Plant hairy roots for the production of extracellular vesicles with antitumor bioactivity

Eleonora Boccia1,7, Mariaevelina Alfieri1,2,7, Raffaella Belvedere1, Valentina Santoro1, Marianna Colella1, Pasquale Del Gaudio1, Maria Moros3,4, Fabrizio Dal Piaz5,6, Antonello Petrella1, Antonietta Leone1 & Alfredo Ambrosone1

Plant extracellular vesicles (EVs) concentrate and deliver different types of bioactive molecules in human cells and are excellent candidates for a next-generation drug delivery system. However, the lack of standard protocols for plant EV production and the natural variations of their biomolecular cargo pose serious limitation to their use as therapeutics. To overcome these issues, we set up a versatile and standardized procedure to purify plant EVs from hairy root (HR) cultures, a versatile biotechnological system, already successfully employed as source of bioactive molecules with pharmaceutical and nutraceutical relevance. Herewith, we report that HR of Salvia dominica represent an excellent platform for the production of plant EVs. In particular, EVs derived from S. dominica HRs are small round-shaped vesicles carrying typical EV-associated proteins such as cytoskeletal components, chaperon proteins and integral membrane proteins including the tetraspanin TET-7. Interestingly, the HR-derived EVs showed selective and strong pro-apoptotic activity in pancreatic and mammary cancer cells. These results reveal that plant hairy roots may be considered a new promising tool in plant biotechnology for the production of extracellular vesicles for human health.
Extracellular vesicles have been described in all three domains of the tree of life, suggesting that vesicular transport is a universal biological process through which cells exchange important biomolecules and genetic information, thus establishing an intercellular communication in the same or even between different organisms\(^1,2\). The International Society of Extracellular Vesicles (ISEV) consensus recommends using the term extracellular vesicles for particles, delimited by a lipid bilayer and which cannot replicate, naturally released from cells\(^3\).

Plant-derived nano and microvesicles have been surprisingly almost neglected for a long time. In fact, nano-sized vesicles in plants have been observed in the '60s\(^4,5\), earlier than animal exosomes\(^6,7\), but their biological role remained largely unexplored. In the last decade, distinct classes of plant-derived nano and microvesicles (PDVs), including extracellular vesicles (EVs) have been obtained from tissues, organs, apoplastic fluids, and juices of several plant species. Some of these studies have shown that PDVs are excellent nanocarriers able to transport bioactive molecules, including lipids, proteins, small non-coding RNAs, and other metabolites to target cells, driving cell-cell communication, even between different organisms and species. Proteomic studies have demonstrated that PDVs contain typical EV-associated proteins together with numerous proteins with antifungal, antimicrobial and hydrolytic activities, suggesting that EVs are important components of the innate immune system in plants and participate in cell wall organization\(^8\)–\(^11\). Besides their physiological role in plants, it has been recently observed that PDVs may provide beneficial effects for human health\(^12,13\). For instance, PDVs derived from edible plants such as grapes, grapefruit and ginger have been demonstrated to contribute to the maintenance of the intestinal tissue homeostasis and repair mechanisms in animal models, favouring the process of renewal of enterocytes and alleviating inflammation\(^14\)–\(^16\). Citrus-derived vesicles, especially those obtained from lemon and grapefruits, have shown antineoplastic effects by inhibiting cancer cell proliferation through the activation of the programmed cell death mechanisms both in vitro and in vivo\(^17,18\). Also, a few clinical studies have been started to assess the therapeutic role of the plant-derived nano and microvesicles in the clinical practice (ClinicalTrials.gov Identifier: NCT01668849; ClinicalTrials.gov Identifier: NCT01294072). More recently, the preliminary results of a pilot open label study demonstrated that EVs derived from C. limon juice exert positive effects reducing waist circumference and LDL cholesterol in healthy subjects\(^19\).

Despite their promising potential, the safe and proper use of plant EVs for biomedical purposes is still limited due to the lack of standardization during the EV isolation and characterization. For instance, due to the natural diversity of plant-derived nano and microvesicles, their cargo and bioactivity may change substantially according to the sources (e.g., plant species, tissue and organs), as well as to the plant physio-pathological conditions. PDV availability and characteristics are also affected by the production seasons and accessibility of plant materials.

Moreover, the current PDV purification procedures are mostly based on destructive methods of tissues and organs which do not allow discriminating between true extracellular vesicles and other types of small vesicles released or artificially created after cell destruction\(^20\). This also contributes to the lack of a clear classification of plant-derived vesicles. Therefore, the development of a plant-based biotechnology system ensuring a standardised purification of true extracellular vesicles with a conserved biomolecular cargo and reproducible bioactivity is highly demanded.

As proof of concept, in this work we focused on setting up a reliable and reproducible procedure of purification and characterization of extracellular vesicles from the hairy root cultures of Salvia dominica, a medicinal plant known for the several biological activities of its extracts\(^21,22\).

Hairy roots (HR) are neoplastic growth that originates from the wounding site of plants infected by Agrobacterium rhizogenes, a soil symbiotic bacterium currently taxonomically renamed Rhizobium rhizogenes. During the infection mechanism, specific bacterial DNA fragments (T-DNA) transfer from its root-inducing plasmid into the plant genome, stimulating the differentiation of hairy roots in many dicotyledonous plants\(^23\).

Nowadays HR cultures have become major and versatile plant biotechnological tools attracting the interest of different industries in the pharmaceutical, nutraceutical and cosmetics fields\(^24\). Compared to other plant cell cultures, the hairy roots are stable under hormone-free culture conditions and offer additional advantages such as fast growth, low doubling time, ease of maintenance, absence of pathogen contaminants as well as the ability to synthesize a wide range of natural chemical compounds. Besides, strategies of metabolic engineering to control the bio-synthetic pathways and to push the metabolic flux toward the desired compounds are viable routes to fully harness the potential applications of hairy root cultures\(^25,26\). Hairy roots obtained from many plant species have also been used to produce recombinant proteins\(^27\)–\(^29\) (e.g., human acetylcholinesterase, murine interleukin-1, human interferon alpha-2b, human erythropoietin) and specialized metabolites such as camptothecin, naphthoquinones, stilbenes, tanshinones, anthocyanins and many others\(^30\)–\(^35\).

Herein, we report the purification of round-shaped extracellular vesicles by differential ultracentrifugation from S. dominica HR-conditioned medium. They share biophysical characteristics and a pattern of associated proteins as previously described in animal and plant EVs, such as actin, chaperones and many integral components of plasma membranes. We also demonstrated that S. dominica HR-derived EVs have a selective and strong pro-apoptotic activity in pancreatic and breast cancer cells. The results of this work pave the way for the exploitation of hairy roots as novel biotechnological platforms for the standardised production of plant bioactive EVs to be used as pharmaceuticals and nutraceuticals.

**Results**

**Hairy root induction and characterization.** Hairy roots (HRs) were obtained by infecting leaf explants excised from 20-days old S. dominica plantlets with Agrobacterium rhizogenes (Fig. 1a, d). Four week-old healthy and differentiated HRs were transferred into a sterile flask containing the liquid medium without phytohormones and grown in the dark under continuous agitation (Fig. 1e). The HRs were characterized for the stable genomic integration of the T-DNA region of the Root Inducing plasmid (pri) of A. rhizogenes in HRs by rolB gene amplification using a polymerase chain reaction (PCR) (Fig. 1f). Elimination of Agrobacterium in the HR cultures after antibiotic treatment (Fig. S1) was verified by amplification of virD2 virulence gene, carried by the T-helper plasmid of A. rhizogenes, which is not transferred into the plant genome. Only stable and Agrobacterium-free S. dominica HR cultures were then used to isolate EVs from their conditioned media, as schematised in Fig. 1g.

**Isolation and biophysical characterization of HR-derived EVs.** EVs were isolated by differential ultracentrifugation from the conditioned medium (~40 mL) collected from 10-day freshly inoculated HR cultures, which yielded an average of 2 grams (fresh weight) of HRs. Particle size distribution and morphology of HR-derived extracellular vesicles were characterized by different techniques. Dynamic light scattering (DLS) analyses revealed that EVs ranged in size prevalently from 140 to 210 nm (Fig. 2a) with the major peak for the particle hydrodynamic
diameter at ~180 nm. As detergents are able to solubilize and destroy lipid bilayers of EVs, sensitivity to detergents has been used as a method to validate the vesicular nature of the isolated nanostructures.\textsuperscript{36,37} DLS analysis of HR-derived EV preparations after 1 h incubation with 1% sodium dodecyl sulphate (SDS) showed the destruction of HR-derived EVs confirming that they are lipid membrane enclosed vesicles (Fig. S2).

Complementary approaches, such as Nanoparticle Tracking Analysis (NTA) and electron microscopy confirmed these measurements. NTA is a powerful technique that combines both laser light scattering and Brownian motion in order to characterize the hydrodynamic diameter and concentration of particles, including extracellular vesicles in liquid suspensions. Also, NTA provides a better peak resolution than DLS offering an accurate size estimation of heterogeneous EV populations.\textsuperscript{38,39} NTA revealed that 81.8% of detectable particles were within the size range of 100–200 nm with a mean diameter of around 153 nm ± 1.8 nm. We noticed also the presence of nanovesicles (about 9% of the total particles analysed) with a diameter smaller than 100 nm (Fig. 2b and Fig. S3), not found in DLS measurements. Moreover, NTA detected the presence of particles with a hydrodynamic diameter higher than 250 nm (approx. 4%) probably generated by the formation of EV aggregates. According to NTA estimation, EV mean concentration was $1.89 \times 10^9 + 8.6e + 07$ particle/mL. Transmission Electron microscopy (TEM) was used for wide-field and close-up imaging of EVs, respectively.

**Fig. 1** Experimental design for the production and characterization of *S. dominica* hairy root derived EVs. a Plantlets of *Salvia dominica* grown in vitro. b Sterile leaf sections infected by *A. rhizogenes*. c Hairy roots (HRs) development from infected leaves. d Subcultures of excised hairy roots in solid hormone free medium. e HR in the hormone-free liquid MS medium. f Stable integration of the rolB gene in HRs assessed by polymerase chain reaction (PCR) in three independent transformation events. Amplicon length: 451 base pairs (bp) and 142 bp for rolB and actin genes, respectively. NTC, no template control. g Workflow planned for the purification and characterization steps of extracellular vesicles released by *S. dominica* HRs.

**Fig. 2** Biophysical characterization of HR-derived EVs. a EVs size distribution curve obtained by dynamic light scattering (DLS). b Nanoparticle Tracking Analysis (NTA) measurements show the distribution of the EV size with the major peak at 151 nm. c TEM image of isolated vesicles exhibiting round-shaped morphology. d, e Close-up view images of individual HR-derived EVs. f Protein pattern of HR-derived EVs and EV depleted-medium (EDM) visualized by SDS-PAGE and silver staining. Silver staining of EDM did not show detectable proteins. Scale bars: 500 nm in c, 50 nm in d, 50 nm in e.
TEM images revealed the presence of intact round-shaped EVs, heterogeneous in size and morphology (Fig. 2c–e), mostly ranging between 70 nm and 160 nm, as expected.

The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of S. dominica EVs showed a characteristic and reproducible protein pattern (Fig. 2f), indicating that the purification method does not affect the EV cargo stability and integrity.

Finally, any potential A. rhizogenes contamination in the EV preparations was excluded by PCR amplification of rolB, rolC and virD2 genes as reported in Fig. S4.

Proteome analysis of hairy root-derived extracellular vesicles. EVs carry a distinct repertoire of luminal and membrane proteins implicated in their formation, release and cell uptake together with numerous molecular signal components able to control biological functions in the recipient cells. In order to provide a detailed protein characterization of HR-derived EVs and identify typical EV-associated biomarkers, we performed a classical gel-based proteomic approach in three distinct preparations of HR-derived EVs. To obtain a list of proteins enriched in the EV preparations, we also characterised the proteins present in the pellet fractions after low-speed centrifugation steps and subtracted them to the EV protein dataset. As the information available in the database of S. dominica proteome is rather limited, proteins were identified against the Green Plant (Viridiplantae) protein database.

The proteins commonly found in the EV preparations and in the non-vesicular fractions, reported as supplementary data 1, were not considered further in this work. Conversely, in two replicate analyses performed on two independent experiments, we found 143 proteins exclusively associated to S. dominica HR-derived EVs (table S1). The proteins with the highest Mascot score among these are listed in Table 1.

Gene Ontology term enrichment of cellular components showed the presence of numerous proteins in the categories “membrane”, “plant -type cell wall organization” and “extracellular components” (Fig. S5a) confirming again the vesicular nature of EVs released from HRs.

Moreover, a wide range of molecular functions can be covered by these proteins (Fig. S5b). Remarkably, we found the presence of a large number of ribonucleotide binding proteins (Fig. S5c) that may be involved in the nucleic acid packaging and extracellular transport, relevant for the intercellular and interkingdom signalling role of EVs1,40–42.

Interestingly, proteomic data reported in Table 1, table S1, and in Supplementary data 1 evidenced also that S. dominica EV carry many proteins frequently found in mammalian extracellular vesicles according to Vesiclepedia database13, such as cytoskeletal components (actin, tubulin, kinesin proteins), chaperon proteins (HSP70s, HSP 80 and HSP90), glycolytic enzymes (enolases and glyceraldehyde-3-phosphate dehydrogenases), annexin-like protein and elongation factors. It is worthy to evidence that HR-derived EVs carry tetraspanin-7 (TET7), a protein with high homology to A. thaliana TET8, which is considered a specific plant exosomal marker.

Finally, numerous integral components of membranes, commonly described in the vesicles isolated in animals and plant species were also found in HR-derived EV protein dataset. Among them, we identified an ATP synthase subunits, a LRR receptor-like serine/threonine-protein kinase HSL2, the Aquaporin TIP3-1, the probable phospholipid-transporting ATPase 8, the Ras-related proteins RABA4a and Rab11A.

Salvia dominica HR-derived Extracellular Vesicles have antitumor activities. Antibacterial, antioxidant, anti-diabetic, cardiovascular and antitumor activities of natural products obtained from Salvia species have been largely documented44. In particular, S. dominica extracts exhibited antiproliferative activity against different cell tumor lines21,22. Hence, we evaluated whether EVs released from HR cultures of this Salvia species might also have antitumor activity.

Firstly, we examined the EV uptake in the HaCaT cells (human keratinocytes), already employed by our group as non-malignant cells to test anticancer activity of several plant-derived

Table 1 List of the 20 vesicle proteins showing the highest score in the Mascot-based bioinformatics analysis carried out on proteomic data.

| Accession   | Score | Mass       | Num. of significant matches | Num. of significant sequences | Description                                                                 |
|-------------|-------|------------|-----------------------------|-------------------------------|-----------------------------------------------------------------------------|
| G3PC_IDACA  | 475   | 37105      | 28                          | 11                            | Glyceraldehyde-3-phosphate dehydrogenase                                  |
| G3PC2ORYSJ  | 389   | 36921      | 25                          | 11                            | Glyceraldehyde-3-phosphate dehydrogenase 2                                 |
| EF1A1DAUCA  | 366   | 49613      | 17                          | 8                             | Elongation factor 1-alpha                                                  |
| HSP70SOYBN  | 309   | 71291      | 17                          | 9                             | Heat shock 70 kDa protein                                                 |
| METE1ORYSJ  | 307   | 84874      | 12                          | 5                             | 5-methyltetrahydropteroylglutamate–homocysteine methyltransferase 1          |
| BIP1ARATH   | 292   | 73869      | 15                          | 8                             | Heat shock 70 kDa protein BIP1                                             |
| ALF2PEA     | 280   | 38638      | 10                          | 6                             | Fructose-bisphosphate aldolase                                              |
| METE3ARATH  | 249   | 90993      | 10                          | 4                             | 5-methyltetrahydropteroylglutamate–homocysteine methyltransferase 3         |
| API3SOLTU   | 226   | 18909      | 12                          | 6                             | Aspartic protease inhibitor 3                                              |
| PGKH2ARATH  | 208   | 50022      | 6                           | 4                             | Phosphoglycerate kinase 2                                                  |
| 14338TOBAC  | 207   | 29037      | 6                           | 4                             | 14-3-3-like protein B                                                      |
| 14337ARATH  | 201   | 29920      | 16                          | 6                             | 14-3-3-like protein GF14 nu                                                |
| CDC48SOYBN  | 190   | 90512      | 10                          | 6                             | Cell division cycle protein 48 homolog                                    |
| ATPAMSOYBN  | 185   | 55581      | 7                           | 5                             | ATP synthase subunit alpha                                                |
| LOX1LENCU   | 177   | 96807      | 7                           | 2                             | Linoleate 95-lipoxygenase                                                  |
| 14331SOLTU  | 174   | 29491      | 16                          | 6                             | 14-3-3-like protein                                                        |
| MDHM_CITLA  | 164   | 36406      | 6                           | 2                             | Malate dehydrogenase, mitochondrial                                       |
| ENPLCATRO   | 154   | 93605      | 5                           | 3                             | Endoplasmic homolog                                                       |
| VCL1PEA     | 152   | 14031      | 4                           | 4                             | Viciin, 14 kDa component                                                   |
| ENO2ARATH   | 152   | 47974      | 7                           | 3                             | Bifunctional enolase 2/transcriptional activator                           |
vesicles\textsuperscript{18}, and MIA PaCa-2 pancreatic carcinoma cells, characterised by high invasiveness and drug-resistance\textsuperscript{45}. To this aim, the cell lines were incubated for 24 h with HR-derived EVs (1.2 \times 10^8 particles/mL) labelled with the lipophilic fluorescence dye Bodipy FL (Fluo-EVs). A confocal microscopy analysis showed that Fluo-EVs mostly enter and accumulate into the cytoplasm, preferentially in cancer cells (Fig. 3a–d). Longer incubation times up to 48 h did not result in an increased accumulation of Fluo-EVs in cancer cells (Fig. S6). To check the stability of EVs in different culture conditions, we evaluated the uptake levels in cells cultured in fetal bovine serum (FBS)-free media. Confocal images confirmed that the Fluo-EV internalization levels were comparable to those observed in cells grown in complete culture medium (Fig. S7), indicating that HR-derived EVs stability and uptake are not affected by the presence of exogenous FBS proteins that could cause extensive EV aggregation during the incubation.

Subsequently, we evaluated the impact of HR-derived EVs on the growth of HaCaT and MIA PaCa-2 cell lines. In particular, cells were incubated for 24 h and 48 h with increasing concentrations of EVs (from 6 \times 10^7 to 3 \times 10^8 particle/mL). EV effects were compared to those of gemcitabine, a chemotherapeutic drug widely used for pancreatic cancer treatment known to inhibit DNA synthesis and promote cell apoptosis\textsuperscript{46,47}. Additionally, both cell lines were incubated with heat-denatured EV preparations, which have been previously used as negative controls\textsuperscript{48–50}. Cell viability was determined by the tetrazolium-based cytotoxicity assay (MTT). As shown in Fig. 3e–f, S. dominica EVs did not alter HaCaT viability after 24 h and 48 h treatment, indicating a safe profile for non-cancerous cells. Conversely, HR-derived EVs severely affected the cell viability of MIA PaCa-2 cell line in a dose-dependent manner with the highest cell viability reduction (>40%) when used at a concentration of 3 \times 10^8 EVs/mL for 24 h (Fig. 3e). Under these conditions the cytotoxic effects of EVs in cancer cells were significantly higher than those in cells treated with gemcitabine. Longer incubation with EVs did not reduce further the cell viability of MIA PaCa-2 cells (Fig. 3f). Heat-denatured EVs induced only non-significant slight fluctuations of cancer cell viability (Fig. S8). Taken together, these data prove that intact HR-derived EVs exert selective and early cytotoxic effects in cancer cells.

To investigate deeper the bioactivity of HR-derived EVs on tumor cells, MIA PaCa-2 cells exposed to HR-derived EVs for 24 h and 48 h were analysed by fluorescence-activated cell sorting (FACS) as reported in Fig. 3g–h, respectively. FACS analyses showed that the treatment with HR-derived EVs induces a dose-dependent increase of cell apoptosis compared to the control cell in tumor cells, but not in HaCat cells (Fig. S9). Remarkably, we found that pro-apoptotic activity of EV treatments was comparable to that observed in cancer cells treated with different concentrations of gemcitabine. Western blot analyses (Fig. 3i and Fig. S12) were carried out to further confirm at molecular level the programmed cell death execution through the activation of procaspase-3 and subsequent cleavage of poly (ADP-ribose) polymerase-1 (PARP-1), which is one of the several known cellular substrates of caspases. In particular we found that the 32 KDa band of pro-caspase 3 was significantly higher than those in cells treated with gemcitabine. This was confirmed at molecular level by the relative increase of the PARP-1 cleaved band, which is one of the several known cellular substrates of caspases. Western blot analyses (Fig. 3i and Fig. S12) were carried out to further confirm at molecular level the programmed cell death execution through the activation of procaspase-3 and subsequent cleavage of poly (ADP-ribose) polymerase-1 (PARP-1), which is one of the several known cellular substrates of caspases. In particular we found that the 32 KDa band of pro-caspase 3 was cleaved in EV-treated MIA PaCa-2 cells in a dose dependent manner. As expected, partial degradation of PARP1 was detected after 24 h EV treatment. To confirm the antitumor activity of S. dominica HR EVs, we evaluated their effects also in MCF-7 breast cancer cell line, as reported in Fig. 3i. These data revealed that MCF-7 cells were not affected by the presence of HR EVs, confirming their selective effects in cancer cells.
cancer cell line. As reported in Figs. S10a and S10b, 24 h and 48 h EV treatments reduced up to 60% the MCF-7 viability and promoted programmed cell death in a dose-dependent manner, respectively. Selective procaspase-3 and PARP-1 cleavage in MCF7 cells confirmed the activation of the apoptotic molecular cascade upon 24 h EV treatment (figs. S10c and S13). As expected, heat-denatured EV did not affect MCF-7 viability (Fig. S11).

Altogether, these data clearly indicate that S. dominica HR-derived EVs play a positive role in restoring programmed death in cancer cells, likely triggered through the localised release of anticancer molecules carried by EVs.

Discussion
Most of the plant-derived nano and microvesicles so far isolated have been obtained through destructive methods that lead to the purification of heterogeneous vesicle populations. In these preparations, EVs and intracellular nano or microvesicles derived from the rupture of plant tissues and organs are mixed together. Not least, artificial vesicles unintentionally produced during the invasive extraction procedures may contaminate the EV samples. Actually, only a handful of works have described true EVs collected from extracellular apoptotic fluids of plants. More recently, we reported that tomato plants, grown in hydroponic culture, release EVs with antifungal properties in the surrounding environment through the root system. These previous findings inspired the present study aimed at setting up a reliable biotechnological platform for the production and purification of biologically active plant EVs based on hairy root (HR) technology.

In particular, to the best of our knowledge here we demonstrated for the first time that HRs obtained from Salvia dominica secrete round-shaped extracellular vesicles ranging in size prevalently between 100–200 nm. The lipidic membranous nature of HR-derived vesicles was confirmed through the staining with the specific hydrophobic dyes BODIPY FL able to bind lipid membranes and through the detergent SDS sensitivity assay. In addition, proteomic analysis of the HR-derived EVs revealed the presence of a consistent number of membrane and extracellular proteins and other reported EV-associated proteins. We also noticed in the HR-released EV proteome the absence of the category “endoplasmic reticulum” (Fig. S5A). According to a recent comparative proteomic study of plant-derived nanovesicles and small EVs in A. thaliana, this could be a distinctive feature of the true EV proteome.

S. dominica HR-released EVs cargo contains proteins such as elongation factors, heat shock proteins, glyceraldehyde 3-phosphate dehydrogenases and integral membrane proteins, also reported in the proteome of EVs isolated from important plant species. Interestingly, HR-derived EVs also carry TET-7, a tetraspanin with high homology to the plant exosomal marker TET-8. Finally, the protein cargo of EVs released by S. dominica HRs is comparable also to those reported in a recent survey on the protein families most frequently found in plant EV proteomes. This indicates that this set of proteins might be considered as reliable markers of plant EVs, although it is necessary to extend the analysis to EVs purified from a larger number of plant species, to establish a general consensus of the scientific community on bona fide protein markers of plant EVs. Taken together these data indicate that HR-released vesicles are true plant EV, however the presence of a small number of vesicles generated by accidental rupture of soft structures (e.g. root hairs) in the growth phase cannot be completely excluded.

Remarkably, the size, the morphology and the protein cargo of HR-derived EVs significantly diverge from EVs that we have previously isolated from root exudates of hydroponically grown tomato plants, suggesting that plant hairy roots may release in the environment EVs with peculiar biophysical and biochemical characteristics compared to in vivo roots.

An overwhelming number of recent data has largely proved that the biological activity of plant EVs is strictly associated to their biomolecular cargo which contains high concentrations of bioactive molecules (e.g., proteins, specialised metabolites and small-non coding RNAs) packed in nano and microstructures and is due to their innate delivery function. For these peculiar features, plant EVs have attracted great attention as new candidates for the development of innovative therapeutics. Their potential biomedical applications have been supported in the last few years by a growing body of evidence that has shown how PDVs play an important role in the regulation of gut microbiome and exert important antioxidant, anti-inflammatory and anticancer properties. For instance, it has been proved that lemon-derived vesicles were able to inhibit cell proliferation of tumor cell lines in part by inhibiting the lipid metabolism of cancer cells. Recently we have demonstrated that grapefruit-derived nano and microvesicles carry antioxidant enzymes and anticancer compounds conferring antiproliferative and pro-apoptotic activities in a melanoma cell line. Moreover, PDVs have shown a low immunogenicity and can be virtually obtained at low cost from a considerable number of plant sources. Despite their great promise, the standardisation of purification processes and biomedical applications of PDVs struggle to keep up. Data presented herewith have demonstrated that EVs are released from HRs of S. dominica and can be easily purified from the growth medium. This constitutes an important biotechnological advance for ensuring an easy, cost-effective, scalable and rapid purification of bioactive plant EVs. A HR-based production of plant EVs avoids invasive extraction procedures from plant tissue and organs and contributes also to achieve a standardised EV quality, abolishing the effects on PDVs variation of multiple factors, such as the developmental stages, the environmental conditions and genetic diversity of plant sources. At the same time it ensures the sterility of EV preparations required for biomedical applications.

In this work, we have used HRs of Salvia dominica as EV source. The genus Salvia is characterised by high biodiversity including over 900 species in the Lamiaceae family. Besides their use in food consumption, many of these species are routinely used in traditional medicine to treat cardiovascular diseases and infections and are included in the official pharmacopoeia lists of different countries. Most of the therapeutic effects of Salvia species are probably determined by the elevated content of polyphenols, flavonoids and terpenoids. Cell and organ cultures of several Salvia species have been set up to produce interesting compounds such as rosmarinic acid, aethiopinine and tanshinone. Our data have shown that that HR-released EVs of S. dominica possess a strong and selective pro-apoptotic activity in pancreatic and breast cancer cells with a safe profile for non-cancer cells. It is worthy to note that the antiproliferative and pro-apoptotic properties of HR-derived EVs in Mia PaCa-2 cells were comparable or even higher than those of gemcitabine, a chemotherapy drug used to treat several solid tumors. Further studies are necessary to establish which components of the EVs released from S. dominica HRs are responsible of this clear and strong antitumor activity we have observed in pancreatic and breast tumor cells. Although the therapeutic properties of S. dominica have not yet been fully elucidated, previous works have reported that the extract of this species may exert anticancer activity in part mediated by sesterterpene lactones which interact with the tubulin−tyrosine ligase (TTL), an enzyme involved in the tyrosination cycle of the C-terminal of tubulin.
Furthermore, we proved that fluorescently-labelled EVs are internalized and accumulate in the cytoplasm of keratinocytes and cancer cells within 24 h. This timing of internalization is similar to that observed in mammalian exosomes and other plant EVs, which is normally completed in a few hours. The mechanisms of plant EVs uptake, specific surface interactions and molecular players involved in mammalian cell/plant EV recognition are only marginally known. For instance, surface protein interactions between a mannose-specific binding protein II lectin and CD98 may play an important role as reported for the molecular players involved in mammalian cell/plant EV recognition.

Similarly to that observed in mammalian exosomes and other plant internalized and accumulate in the cytoplasm of keratinocytes ultimately may result in an expected high therapeutic effect and locally deliver one or more co-acting biomolecules, which clearly indicated by our first report on the EV purification from a hairy root biotechnological platform. In particular, we demonstrated that hairy roots might be used as biofactories of extracellular vesicles with interesting biological properties to be explored in more depth in the nutraceutical and pharmaceutical sectors. It appears appealing the logical platform. In particular, we demonstrated that hairy roots developing from the hairy roots of *Salvia dominica*.

In conclusion, to the best of our knowledge, this study is the first report on the EV purification from a hairy root biotechnological platform. In particular, we demonstrated that hairy roots might be used as biofactories of extracellular vesicles with interesting biological properties to be explored in more depth in the nutraceutical and pharmaceutical sectors. It appears appealing the logical platform. In particular, we demonstrated that hairy roots developing from the hairy roots of *Salvia dominica*.

**Methods**

**Plant material and growth conditions.** *Salvia dominica* seeds were kindly provided by Ammar Bader (Department of Pharmacognosy, Umm Al-Qura University, Saudi Arabia). The seeds were surface sterilized with 70% (v/v) ethanol for 1 min and then with 2% (v/v) sodium hypochlorite solution for 10 min, rinsed five times with sterile distilled water for 1 min each and then sown under aseptic conditions in autoclaved Murashige & Skoog (MS) medium (pH 5.8) containing 30 g/L sucrose and 9 g/L agar. The plants were grown in vitro at 23 °C under a 140 μmol m⁻² s⁻¹ at the leaf level.

**Hairy root cultures production and characterization.** The protocol to obtain hairy roots of *Salvia dominica* was adapted from our previous work. Briefly, the Agrobacterium rhizogenes virulent strain ATCC 15834 obtained from a single colony was grown (OD₅₉₅ = 0.3–0.5) in Yeast Extract Broth (YE) liquid medium (0.1% Yeast extract, 0.5% Beef extract, 0.5% Peptone, 0.5% Sucrose, 0.04% MgSO₄) at 26 °C. Before transformation, bacteria were collected from liquid cultures at 5000 rpm for 5 min and resuspended at a cell density OD₆₀₀ = 0.5 in YE and were shaken for 1 h in the rotary shaker before inoculation. *S. dominica* sterile leaf explants from 20-day-old plants aseptically grown in vitro were submerged in the bacterial suspension for 30 min. Then, leaf explants were dried and treated under laminar flow hood for 15 minutes and co-cultivated at 26 °C for 3 days in MS30 solid medium and then transferred to fresh MS medium containing cefotaxime (100 mg/L) and cephalexin (100 mg/L). Hairy root cultures were then propagated on MS30 solid medium containing cefotaxime (100 mg/L) and cephalexin (100 mg/L) and then sub-cultured in MS medium and kept for additional two weeks in the dark at 23 °C.

**Dynamic Light Scattering (DLS).** Size distribution of extracellular nanovesicles was measured by dynamic light scattering (DLS) using a Zetasizer Ver. 7.01, Malvern Instrument (Malvern, UK). Ten microliters of EV preparation were dispersed in deionized MilliQ water and the intensity of the scattered light was measured with a detector at 90° angle at room temperature. To prove the EV sensitivity to detergents, HR-derived EVs were incubated with 1% SDS and kept at room temperature for 1 h. Then the samples were analysed by DLS as above mentioned. Measurements were carried out in three different EV preparations.

**Transmission Electron Microscopy (TEM).** Before using a grid of carbon film supported on a 300 mesh copper grid, a glow discharge was performed on it (30 s 15 mA). The samples were fixed using glutaraldehyde 2.5% in PBS and deposited on a parafilm. The grid was placed on the drop for 5 min, washed with distilled water for 1 min, and placed for 1 min in a drop of 3% glutaraldehyde (10% aqueous) and then dehydrated in a graded ethanol series (50%, 70%, 85%, 95%, 100%) and dried it in a critical point drier (HMK211) by replacing it with water for 1 min. TEM images were collected using a FEI Tecnai T20 (FEI Europe, Eindhoven, Netherlands) in the Laboratorio de Microscopias Avanzadas at
Universidad de Zaragoza (Spain). TEM observations have been carried out in three independent EV preparations.

**Proteomic and Bioinformatic analyses.** Protein extracts from purified HR-derived EVs and from pellet fractions collected at low-speed centrifugation were separated by 12% SDS-PAGE. Resulting gels were divided into 10 pieces, and each underwent a trypsin digestion procedure. Peptides were analysed on a Orbitrap Q-Exactive Classic Mass Spectrometer (Thermo Fisher Scientific) coupled with a nanoUltimate300 UHPLC system (Thermo Fisher Scientific). Peptide separation was performed on a capillary EASY-Spray PepMap column (0.075 mm x 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 minutes and a 300 nL·min⁻¹ flow rate. Mass spectra were acquired over an m/z range from 375 to 1500. To achieve sequence confirmation, MS and MS/MS data underwent Mascot software (v2.5, Matrix Science) analysis using the non-redundant Data Bank UniprotKB/Swiss-Prot (Release 2021_03), limited to green plants proteins (Viridiplantae). Parameter sets were: 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 three different vesicle preparations and only the proteins commonly identified in all the experiments were considered. Gene ontology analysis of identified proteins was carried out using the specific tool in UniProt Knowledgebase (UniProtKB; http://www.uniprot.org).

**Human cell culture.** MIA PaCa-2 (CRL-1420™) are immortalized epithelial cells of human pancreatic carcinoma. They were purchased from ATCC (Manassas, VA, USA) and cultured as reported48. HaCaT cells are human immortalized keratinocytes. They were purchased from CRL (Cell Lines Service GmbH (Germany)) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). MCF-7 (HTB-22™) are human breast cancer cells and were purchased from ATCC (Manassas, VA, USA). Both cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS).

**MTT assay.** HaCaT, MIA PaCa-2 and MCF-7 cells were seeded in 15 x 10⁷ cells/well in triplicate in 96 well-plates and incubated for 24 and 48 h with freshly prepared S. dominica EVs at three different concentrations, namely 6 x 10⁷, 1.2 x 10⁸ and 3 x 10⁸ particles/mL. Cells treated with heat-inactivated EVs were used as negative controls. To this aim, EV preparations were kept at 95 °C for 1 h, followed by 30 min at room temperature, EVs were isolated by ultracentrifugation as described before. HR conditioned medium and mixed for 30 sec. After 5 min incubation at 37 °C, cells were then lysed and the dark blue formazan (Titertek Multiskan MCC/340) density (OD) of each well was measured in a microplate spectrophotometer (BIO-RAD) as described in71. Protein cell lysates (20 µg) were separated by SDS-PAGE under denaturing conditions, transferred onto a nitrocellulose membrane and visualized with goat serum (20% v/v in PBS; Lonza; Basel, Switzerland) and then incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Thermo Fisher Scientific). The proteins recognized by Abs were detected by ECL (Amersham biosciences) and exposed to Las4000 (GE Healthcare Life Sciences) and the relative band intensities were determined using ImageQuant software (GE Healthcare Life Sciences). The corresponding autoradiograms were then scanned and the intensity of each band divided by the intensity of the actin band (internal standard) and the final value is expressed as percentage of untreated control.

**Cell uptake and confocal microscopy.** EVs were labelled with BODIPY™ FL N-((2-aminoethyl)maleimide. Briefly, 10 µL of Bodipy dye was added to 13.5 mL of S. dominica HR conditioned medium and mixed for 30 sec. After 5 min incubation at room temperature, EVs were isolated by ultracentrifugation as described before. After 24 and 48 h EV treatment, MIA PaCa-2 cells were harvested and used for confocal analysis performed as reported in a previous work97. Briefly, cells were fixed in p-formaldehyde (4% v/v in PBS; Lonza; Basel, Switzerland) and then incubated with anti-annexin A1 antibody (rabbit polyclonal; 1:100; Thermo Fisher Scientific; Walham, MA, USA), overnight at 4 °C. After two washing steps, cells were incubated with anti-rabbit AlexaFluor 555 (1:500; Thermo Fisher Scientific; Walham, MA, USA) for 2 h at RT in the dark. To detect the nuclei, 4’6-diamidino-2-phenylindole (DAPI; 1:1000) was used. Samples were vertically scanned from the bottom of the coverslips with a total depth of 5 μm and a 63X (1.40 NA) Plan-Apochromat oil-immersion objective. Images and scale bars were generated with Zeiss ZEN Confofocus Software (Carl Zeiss Micro Imaging GmbH) and presented as single stack. Confocal microscopy analyses were carried out in three independent experiments.

**Apoptosis analysis by Flow Cytometry.** The percentage of hypodiploid nuclei was estimated by propidium iodide (PI) incorporation into permeabilized cells, as described by Belvedere et al48. Briefly, cancer cells were harvested after treatment with S. dominica EVs and incubated with a PI solution (0.1% sodium citrate, 0.1% Triton X-100) and 50 g/mL of PI (Sigma-Aldrich, St. Louis, MO, USA), for 30 min at room temperature. Data from 10,000 events for each sample were collected by a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest program evaluation. FACS analyses were conducted in technical triplicate at least in three independent biological experiments.

**Western blot analysis.** Cells were lysed in a RIPA buffer containing 10 mM Tris HCl, pH 7.5, NaCl 150 mM, and Triton X-1% supplemented with Protease Inhibitor Cocktails (Sigma-Aldrich) and the protein content was measured by a colorimetric assay (BIO-RAD) as described in71. Protein cell lysates (20 µg) were separated by SDS-PAGE under denaturing conditions, transferred onto a nitrocellulose filter (BIO-RAD), blocked with 5% milk and probed with primary antibodies: mouse anti-PARP1 (1:500), rabbit anti-proapoptase-3 (1:500) and mouse anti-β-actin (1:1000) (Santa Cruz Biotechnology). After primary antibody incubation, the blots were washed and incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Thermo Fisher Scientific). The proteins recognized by Abs were detected by ECL (Amersham biosciences) and exposed to Las4000 (GE Healthcare Life Sciences) and the relative band intensities were determined using ImageQuant software (GE Healthcare Life Sciences). Pre-stained protein markers (Thermo Fisher Scientific) were used as molecular size standards. All experiments were performed in triplicate.

**Statistics and reproducibility.** Sample size and number of replicates for each experiment were described in figure legends, where possible. Otherwise these data were properly reported throughout the materials and methods section. Error bars in the graphical data represent standard deviations. Student’s t-test was used for statistical analysis, and P-values were considered to be statistically significant.

**Data availability.** Proteomic data generated in this study are available via ProteomeXchange with identifier PXD035119. They have been included also in Supplementary Data 1. The source data underlying the graphs and charts in the main manuscript are available as Supplementary Data 2. Unsupervised gels have been included in the supplementary information. We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV230201)72. All other data from this study are available from the corresponding author upon reasonable request.

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**References.**

1. Van Niel, G., D’Angelo, G. & Raposo, G. Shedding light on the cell biology of extracellular vesicles. Nat. Rev. Mol. Cell Biol. https://doi.org/10.1038/nrm.2017.125 (2018).
2. Cai, Q. et al. Message in a Bubble: Shuttling small RNAs and proteins between cells and interacting organisms using extracellular vesicles. Annu. Rev. Plant Biol. 72, 497–524 (2021).
3. Thery, C. et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018). J. Extracell. Vesicles 7, 1535750–1535796 (2018).
4. Halperin, W. & Jensen, W. A. Ultrastructural changes during growth and differentiation. J. Cell Biol. 104, 974–987 (1986).
5. Politis, D. J. Localized cell wall appositions: incompatibility response of tobacco leaf cell pseudomomas pisi. Phytopathology https://doi.org/10.1094/phyto-68-309 (1978).
6. Harding, C. & Stahl, P. Transferrin recycling in reticuloctyes: pH and iron are important determinants of ligand binding and processing. Biochem. Biophys. Res. Commun. https://doi.org/10.1016/s0006-291x(83)90040-5 (1983).
7. Pan, B. T. & Johnstone, R. M. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: Selective externalization of the cell membrane. Cell. https://doi.org/10.1016/0092-8674(83)90040-5 (1983).
8. de la Canal, L. & Pinedo, M. Extracellular vesicles: A missing component in plant cell wall remodeling. J. Exp. Bot. 69, 4655–4658 (2018).
9. Rutter, B. D. & Innes, R. W. Transferrin extracellular vesicles as key mediators of humoral immune interactions. Curr. Opin. Plant Biol. https://doi.org/10.1016/j. ribmb.2018.01.008 (2018).
10. Rutter, B. D. & Innes, R. W. Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins. Plant Physiol. 173, 728–741 (2017).
11. De Palma, M. et al. Plant roots release small extracellular vesicles with antifungal activity. Plants https://doi.org/10.3390/plants9121777 (2020).
12. Alferi, M., Leone, A. & Ambroso, A. Plant-derived nano and microvesicles for human health and therapeutic potential in nanomedicine. Pharmaceuticals 13, 498–518 (2021).
13. Daude, M. A., Gu, T. W., Zhi, A. Q., Huang, L. Q. & Peng, L. H. Plant exosome-like nanovesicles: emerging therapeutics and drug delivery nanoplatforms. Mol. Ther. https://doi.org/10.1016/j.ymthe.2020.11.030 (2021).
14. Ju, S. et al. Grape exosome-like nanoparticles induce intestinal stem cells and protect mice from DDS-induced colitis. Mol. Ther. https://doi.org/10.1038/mtna.2013.64 (2013).
15. Zhang, M. et al. Visible-light-responsive vesicles functionalized for specific target cell membrane binding and chemotherapy. Biomaterials https://doi.org/10.1016/j.biomaterials.2016.06.018 (2016).
16. Wang, B. et al. Targeted drug delivery to intestinal macrophages by biodegradable nanovesicles released from grapefruit. Mol. Ther. https://doi.org/10.1038/mt.2013.190 (2014).
17. Raimondo, S. et al. Citrus limon-derived nanovesicles inhibit cancer cell proliferation and suppress CML xenograft growth by inducing TRAIL-mediated cell death. Oncotarget 6, 19514–19527 (2015).
18. Stanly, C. et al. Grapefruit-derived micro and nanovesicles show distinct membrane protein and anticancer activities in the A375 human melanoma cell line. Cells https://doi.org/10.3390/cells9122722 (2020).
19. Raimondo, S. et al. Preliminary results of CitraVes™ effects on low density lipoprotein cholesterol and waist circumference in healthy subjects after 12 weeks: a pilot open-label study. Metabolites 11, 276 (2021).
20. Pinedo, M., Canal, L. de la & Lousa, C. de M. A call for Rigor and standardization in plant extracellular vesicle research. J. Extracell. Vesicles 10, e12048 (2021).
21. Abu-Dahab, R., Abdallah, M. R., Kasabri, V., Mhaidat, N. M. & Affif, F. U. Mechanistic studies of antiproliferative effects of Salvia triloba and Salvia domina (Lamiaceae) on breast cancer cell lines (MCF7 and T47D). J. Natuforsch. C 69, 443–451 (2014).
22. Fiore, G. et al. In vitro antiproliferative effect of six Salvia species on human tumor cell lines. Phytother. Res. 20, 701–703 (2006).
23. Christey, M. C. & Braun, R. H. Production of hairy root cultures and metabolite production. Phyther. Res. 29, 276 (2015).
24. Vaccaro, M. C. et al. Hairy root cultures: factories for secondary compounds. Biotechnol. Rep. 8, e12048 (2021).
25. Christey, M. C. & Braun, R. H. Production of hairy root cultures and metabolite production. Phyther. Res. 29, 276 (2015).
26. Alfi, M. C., Mariaevelina, A., Malafronte, N., De Tommasi, N. & Leone, A. Coactivation of MEP-biosynthetic genes and accumulation of abietane diterpenes in S. sclarea hairy roots by engineering the GGPPS and FPPS genes. Curr. Med. Chem. 36, 4718–4739 (2019).
27. Woods, R. R., Geyer, B. C. & Mor, T. S. Hairy-root organ cultures for the production of high-value compounds. Biotechnol. Lett. 22, 437–441 (2004).
28. Liu, C., Towler, M. J., Medrano, G., Cramer, C. L. & Weathers, P. J. Production of mouse interleukin-12 is greater in tobacco hairy roots grown in nutrient-rich media than in nutrient-poor media. J. Exp. Bot. 56, 11085–11094 (2015).
29. Liu, Y. et al. Characterization of and isolation methods for plant leaf nanovesicles and small extracellular vesicles. Nanomed. Nanotechnol., Biol. Med. 12, 102271 (2020).
30. Di Gioia, S., Hossain, M. N. & Conese, M. Biological properties and therapeutic effects of plant-derived nanovesicles. Open Med. 15, 1096–1122 (2020).
31. Rome, S. Biological properties of plant-derived extracellular vesicles. Food Funct. https://doi.org/10.1039/c8fo0033f (2019).
32. Raimondo, S. et al. Label-free quantitative proteomic profiling of colon cancer cells identifies acetyl-CoA carboxylase alpha as antitumor target of Citrus limon-derived nanovesicles. J. Proteom. 173, 1–11 (2018).
33. Pocsfalvi, G. et al. Protein biocargo of citrus fruit-derived vesicles reveals heterogeneous transport and extracellular vesicle populations. J. Plant Physiol. https://doi.org/10.1016/j.jplph.2018.07.006 (2018).
34. Wang, Q. et al. Delivery of therapeutic agents by nanoparticles made of grapefruit-derived lipids. Nat. Commun. https://doi.org/10.1038/s41467-018-06742-7 (2018).
35. Walker, J. B. & Sytsma, K. J. Staminal evolution in the genus Salvia (Lamiaceae): molecular phylogenetic evidence for multiple origins of the staminal lever. Amm. Bot. 100, 375–391 (2007).
36. Vaccaro, M. C. et al. Boosting the synthesis of pharmacologically active abietane diterpenes in S. sclarea hairy roots by engineering the GGPPS and CPPS Genes. Front. Plant Sci. 11, 924 (2020).
64. Trócsányi, E., György, Z. & Zám boriné-Németh, É. New insights into rosmarinic acid biosynthesis based on molecular studies. *Curr. Plant Biol.* **23**, 100162 (2020).

65. Dal Piaz, F. et al. Sesterterpenes as tubulin tyrosine ligase inhibitors. First insight of structure-activity relationships and discovery of new lead. *J. Med. Chem.* **52**, 3814–3828 (2009).

66. Song, H. et al. Internalization of garlic-derived nanovesicles on liver cells is triggered by interaction with CD98. *ACS Omega* [https://doi.org/10.1021/acsomega.0c02893] (2020).

67. Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. Mass Spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–858 (1996).

68. Belvedere, R., Bizzarro, V., Parente, L., Petrella, F. & Petrella, A. Effects of Prisma® Skin dermal regeneration device containing glycosaminoglycans on human keratinocytes and fibroblasts. *Cell Adh. Migr.* **12**, 168–183 (2018).

69. Bizzarro, V. et al. Annexin A1 is involved in the acquisition and maintenance of a stem cell-like/aggressive phenotype in prostate cancer cells with acquired resistance to zoledronic acid. *Oncotarget* **6**, 25076–25092 (2015).

70. Belvedere, R. et al. Annexin A1 contributes to pancreatic cancer cell phenotype, behaviour and metastatic potential independently of Formyl Peptide Receptor pathway. *Sci. Rep.* **6**, 29660 (2016).

71. Amodio, G., Margarucci, L., Molteno, O., Casapullo, A. & Remondelli, P. Identification of cysteine ubiquitylation sites on the Sec23A Protein of the COPII complex required for vesicle formation from the ER. *Open Biochem. J.* **11**, 36–46 (2017).

72. Van Deun, J. & Hendrix, A. Is your article EV-TRACKed? *J. Extracell. Vesicles* **6**, 1379835 (2017).

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Author contributions
M.A., A.L. and A.A conceived the study; E.B., M.A., M.C. and A.A. set up the protocol for HR induction, EV purification and performed EV characterization. R.B and A.P. designed and carried out EV cell uptake and bioactivity studies in human cell lines. V.S and F.D.P performed proteomic and bioinformatic analyses. P.D.L and A.A. performed DLS analyses. M.M. carried out NTA and TEM experiments. All the authors participated in data analyses. AA wrote the manuscript. All the authors edited and approved the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Alfredo Ambrosone.

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