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Monica De Palma:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vincenzo Vestuto:** Writing – original draft, Methodology, Formal analysis, Data curation. **Emanuele Rosa:** Formal analysis, Data curation. **Marisa Conte:** Visualization, Formal analysis, Data curation. **Maria Rosaria Miranda:** Investigation, Formal analysis, Data curation. **Mariapia Vietri:** Formal analysis, Investigation, Validation. **Francesca Mensitieri:** Methodology. **Pablo Martínez-Vicente:** Investigation, Formal analysis. **Valentina Santoro:** Formal analysis, Data curation. **Ornella Moltedo:** Supervision. **Pietro Campiglia:** Supervision, Resources. **Nunziatina De Tommasi:** Supervision, Resources. **Fabrizio Dal Piaz:** Writing – review & editing, Supervision. **Alfredo Ambrosone:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.118395.

Data availability

Data supporting proteomic analyses are available have been deposited to the ProteomeXchange (PXD059086). All the other data of this study are available on request from the corresponding author

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