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REVIEW

Plant Extracellular Vesicles and Their Potential in Human Health Research, the Practical Approach

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Summary

Extracellular vesicles are small membrane particles (30-1000 nm) released by Bacteria, Eukaryotes and Archaea. They have been shown to play an important role in intracellular and intercellular communication, within and between kingdoms *via* transport of bioactive molecules. Thus, they can be involved in altering gene expression and regulation of physiological and pathological processes of the recipient. Their unique properties make extracellular vesicles a perfect candidate vector for targeted drug delivery or a biomarker. For a long time, animal and mainly mammal extracellular vesicles have been used in research. But for plants, there had been speculations about the existence of nanovesicles due to the presence of a cell wall. Today, awareness of plant extracellular vesicles is on the rise and their research has proved they have various functions, such as protein secretion, transport of bioactive molecules or defense against pathogens. Further potential of plant extracellular vesicles is stressed in this review.

Key words

Plant extracellular vesicles • Exosomes • Plant exosome-like nanovesicles • Drug delivery • Anticancer therapy

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Introduction

Extracellular vesicles (EVs) are nanoparticles surrounded by a lipid bilayer, which are naturally released and taken up by various cells. EVs form a heterogeneous group of vesicles produced by cells. The term "extracellular vesicle" includes exosomes, microvesicles and microparticles, whereas the greatest attention is paid to exosomes. Plant extracellular vesicles (pEVs) carry a heterogeneous cargo containing proteins, lipids, nucleic acids and other molecules, such as plant flavonoids etc. In recent years it has been discovered that plant extracellular vesicles participate not only in intracellular communication, but also in communication between individual organisms. Their specific structure allows controlled and protected transport of information molecules between cells. While extracellular vesicles of mammals (mainly human) have attracted great interest in last years, and are therefore highly characterized, plant vesicles have remained secluded and poorly investigated. A number of studies have dealt with the potential effects of pEVs on human health and with possibilities of their use in drug delivery. Studies focused on isolation methods and basic characterizations of pEVs are less common. In this review we summarize basic information about pEVS, as well as their physical and biochemical characteristics.

Methods of isolation

Despite the interest in pEVs has grown in recent years, a standardized protocol of isolation still does not exist. In order to take advantage of the potential offered by pEVs, it is necessary to choose the fitting method of isolation and a suitable output material. PEVs can be isolated from various types of plant material, including juice, apoplastic fluid, homogenized plant mixate or plant tissue cultures. For apoplastic fluid isolation, using the whole undamaged plant is necessary. For mixate preparation and tissue cultures, the whole plant, but also only stems, leaves, roots, seeds or saps can be utilized [1,2].

A commonly used method of EVs isolation from various plant materials is ultracentrifugation. Although alternative methods of pEVs isolation have emerged in recent years, including precipitation methods using polyethylene glycol, gel chromatography and immunoaffinity methods, ultracentrifugation remains the most widely used method of pEVs isolation [3,4,5].

Ultracentrifugation is a physico-chemical particles separating technique using relative centrifugal force. During centrifugation, each particle of the solution is exposed to centrifugal force due to the rotating device. Particles sediment depending on their physical properties, sample viscosity and the amount of centrifugal force. Given its viscosity and centrifugal force, each particle sediments proportionally to its molecular weight and to the difference between particle density and dispersion density [6]. The process of EVs isolation using differential ultracentrifugation begins with a series of purifying centrifugation steps designed to remove cells and their residues, apoptotic bodies and microvesicles. After the last purification step, a supernatant containing a high concentration of EVs is taken and purified by high-speed centrifugation (40 000-200 000× g). Since ultracentrifugation also sediments contaminants such as proteins, other vesicles or RNA-protein aggregates, there is a variation of this protocol, using ultracentrifugation with density gradient (sucrose or iodixanol) for separation of subtypes of pEVs. [1,7]. Density gradient centrifugation can yield high-quality EVs [8,9].

Ultracentrifugation is sometimes supplemented by ultrafiltration that consists in use of membranes with a specific pore size, through which only particles of certain size can pass. When filtration complements ultracentrifugation, filters with 0.22 μm or 0.45 μm pore size are used [10,11,12].

Storage and stability of plant extracellular vesicles

The stability and physiological functions of isolated pEVs are affected by their proper preservation and storage. Among ectosomes, exosomes and apoptotic bodies, exosomes have been proven as the most stable vesicles [8]. Extracellular vesicles were proven to increase the stability of their cargo molecules, hence, can increase bioavailability of bioactive compounds [12-14]. It was already shown that pEVs can resist enzymes in digestive fluids, so their content remains protected [15,16].

In optimal state, meaning freshly isolated, extracellular vesicles are usually recommended to be stored in -80 °C up to 1 year, or up to 3 months in -20 °C. Repeated freeze-thaw cycles can affect their structural identity and their biofunctions [17]. The study published by Richter *et al.* [18], has shown that the particle recovery was better in case of vesicles stored in -80 °C and 4 °C than in lyophilized vesicles. On the other hand, storage by lyophilization of EVs was recommended when analyzing EV biomarkers, where it is important to maintain vesicle integrity [18].

The storage and isolation method may be related to the formation of undesirable pEVs aggregates [19]. It was established that standard EVs isolation methods, such as ultracentrifugation, tend to induce aggregation of particles [20-22]. Although aggregation is often observed in mammalian EVs, in studies focused on pEVs the aggregation is rarely mentioned [11,23,24]. To warrant the biological activity for downstream applications, it is crucial to preserve physical characteristics of pEVs. Moreover, the presence of pEVs aggregates may make certain analytical methods difficult, or even impossible to use [19]. Bosch *et al.* [19] brought a solution by adding trehalose into starting material during the isolation, which suppressed the formation of aggregates [19].

Characteristics of plant extracellular vesicles

Plant EVs are believed to form a heterogeneous population of vesicles with different origin, including multivesicular bodies (MVBs), autophagosomes, vacuoles and exocyst-positive organelles (EXPOs) [25]. EVs can be characterized by their physical properties, such as size or the surface charge, as well as by their biological properties, predominantly by the content of different biomolecules (proteins, lipids and small RNAs). The characteristics of nanovesicles, such as negative

charge and the lipid and protein content of pEVs, can affect interactions, and also the resulting effects of pEVs on mammalian cells. To maintain pEVs physiological functionalities, it is necessary to keep their membrane intact. The integrity of EVs is sensitive to changes in temperature and pH [5]. Confirmation was provided by research aimed on altering the pH of pEVs solution derived from ginger, grape, grapefruit and carrot, leading to the change of vesicles size and charge [16].

The size of pEVs usually ranges between 30-1000 nm and it depends on the source material and the method of isolation. As an example of pEVs size differences we can mention extracellular vesicles isolated from grapes (*V. vinifera*; 400 nm), orange (*C. aurantium*; 105-396 nm), ginger (*Z. officinale*; 125-250 nm), broccoli (*B. oleracea*; 18-400 nm), carrot (*D. carota*; 100-1000 nm) or cotton (*Gossypium*; 150 nm) [2,13,14,16,21,26-31]. Plant EVs display zeta potential value from -100 mV-30 mV [32-35].

The lipid composition of vesicle membrane is an important component, as it is involved in intercellular interactions as well as maintaining vesicle stability in physiological and pathological conditions [23,31]. Mammalian vesicles are rich in cholesterol, ceramides, glycosphingolipids and phosphatidylserine [24,36,37]. In contrary, pEVs are composed mainly of phosphatidic acid, phosphatidylcholine, digalactosyldiacylglycerol, monogalactosyldiacylglycerol and phytosterols [2,38]. The membrane composition depends on the origin of pEVs. E.g. the membrane bilayer of ginger-derived EVs contains 25-40 % of phosphatidic acid, 25-40 % of galactosyldiacylglycerol and 20-30 % of monogalactosyl-diacylglycerol [39]. Different membrane composition was observed in EVs isolated from grapefruit, containing 45 % of phosphatidylethanolamine, 28 % of phosphati-dylecholine and only 2.5 % of phosphatidic acid [40]. The phospholipid group plays role in vesicle stability and in ability to address target cells [20,41]. Phosphatidic acid was established to participate in the mechanism of membrane fusion and in the release of plant nanovesicles [41,42]. It is a cell-signaling lipid, which is able to activate mammalian target of rapamycin (mTOR), as well as mitogen-activated protein kinase (MAPK) pathways, which could explain the observed effects of pEVs on growth and proliferation of mammalian cells [27,43]. Furthermore, the presence of phosphatidylcholine and phosphatidylethanolamine can enhance antioxidant, anticolitic and anti-inflammatory activities of extracellular vesicles [15,44,45]. It is assumed, there are some specific ligand-receptor pathways between pEVs and mammalian cells, however the mechanisms of delivery and pEVs internalization still remains insufficiently described.

A specific profile of proteins is found in pEVs, depending on the origin of vesicles and on the physiological condition of the plant. It is necessary to mention that to obtain homogeneous population of pEVs from several isolations, it is necessary to cultivate plants (the source of pEVs) in strictly defined and stable conditions, as the presence of individual components may vary depending on cultivating conditions [21,32,46]. In parallel to membrane lipids, proteins also participate in pEVs mediated intercellular communication [20,21,33,34]. The concentration of pEVs proteins is low, including cytosolic and also membrane proteins, such as ion channels and transporters within the membrane [35]. Proteins found in plant vesicles are mainly metabolic enzymes, actins, annexins, aquaporins, coatomers, clathrins, Rab proteins, heat shock proteins (HSP), syntaxins, patellins and ubiquitins [38,47]. A proteomic study made on *C. limon*-derived EVs identified 580 proteins of which 56.7 % matched proteins found in mammalian vesicles [34]. Due to small number of proteomic analysis performed on pEVs there is not many standardized plant EVs markers known. Pinedo *et al.* (2021) summarizes current knowledge about plant EVs markers, including HSP70 (Heat shock protein 70), GAPDH (Glyceraldehyde 3 phosphate dehydrogenase), TET8 (tetraspanin 8) and S-adenosyl-homocysteinase [48]. The further investigations are needed in this area.

As already mentioned, plant extracellular vesicles can serve as a transport mechanism for small RNAs (sRNAs). EVs derived from different plants can contain various small RNA molecules, usually 21 to 25 nucleotides long which are able to regulate biological functions, such as cross-kingdom communication *via* targeting genes in mammalian genome [33,40,49,50]. For example, the investigation of ginger-derived vesicles cargo revealed 125 different miRNAs with 15-27 nucleotides in length. 24 of these small RNAs could potentially target and regulate human gene expression [26]. The mechanism of plant extracellular vesicles RNAs uptake by mammalian cell is not yet fully understood. However, recent finding prove that pEVs are able to deliver sRNAs into target cells [32,47,49,51]. Recently, RNA-binding proteins, such as Argonaut protein 1 (AGO1), DEAD-box RNA helicases and annexins, were found in pEVs. These pEVs-associated

proteins specifically bind to pEVs-enriched small RNAs and colocalize with multivesicular bodies in the cell, where the complex of RNA-binding protein-sRNAs is packed into plant extracellular vesicles [52]. Although the RNAs of mammalian vesicles have been well characterized, the content and the functions of RNAs in pEVs also need further investigation.

In addition to other compounds, pEVs can carry secondary metabolites depending on the source of the vesicle. These naturally occurring active molecules having antioxidant, antitumor or immunomodulatory effects can be transferred *via* pEVs and thus, they can promote some of their beneficial effects in target cells. Plant secondary metabolites are only specific for pEVs, giving them the additional value. Studies have been published to highlight the promotion of pEVs effects, such as reducing inflammation, supporting the intestinal microflora growth or preventing cancer and infection, due to the application of secondary metabolites carried by them [15,35,53]. Great attention is paid to *Z. officinale* (ginger), which is well known for its beneficial effects on human health. Shoagaol and gingerol were identified in ginger-derived EVs and they were shown to be crucial in induction of nuclear factor erythroid 2-related factor 2 (Nrf2), modulating drug metabolism, hepatocyte homeostasis and cell-cycle progression in liver [13,54]. Naringin, and also its metabolite – naringenin, are flavonoids of citrus fruits. Naringenin has anticancer, anti-inflammatory and antioxidant effects [15,53,55]. Both molecules were identified in grapefruit-derived vesicles [15]. Also ascorbic acid, popular antioxidant known for the reduction of cancer and cardiovascular diseases risk factors, was found in extracellular vesicles derived from citrus fruits and strawberries [32]. And finally, broccoli-derived extracellular vesicles carried sulforaphane, which was shown to participate in a prevention of mouse colitis by AMPK-mediated induction of tolerogenic dendritic cells [27]. Due to the presence of secondary metabolites in pEVs, it is possible to predict beneficial effects of these vesicles on mammalian cells, although further research is needed.

Plant extracellular vesicles and their effects on mammalian cells

It was originally assumed that extracellular vesicles are only involved in eliminating of unnecessary compounds from the cell. Today it is clear, that EVs mainly participate in the signal transmission between cells and organisms. It is important to point out that human, as other mammals, consumes pEVs every day as a part of his diet. However their effects are not yet completely understood, it is assumed, they could be involved in regulation of various physiological processes in human body.

As already mentioned, pEVs are involved in the information transfer not only between plant cells, but also between plant and animal cells. A great attention is paid to this ability as it brings new potential nanocarrier, especially for the transfer of therapeutics into mammalian cells. While some studies observed pEVs antitumor effects, due to their antiproliferative and immunomodulatory properties, other studies suggest pEVs as an efficient system of transportation for small molecules with therapeutic effects [14,24,26,43,51,56]. PEVs were shown to have various potential health benefits through animal models and *in vitro* studies, which are summarized in this chapter.

Immunomodulatory effects of plant extracellular vesicle

It is not yet entirely clear what effects can plant extracellular vesicles potentionally have on human health, however over the last few years, a number of attempts have been made to show that pEVs are able not only to enter mammalian cells, but also to regulate their physiological processes, including inflammation. Even though several positive effects were observed, the exact mechanism of action often remains unexplained.

Inflammation is part of the immune response of the organism. If the inflammatory condition is not monitored, it may develop into acute or chronic inflammatory disease. During recurrent inflammatory disease, such as colitis, intestinal dendritic cells may be deprived of their tolerogenic properties. It can be the leading cause of many diseases, such as diabetes, colitis, obesity, cancer etc. Current studies have shown that plant vesicles (originating in ginger, broccoli and grape) can improve intestinal diseases in mice by affecting inflammatory reactions and by inducing healing processes [2,16,40,43,51,56]. It was presented that broccoli-derived nanovesicles can be involved in maintaining intestinal immune homeostasis. The oral administration of broccoli EVs to mice with DSS (dextran sulfate sodium)-induced colitis led to an improvement of the disease. Broccoli nanovesicles were taken up by dendritic cells in mouse colon and mesenteric lymph nodes. Dendritic cells can be differentiated into immunogenic or tolerogenic cells,

depending on the stimuli. In this study, broccoli nanovesicles were shown to mediate the activation of adenosine monophosphate-activated protein kinase (AMPK) in dendritic cells, which led to the induction of tolerant dendritic cells. They also observed, that the expression of inflammatory interferon gamma (IFN-γ), interleukin (IL)-17A and tumor necrosis factor alpha (TNF-α) significantly increased after the administration of DSS in colonic tissue. The subsequent administration of broccoli EVs led to a decrease of DSS-induced IFN-γ, IL-17A and TNF-α and to an increase of anti-inflammatory IL-10 [27]. In another study, phosphatidylcholine lipid content in EVs derived from ginger facilitated the absorption of vesicles by the mice gut bacteria *Lactobacillus rhamnosus*. It stimulated a cascade of reactions, leading to the inhibition of proinflammatory cytokines IL-1β, TNF-α, and in increase of anti-inflammatory IL-22 production in colon of DSS-treated mice. The application of pEVs also induced gut healing of colitis-induced mice *via* the miRNA content of ginger vesicles. It was also observed, that the *Lactobacillus rhamnosus* is required for better protection of mice against DSS-induced colitis, as the germ-free mice did not experience a reduction in colitis severity. The administration of ginger nanovesicles also led to an increase in homeostasis between the immune system and gut microbiota by adjusting the microbiota composition [41]. Moreover, the contribution of grapefruit-derived vesicles on the enhancement of anti-inflammatory effects in mice has been observed. Grapefruit vesicles were taken up by intestinal macrophages, leading to a significant improvement of DSS-induced mouse colitis. Vesicles were internalized by macrophages *via* pinocytosis and clathrin-dependent endocytosis, resulting in the reduction of the expression of pro-inflammatory IL-1β and TNF-α, and in an increase in expression of anti-inflammatory proteins, namely heme oxygenase-1 (HO-1) and IL-10 [15]. Ju *et al.* investigated grape-derived extracellular vesicles, which were able to migrate into the mice gut, where they were taken up by the gut stem cells, leading to the proliferation and up-regulation of Wnt pathway genes. Under physiological conditions, vesicles participated in intestinal homeostasis regulation through the induction of Lgr5+ (leucine-rich repeat-containing G-protein coupled receptor 5) stem cells. Under pathophysiological conditions, such as DSS-induced colitis, oral administration of grape EVs activated BMI1+ stem cells, resulting in the regeneration of the intestinal epithelium. The group observed decreased mortality in mice given grape-derived vesicles [57].

In another study, pre-treatment of human endothelial cells with EVs originating in blueberries was able to revert ROS production and also TNFα-induced cell death in endothelial cells. Moreover, the pre-treatment of endothelial cells with blueberry EVs reversed the effects of TNF-α-induced mRNA expression of IL-6, IL1RL1, MAPK1, ICAM1 (intercellular adhesion molecule 1) and TLR8 (toll-like receptor 8). Blueberry-derived EVs contained miR-162, miR-156e and miR-319d, which can potentially target various genes responsible for their anti-inflammatory and antioxidant effects [33].

Investigation of four pEVs (ginger, carrot, grapes and grapefruit) showed that macrophages treated with ginger pEVs significantly enhanced HO-1, IL-6 and IL-10 expression. In contrary, macrophages treated with carrot-pEVs tend to induce IL-10 only. As the Wnt/TCF4 signaling pathway participates in gut homeostasis, the group examined effects of four investigated pEVs on Wnt/TCF4 (transcription factor 4) pathway. The oral treatment of mice with pEVs led to an induction of intestinal Wnt/TCF4 activation [16].

Xiao *et al.* investigated miRNA composition among the 11 samples of pEVs. Many of miRNAs were predicted to target mammalian genes encoding inflammatory factors, such as IL-6, IL-5, IL-1 or IL-2, suggesting pEVs-derived miRNAs are potentially able to directly target and regulate human immune processes [58].

NLRP3 inflammasome activation is a physiological process involved in the activation of autoinflammatory, metabolic and neurodegenerative diseases. Cheng *et al.* examined the effects of pEVs isolated from various plants (grapefruit, aloe vera, cilantro, turmeric, garlic, dandelion, cactus, lavender and ginger) on NLRP3 inflammasome activation. Only ginger-pEVs were shown to prevent the activation of NLRP3 inflammasome in murine macrophages by inhibiting IL-1β and IL-18 release [59].

Antitumor effects of plant extracellular vesicles

Current chemotherapeutic approaches face two main problems. First of them is an insufficient target specifity of the drug and second, anticancer drugs are often very toxic also for healthy cells of the organism. Both problems could be solved by using naturally biocompatible pEVs, that not only reduce the systemic toxicity of loaded drug, but they can also be specifically

targeted into tissue of interest, and they can naturally have antitumor effects [19,54,60].

It was found that exosomes isolated from *C. limon* juice could inhibit the growth of cancer cells *in vitro* without affecting healthy cells. Vesicles regulated proapoptotic and antiapoptotic pathways, by increasing levels of proapoptotic molecules Bad and Bax, along with decrease of survival molecules, including survivin. To confirm these findings obtained *in vitro*, labeled exosomes were intraperitoneally administered to mice with chronic myeloid leukemia. Accumulation of pEVs at the tumor site was observed 15 min after I.V. administration. Tumor growth was strongly suppressed by apoptosis regulation and by angiogenesis inhibition at the tumor site. Moreover, three weeks intratumoral administration of lemon-derived vesicles led to the tumor growth suppression in mice. It was associated with an increase of proapoptotic molecules through TRAIL/DR5 (TNF-related apoptosis-inducing ligand/death receptor 5) signaling and through the reduction of proangiogenic cytokines VEGF-A (vascular endothelial growth factor A), IL-6 and IL-9 [34]. It was also demonstrated, that lemon-derived EVs can be internalized by human gastric cancer cell lines (AGS, BGC-823, SGC-7901) in 2D and 3D models. Lemon vesicles caused S-phase arrest in all three cell lines and they inhibited their growth. The growth suppression was associated with down-regulation of caspase 3 and up-regulation of cleaved caspase 3, which propagates an apoptotic signal through enzymatic activity on downstream targets. The pEVs treatment also promoted GADD45A expression in all cell lines. GADD45A is a protein involved in cellular response to physiological and environmental stressors, including DNA repair, cell cycle control, and it is considered a tumor suppressor [50]. Stanly *et al.* clarified that extracellular vesicles isolated from four *Citrus* species (*C. limon*, *C. sinensis*, *C. aurantium* and *C. Paradisi*) can specifically inhibit the proliferation of lung, skin and breast cancer cells, with no effect on non-cancer cells. Grapefruit-derived vesicles were shown to participate in the arrest of cell cycle in G2/M checkpoint, associated with reduction of cyclins B1 and B2 expression and the up-regulation of p21, which regulates G2/M phase transition. They also observed that pEVs treatment led to an activation of PARP-1, leading to an apoptosis induction in cancer cell lines [61].

In another study EVs derived from *P. ginseng* were able to induce M1-like polarization in macrophages through the activation of Toll-like receptor (TLR)-4/MyD88 (myeloid differentiation antigen 88) signaling pathway, leading to an increase of ROS production. EVs-treated macrophages were able to induce apoptosis in mouse melanoma cells, by increasing caspase 3/7 expression. The *in vivo* experiment showed that the tumor growth was significantly suppressed from day 14 of the administration of pEVs [62].

It was also proven, that pEVs from ginger could be involved in the expression regulation of proteins essential for tumor growth and development, including the expression of cyclin D or activation of cyclic guanosine monophosphate (cGMP), which participates in tumor suppression. In the mouse model of colitis-associated cancer the level of PKG (cGMP-dependent protein kinase) increased, which is involved in prevention and treatment of colon cancer [63]. Another research group investigated extracellular vesicles from *Dendropanax morbifera* and their effects on tumor and healthy cells. Stem- and leaf-derived pEVs of *Dendropanax* inhibited melanogenesis by reducing the expression of tyrosinase-related proteins (TRP): TYR (tyrosinase), TRP-1, TRP-2 and MITF (microphthalmia-associated transcription factor) in mouse melanoma cells. The melanogenesis was more suppressed by leaf-derived pEVs. Moreover, in human epidermis model, leaf-derived pEVs exerted a stronger inhibitory effect on melanin production that arbutin, a TYR inhibitor [64]. Another group has shown that *Dendropanax*-derived and *pinus*-derived EVs had toxic effect on breast and skin cancer cells, but not on healthy cells. They demonstrated that co-treatment with both of these EVs had synergic effect against tumor cell growth and it also led to the improvement in apoptosis, but the mechanism remains unexplained [65]. The same group also investigated the anti-metastatic effects of pEVs derived from *Dendropanax morbifera* using a cancer metastasis model based on 3D microfluidic system that mimics the *in vivo* tumor environment. They observed the concentration-dependent suppressive effects of pEVs on *in vitro* model of cancer-associated fibroblasts (CAFs), which are mediators of cancer metastasis. *Dendropanax* vesicles caused a decrease in the survival rate of CAFs and changes in gene expression of migration related-genes, such as TGF-β2 (transforming growth factor-beta 2), PDGFC (platelet-derived growth factor C) and ILK (integrin-linked kinase), and also in extracellular matrix-related genes, including PLAU (urokinase-type plasminogen activator), CD44, COL3A1 (collagen type

III alpha 1 chain), COL4A6, ITGA6 (integrin subunit alpha 6) and ITGA 11 [66].

Potesta *et al.* studied nanovesicles isolated from *Moringa oleifera* seed extract. They demonstrated that pEVs contain specific miRNAs targeting apoptosis-related human genes, as well as anti-proliferative genes of tumor cell lines. The increase of apoptosis levels was associated with a decrease in B-cell lymphoma 2 protein expression and reduced mitochondrial membrane potential. The effects observed after pEVs treatment were similar to the effects of the treatment of only RNAs isolated from extracellular vesicles [47].

Other effects of plant extracellular vesicles

In recent years, it has been shown that pEVs have not only antitumor and anti-inflammatory effects, but also a number of other beneficial effects, that are mentioned in following text.

It is thought, that orange-derived EVs could ameliorate obesity. The *in vitro* treatment of CACO and HT29 cells, used as a model of *in vivo* intestinal barrier with pEVs, decreased the amount of triglycerides and increased their association with chylomicrons. These data were confirmed by *in vivo* experiment, where mice on high-fat and high-sucrose diet were treated with orange-derived EVs, which were shown to accumulate mainly in jejunum of mice, leading to an enlargement of villus size. It also led to an increase of triglycerides association with chylomicrons [2].

Experiments with melon-derived vesicles revealed miRNAs, lipids and proteins that are involved in fruit ripening, sugar metabolism and ROS degradation in plants. They also internalized into human intestinal cells, promoting proliferation [67].

The *in vitro* and *in vivo* studies have suggested that ginger-derived EVs are selectively taken up by *P. gingivas*, which is a key pathogen in the periodontitis development. The treatment with pEVs led to *P. gingivas* growth inhibition. Lipids and miRNAs of ginger EVs also inhibited gingivas virulence-related genes expression, such as AraC (arabinose operon regulatory protein), OmpA (outer domain protein A), HagA (hemagglutinin A), as well as bacterial attachment and invasion of gingival epithelial cells. Thus, ginger pEVs can ameliorate or prevent chronic state of the disease and minimalize the inflammation [23].

Adipose-derived mesenchymal stem cells treated with strawberry pEVs, stimulated with H_2O_2, showed decreased levels of ROS and reduced cell death *in vitro* [32].

Yepes-Molina *et al.* [30] dealt with EVs derived from broccoli and their possible use in transdermal applications. After the incubation of keratinocytes with EVs, the cell enlargement occurred, which could be due to the uptake of large amount of vesicles. EVs were able to penetrate the stratum corneum and they were able to migrate into inner layers of the skin. This finding suggest that pEVs might be appropriate candidate for transport of transdermal drugs, as well as active chemicals that are part of cosmetic products [30].

Extracellular vesicles derived from wheat were shown to induce skin regeneration by triggering migratory and proliferative actions in epithelial, endothelial and dermal fibroblasts *in vitro*. Moreover, the expression of collagen type I was significantly elevated. Wheat vesicles were able to trigger tube-like structure formation in umbilical vein endothelial cells, which suggested they have the capability to induce vascular formation during the wound healing processes [31].

Plant extracellular vesicles as drug delivery tool

Goia *et al.* [26] summarizes specific properties of pEVs that are useful to overcome the limitation of traditional administration routes of various healing compounds. (1) EVs can improve biological availability of transported compounds and (2) they are able to transport drugs in high concentration. Due to their unique size and composition, (3) they can target specific organ, which improves selectivity, drug delivery, safety and efficiency of the transportation. (4) EVs can also increase the permeation (through barriers etc.) and retention. Moreover, they can also (5) perform passive targeting to tumors and (6) reduce the side effects that occur in other types of therapies [26]. Even though a several researches were done, there were no cytotoxic effects of pEVs observed on healthy cells. Plant vesicles show a great biocompatible nature, allowing them to enter various cells without triggering an immune response. Wang *et al.* quantitatively measured several markers, such as pro-inflammatory cytokines, serum levels of liver enzymes, including aspartate transaminase (AST) and alanine transaminase (ALT). Mice were orally treated with grapefruit-derived nanovesicles and with liposomes. Pro-inflammatory cytokine levels, ALT and AST levels were elevated in liposomal-treated mice, but no elevation was observed in mice treated with grapefruit nanovesicles. Moreover, no pathological modifications or

necrosis were observed in histological samples of spleen, liver and lungs of mice treated with pEVs [51]. Similarly, Zhang *et al.* observed the lower impact on viability of grapefruit nanovesicles treated RAW 264.7 and colon-26 cell lines, in comparison to liposomes [14]. These findings suggest that artificially synthesized nanovectors can induce immune responses in the host organism, in contrary plant EVs with non-immunogenic, but specific actions that help to establish cell homeostasis. It gives them the ability to cross physiological barriers, which are often impermeable for other drug carriers or drugs themselves. It makes plant extracellular vesicles excellent candidates for the improvement of therapeutic agents delivery. PEVs may be internalized by a non-specific processes, such as micropinocytosis or macropinocytosis, or by a specific processes dependent on ligand-receptor interaction. It is also assumed that the internalization of pEVs may occur *via* clathrin-dependant and clathrin-independant pathways. Process of internalization and cargo transportation by pEVs is not yet fully understood and more research is needed in this area [68].

There are two types of plant nanoplatforms used for drug and therapeutic delivery, with one being natural pEVs and the other being pEVs-derived nanocarriers, which are usually formed by using pEVs membrane. PEVs-derived nanovectors offer the possibility of tailoring their surface, which can broaden the scope of their natural target specifity. The specific molecules can be immobilized on the pEVs-derived nanovectors to develop displays more compatible to cellular surface receptors [68]. Recently, a few studies have dealt with bioengineering of natural pEVs to improve their uptake by mammalian cells. The surface modification can be done by genetic engineering or by chemical modifications. Wang *et al.* examined the drug delivery potential of grapefruit-derived vesicles conjugated with methotrexate (MTX), an anti-inflammatory agent and immunosuppressant. The orally administrated vesicles conjugated with MTX were able to target intestinal macrophages of mice with DSS-induced colitis, leading to an improvement of colon shortening and body weight loss. The presence of pEVs significantly enhanced the anti-inflammatory effects of MTX, and also MTX-induced side effects were decreased. In contrary, commercially available liposomes were much less efficient at transfecting intestinal macrophages and the uptake of liposomes by intestinal macrophages was hardly visible [15]. As activated immune cells are capable to target inflammatory sites, another group coated grapefruit-derived vesicles with inflammatory related receptor enriched membranes of activated leukocytes. Plant nanovesicles were than enhanced for homing to inflammatory breast and colon cancer tissues. The I.V. injection of grapefruit-coated vesicles loaded with doxorubicin significantly enhanced the inhibition of breast and colon tumor growth [69]. Teng *et al.* investigated the role of miR-18a loaded into grapefruit-derived vesicles in the induction of mice liver M1 macrophages. Encapsulated miR-18a mediated the activation of macrophages IFN-γ by targeting IRF2, leading to the induction of IL-12, which activates natural killer (NK) and natural killer T (NKT) cells, inhibiting a liver metastasis of colon cancer [70]. Similarly, the film of lipids extracted from grapefruit-derived vesicles was coated with folic acid. Vesicles were enhanced for targeting folate receptor positive GL26 brain tumor. To enhance the capacity to carry RNAs, the toxic polyethylenimin was added into pEVs. The toxicity was eliminated by using grapefruit vesicles lipids. Intranasal administration of therapeutic miR17 loaded into modified vesicles led to a rapid delivery of miR17 into the brain, where it was selectively taken up by tumor cells. The groups have proven that miR17 mediated the induction of NK cells through down-regulation of MHCI (major histocompatibility class I) expressed in tumor cells. Treated mice provided delayed brain tumor growth. Besides miR17, also hydrophobic curcumin, Zymosan A and proteins were transported into different cell types *via* grapefruit nanocarriers [29]. The same group evaluated the absorption of grapefruit nanocarriers by different cell types. Each of the selected animal cell type was cultured with labeled grapefruit-derived nanocarriers. Their presence in cells was examined by confocal microscopy and FACS (fluorescent activated cell sorting). The results showed that pEVs were internalized in mouse glioma cell line (GL26), colon metastases (SW620), mouse colorectal cancer (CT26) and in breast cancer (4T1) cells. Also 20 % of B-cells and 14 % od T-cells absorbed grapefruit nanovectors within 12 h. Grapefruit-derived nanocarriers loaded with paclitaxel and folic acid were also able to transport the drug into mice tumors and to reduce tumor size with a high efficiency [51].

Li *et al.* investigated ginger-derived EVs functionalized with arrowtail pRNA-3WJ and folic acid for ligand display, which were able to deliver surviving siRNA to a KB cancer model. Also increasing folate-arrowtail displaying ratio on nanocarriers led to an enhanced binding to KB cancer cells. In mice they

observed tumor growth inhibition after I.V. administration of designed nanocarriers [71].

Surface proteins were reported to be crucial in the pEVs uptake by liver cancer cells (HepG2). EVs derived from garlic were modified by removing all the surface proteins, leading to a significantly lower uptake compared to the uptake of non-modified vesicles, confirming that the surface proteins participate in endocytosis of plant extracellular vesicles. Also blocking the CD98 receptors on liver cancer cells led to the reduction of garlic-derived vesicles uptake [60].

Similarly, doxorubicin loaded into nanocarriers prepared from ginger-pEVs lipids was transported into mice colon tumor cells. EVs were adjusted to achieve accurate specifity by incorporating folic acid into their membrane. Folic acid is a ligand of FR-receptors that are over-expressed in many tumors. PEVs successfully inhibited tumor growth, along with the reduction of systemic drug toxicity and with the prolongation of circulation time [72].

Clinical trials

Several clinical trials were done on mammalian exosomes, but only recently plant extracellular vesicles entered clinical trials as well. Despite the growing interest in plant extracellular vesicles, no clinical trials results were published yet. To our knowledge, there are currently two active clinical trials aimed on plant extracellular vesicles and their potential effects on human health. One of them is investigating the ability of plant exosomes to deliver curcumin into normal and colon cancer cells. Curcumin is a natural phenol originating in rhizomes of *Curcuma longa* that has anti-inflammatory, antioxidant, antineoplastic and chemoprotective effects. Its hydrophobic properties (and therefore poor solubility and preferential interaction with membranes) remain major barrier to its use. Curcumin has been also shown to interfere with colon carcinogenesis in a variety of chemical and genetic models. No results of the clinical trial have been published yet (ClinicalTrials.gov Identifier: NCT01294072). The second ongoing trial is focused on the ability of edible plant exosomes to prevent mucositis associated with head and neck cancer chemoradiation treatment. The purpose of the study is to investigate the ability of grape exosomes to prevent oral mucositis, which is a common complication of cancer chemotherapy. Also, effects of grape vesicles on the production of cytokines and immune responses to tumor exosomal antigens, metabolic and molecular markers in patients will be examined (ClinicalTrials.gov Identifier: NCT01668849).

Conclusions

In last few years, rising evidence of the specific properties of pEVs, related mainly to their biological content, healing effects and drug delivery potential, makes the topic of plant extracellular vesicles very attractive. The possibility of isolating pEVs from a large amount of material (including sterile plant tissue cultures), the presence of naturally present substances with potential beneficial effects on human health, the biocompatibility with mammalian cells and the natural ability to target specific cell types makes the greatest advantages of plant-derived vesicles.

However, even with a new studies constantly emerging, further research of plant vesicles is still needed. Due to the absence of standardized protocol of isolation, obtained results often vary, depending on the plant source, isolation method or the physiological condition of the plant. By using the same isolation method, the results in particle size and charge are can be different, so is the content of isolated nanoparticles. Also the lack of information about proteomics often impedes researchers, as there are no many specific pEVs protein markers. The use of pEVs as therapeutics or dietary supplement requires further detailed analysis, *in vivo* studies and also clinical trials to provide more detailed information about the effects, stability and properties of plant extracellular vesicles.

Conflict of Interest

There is no conflict of interest.

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