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Plant derived exosome- like Nanovesicles: an updated overview

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ABSTRACT

Exosomes are nanovesicles in the size range of 30–150 nm, produced by mammalian and plant cells. They have the lipid bilayer enclosing a unique mix of biologically active components including proteins, nucleic acids, metabolites and lipids, that depend on their source of origin. The plant derived nanovesicles are gaining considerable research attention due to their ability to be absorbed from the dietary sources. Their bioactive components impart many therapeutic properties to them such as anti-tumorigenic, anti-oxidative, anti-inflammatory, hepatoprotective effects and tissue regeneration. The possibilities of using plant derived exosomes for drug delivery is also promising. This review focusses on the biogenesis and biological nature of exosomes derived from plants and describes their various inherent therapeutic properties. It sheds light on the recently developed methods to study them. The existing challenges in the exosomal research as well as the future prospective are highlighted. Overall, it is an update on the advancements on the research in plant exosomes which can provide a replacement for toxic synthetic drugs and possibilities for disease therapy.

Introduction

Exosomes are membrane bound vesicles secreted into the extracellular space in both mammalian and plant cells mainly through the Multivesicular body (MVB) sorting pathway. They are biological nanostructures of size range 30–150 nm, with the lipid bilayer enclosing unique biologically active ingredients. Previously, these vesicles were considered as artifacts or as delivery vehicles for the removal of waste from the cells (H Rashed et al. 2017). But currently their role in cell-cell communication through transfer of biologically active material and even gene regulation have been established (Ju et al. 2013; Zhang et al. 2016a). The plant derived exosomes began to claim attention once their crucial role in plant immunity against pathogen invasion was identified (An et al. 2006; Nielsen et al. 2012). Another unique aspect of plant derived exosomes is their ability to establish cross kingdom communication through absorption from dietary sources (Mu et al. 2014). While mammalian exosomes are highly regarded for their role as biomarkers in diseases (Samanta et al. 2018), research interest on the plant derived nanoparticles is mounting due to their innate therapeutic properties that can be translated for human health (Zhang et al. 2016b). The biological constituents of the exosomes which includes proteins, miRNA, RNA and metabolites are unique in each case and depends upon the cell of origin (Pegtel and Gould, 2019).

In addition to the benefits from nutritional uptake of the plant derived exosomes, they have great potential for efficient delivery of therapeutics. Plant-based, non-immunogenic, non-toxic nano-carriers for delivery of drugs and other substances to the target cells are always sought-after. Considerable amount of research effort is directed towards identifying and developing the nano-carrier systems out of plant-exosomes (Man et al. 2020; Claridge et al. 2021; Dad et al. 2021; Karamanidou and Tsouknidas, 2021). Growing evidence suggest that plant exosomes will find use in different fields including food and pharmaceutical as biologically active substances.

In this review we endeavor to summarize the recent knowledge on exosome biology, its biogenesis, composition and purification. We then proceed to focus on the various innate therapeutic properties of exosomes extracted from different plant sources. The various challenges involved in purification, scale up and in translation of these biological properties are also discussed.

Biogenesis of exosomes

Exosomes are a type of extracellular vesicles, which are lipid membrane enclosed nano particles enclosing numerous biologically active components. Exosomes are derived from the endocytic pathway, when membrane invagination during late endocytosis results in intraluminal vesicles (ILVs) within Multivesicular Bodies (MVBs) (Zhang et al. 2019). The ILVs are generated when the early endosomes integrates with the surrounding membrane layer. They encapsulate exosomes within the MVBs, which subsequently fuse with plasma membrane and release the

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contents into the extracellular space (Pegtel and Gould, 2019). The contents of the exosomes are not random and are packed in a tightly regulated process, due to which the proteins often contain post-translational modifications such as ubiquitination (Sahu et al. 2011; Joshi et al. 2020). Exosomes are extensively synthesized by mammalian cells and the cell machinery involved in the process is well-studied. The endosomal sorting complex required for transport (ESCRT) plays a crucial role in transport and sorting of the cargo into the ILVs. ESCRT includes four complexes (ESCRT 0, -I, -II, -III) which in turn are composed of multiple proteins. ESCRT-0 plays a key role in isolating the ubiquitinylated proteins by binding and grouping them on the surface of the cell membrane. ESCRT-0 is composed of two subunits namely Hepatocyte growth-factor regulated tyrosine kinase substrate (Hrs) and Signal Transducing Adaptor Molecule (STAM) which can bind with the ubiquitinated cargo. It also interacts with TSG101 protein of ESCRT-1 and brings the complex to the site. ESCRT-1 is a heterotetrametric complex, responsible for sorting of the cargo into the MVBs and it causes a concentration of the cargo proteins through interaction with ESCRT-II complex. The ESCRT-II is also a hetero-tetramer composed of the proteins EAP45, EAP30 and EAP 20. ESCRT-III is an oligomer of Charged multivesicular body protein (CHMP) and is the most important functional component in driving the release of exosomes. Together with an associated ATPase called VpS4p/SKD1, it triggers formation of membrane binding spirals causing neck constriction and release of the vesicle buds from the membrane. This ATPase provides the energy for the whole process of release of exosomes. The vesicles interact with a specific combination of N-ethylmaleimide sensitive factor attachment protein receptors (SNARE) and get released (Colombo et al. 2013; Gurung et al. 2021).

The endocytic pathway in plant cells is not as clearly characterized as in mammalian cells. The proteins are internalized by invagination, budding and formation of transport vesicles at the plasma membrane and are directed as Early Endosomes (EE) to the Trans Golgi Network (TGN), a subunit of which matures into MVBs (An et al. 2007). Most of the ESCRT complexes responsible for the formation and release of ILVs of MVBs are highly conserved in plants as well and perform similar functions. Homologues of ESCRT-I, -II and -III have been identified in *Arabidopsis* genome, with exception of ESCRT-0. Translocase of outer-membrane (TOM)− 1 proteins are present in almost all higher eukaryotes and they function in conjunction with ESCRT complexes during ILV formation. Orthologue of TOM-1, namely TOM-I like proteins (TOL) have been recognized in *Arabidopsis*. Owing to their conserved N-terminal VHS domain and ability to localize in early endosomal structures closer to plasma membrane, it is believed that they substitute for the ESCRT-0 in plants. FREE-1 is a unique ESCRT component in plant, which localizes to the MVBs and interact with ESCRT-I causing its incorporation into MVBs. Its ability to bind ubiquitin as well, hints on additional role for this protein in plant exosome genesis (Cui et al. 2016). Apart from the few differences, the mechanism for exosomes generation is conserved in plants and are generated mainly by the activity of ESCRT protein complexes in sorting and maturation of MVBs (Colombo et al. 2014; Kowal et al. 2014).

Plants employ a unique pathway as well to produce exosomes. Double membranous structures resembling autophagosomes termed as EXPO (EXocyst Positive Organelles) have been identified in *Arabidopsis* and Tobacco. Though they share similarity with autophagosomes, they follow endocytic pathway or fuse with any lytic compartments. They fuse with plasma membrane and release single membraned vesicles into the cell wall. These vesicles can be considered as exosomes produced by EXPO unlike in animals where it happens exclusively through MVBs (Wang et al. 2010). The difference in the exosome generation pathways between mammalian cells and plant cells is highlighted in Fig. 1.

Biological composition of exosomes

Exosomes can be considered as membrane enclosed signalosomes which carry their unique biologically active cargo. Lipids, proteins, miRNA and secondary metabolites are among the important components of these extracellular vesicles. The composition of the exosomes being unique to each plant, determines the bio-activity and the functionalities such as uptake, targeting and altering gene expression.

(a) Proteins:

Typically the protein concentration in plant exosomes is quite low and most of them identified are of cytosolic origin. Few membrane proteins such as chloride channel proteins and aquaporins have been identified in the proteomic studies on ginger exosomes (Zhang et al. 2016a). Proteomic studies on apoplastic fluid of *N.tabacum* identified 40 S ribosomal proteins S4, S6 and a 85KDa hydrolase ß-xylosidase/alpha -L-arabinofuranosidase 2 like protein as exosome specific protein. Cell wall related proteins and stress related proteins dominated in the studies on nanovesicles from *Craterostigma plantagineum* (Woith et al. 2021). The proteomic studies on the plant exosomes are limited by the contaminations from abundant enzyme proteins such as RuBisco and lack of proper databases for comparison (Woith et al. 2021). Moreover the proteomic concentration of the plant exosomes change drastically in response to abiotic and biotic stresses (Rutter and Innes, 2017).

(b) Secondary metabolites:

Secondary metabolite analyses in several plant exosomes revealed that they contain mostly lipophilic molecules such as curcuminoids and chlorophylls (Woith et al. 2021). Norditerpene alkaloids such as aconitine, mesaconitine and hypaconitine have been identified in Aconiti tuber. Flavanoids such as naringin, naringenin and Shogaol were identified in the vesicles from grape fruit and ginger respectively (Wang et al. 2014; Zhuang et al. 2015). The studies on the secondary metabolites in exosomes is often biased by the acidic/basic conditions of the buffer system used for extraction. The presence of secondary metabolites in the exosomes can attribute for its anti-microbial activity and its increased synthesis during infections (Woith et al. 2021).

(c) Lipids:

Lipids are essential components of the exosomes as they play a critical role in their uptake by specific cells. The plant exosomes predominantly contain phospholipids in contrast to their mammalian counterparts which are composed mostly of cholesterol and sphingomyelin. HPTLC studies on plant exosomes recognized many phospholipids, which play crucial roles in nanovesicle stability and in targeting specific cells (Zhang et al. 2016b). Nanovesicles extracted from grapes contain about 98 % phospholipids and 2 % of typical plant lipids such as mono and di-galactosyl diacylglycerol (Ju et al. 2013). Exosomes isolated from grapefruit were enriched with phosphatidyl-ethanolamine and phosphatidylcholine. Nano-vectors constructed with grapefruit particle derived lipids have been successfully used for delivery of chemotherapeutic agents and siRNA (Wang et al. 2014). The lipid composition of the exosomes serve as signals for their selective absorption into certain gut bacteria. PA (Phaosphatidic Acid) lipids are known to maintain the duration and accumulation of these particles in the gut and modulates their uptake by members of *Lactobacillaceae* (Teng et al. 2018). Studies on ginger derived edible nanoparticles also indicate that the lipid composition of these nanoparticles affect the recipient cells localized in gut and liver (Zhuang et al. 2015).

(d) MiRNAs:

MicroRNAs are short non coding RNAs of average size of about 22 nt capable of regulating gene expression by specifically binding and cleaving the target mRNAs or by inhibiting the translation process. Previous reports suggest that miRNAs are an integral part of exosomal cargo in plants (Xiao et al. 2018). Cross-kingdom functions of plant exosomes can be attributed to the presence of biologically active factors such as miRNAs (Zhang et al. 2012; Chin et al. 2016). The absorption of miRNAs from dietary sources has been demonstrated to occur via the delivery of exosomes (Ito et al. 2021). Subsequent studies have proven that dietary miRNAs can not only enter the system, but can also regulate human genes (Zempleni et al. 2015; Lukasik and Zielenkiewicz, 2016; Li et al. 2021a). A large scale study to identify the miRNAs associated with exosomes derived from edible plants has revealed about

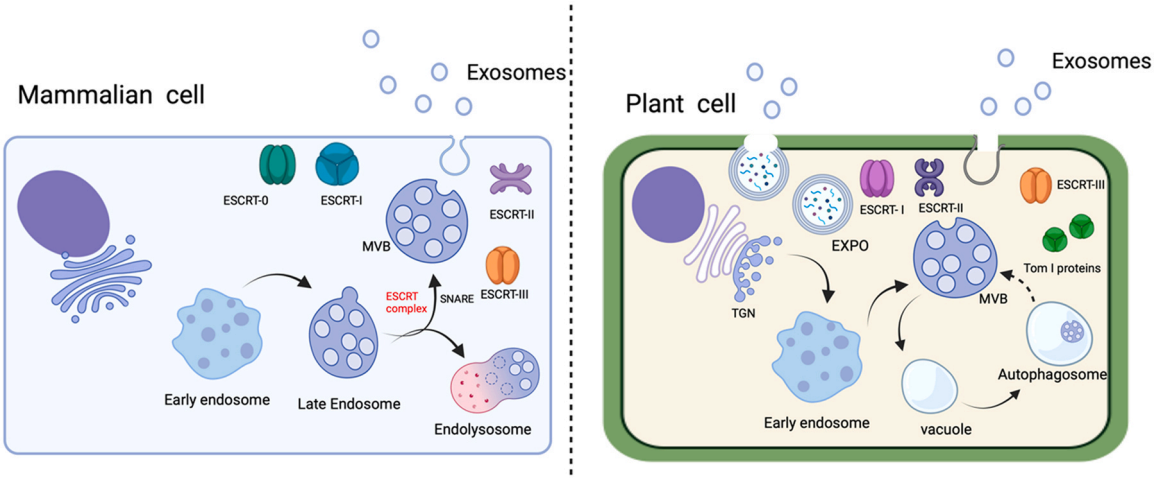

Fig. 1. Biogenesis of exosomes in mammalian cells and plant cells.

418 miRNAs ranging from 32 to 127 per species. Target prediction and functional assays on these miRNAs mostly identified genes from inflammatory and cancer related pathways (Xiao et al. 2018). A recent study on exosome associated miRNAs in ginger identified 27 miRNAs that are highly expressing and targeting many cytokine genes associated with inflammation (Yin et al. 2022). Several studies support the fact that the plant derived exosome like nanoparticles target the digestive tract of the consuming organism and is capable of a physiological regulation role mediated through micro RNAs.

Isolation of plant derived exosomes

Several methods are available for the purification of exosomes based on their size, flotation density, morphology and the presence of unique molecular markers on their surface. Exosomes can be extracted from the cell culture media or homogenate through conventional methods such as ultracentrifugation, differential centrifugation based on its molecular weight, immunoaffinity, size exclusion chromatography and commercially available kits (Konoshenko et al. 2018). Currently, several novel technologies exploiting the physiochemical and hydrodynamic properties of the exosomes have also been developed. The method of purification is determined by the nature of the source material from which exosomes are to be extracted and the downstream applications of the final preparation.

Conventional methods for exosome isolation

Ultracentrifugation is the most preferred method for isolation of exosomes from plant sources. It relies on the size and density difference of exosomes from the rest of the material. The homogenate or the juice of the plant material of interest is subjected to a series of centrifugation steps of increasing speed. The pellet obtained from the centrifugation steps below 100000xg do not encompass exosomes and are discarded. The pellet obtained thereafter is washed with buffers multiple times recentrifuged and collected. The method is usually effective for most of the samples but has the disadvantages of requiring expensive lab equipment and time consuming. Exosomes prepared by ultracentrifugation are usually contaminated with protein aggregates and this can be circumvented by using a density gradient during centrifugation. On a 30 % sucrose gradient, the exosomes float due to their mass and density differences while most of the contaminants do not get retained in these fractions. Studies have shown that increasing the centrifugation speed to 135000xg with sucrose fractionation provides pure preparation of exosomes. However, these extreme treatments reduce the yield considerably (Stanly et al. 2016).

Isolation by immunoaffinity is based on the usage of magnetic beads coated with antibodies specific to the exosome specific markers. The transmembrane markers such as CD81, CD82, CD63 and annexin are made use of for the specific isolation of selected exosomes. The preparation is usually ultrapure and is a method of choice for studying the immunogenic and therapeutic properties of the exosomes. The limiting factor with this method is the requirement for unique markers which are not well characterized in some systems, especially plants. Moreover, this method is unsuitable for large scale extraction of exosomes due to the downstream processing required to remove the bound antibodies (Konoshenko et al. 2018).

Ultrafiltration is an alternative method for large scale isolation of exosomes of high purity. It separates out exosomes on the basis of size and is a faster alternative to ultracentrifugation. It makes use of ultrafine nano-membranes with different molecular weight cut-off to isolate extracellular vesicles of different sizes. Sequential ultrafiltration is a modified method for extraction of exosomes in which the biological material is first passed through membranes of larger cut-off (approximately 1000 nm) to remove larger contaminants and the filtrate again undergoes the process through membranes of smaller size (500 nm) for fine purification. Limitations of this method include clogging of membrane and disruption of the vesicles due to the pressure applied during filtration (Yang et al. 2020a).

Size exclusion chromatography is a simple method that makes use of the difference in size to separate molecules. As it uses a passive gravity flow, the structural integrity of the exosomes is maintained in contrast to ultrafiltration techniques. The most positive aspect of the method is that the exosomes isolated by this method retain their biological properties and the system can work with minimal sample volumes. Limitations include contamination of the preparation with lipoproteins and protein aggregates (Yang et al. 2020a).

Polymer induced precipitation is a cost-effective strategy for separation of exosomes. Highly hydrophilic polymers such as poly ethylene glycol, interact with the water molecules surrounding the exosomes and create a hydrophobic microenvironment which leads to the precipitation of the exosomes. PEG within the molecular weight range of 6000 and 20000, is most preferred for the purpose, owing to its non-toxic and inert nature. Though the method is characterized by high yield, the purity of the preparation is often compromised, as PEG would precipitate out some hydrophilic extracellular contaminants as well (Rider et al. 2016).

Novel methods for exosome isolation

Affinity capture with biopolymers

Affinity capture can be used not only for the isolation but also for the enrichment of exosomes. The method is based on using specific antibodies against the proteins and markers expressed on the surface of

the exosomes. On incubating the exosome containing sample with the antibody conjugated solid matrices, exosomes were found to be enriched on the matrices and were eluted out. The selection of appropriate antibodies can also make the use of alternative methods such as microbead assisted flowcytometry for enrichment, a possibility (Li et al. 2018). In-spite of many advantages such as high specificity and affinity, use of antibodies have some drawbacks such as high cost, low stability and requisite for skilled labor. Use of aptamers and peptides in the place of antibodies can counter some of these demerits, as they are more stable and less expensive to make. A novel thermophoretic aptasensor method has been recently developed using microbeads conjugated with CD63 aptamers for the effective separation of exosomes (Li et al. 2021b). Similarly, CD63 aptamer based graphene composites have been used to capture and enrich exosomes from cell culture media (Chang et al. 2020). Methods based on phosphatidyl serine, which is highly expressed on exosomes are also being explored. TiO2 modified on the surface of the magnetic beads can be used for isolation of exosomes through covalent bonding between TiO2 and phosphatidylserine (Pang et al. 2020). Magnetic beads immobilized with Tim4, a protein that specifically binds phosphatidylserine has also been developed for isolation of exosomes (Xu et al. 2018).

Molecular Imprinted Polymers (MIP)

MIP provides nanocavities that match the shape and also the charge based binding features of the exosomes. Purification occurs via a synergistic effect of electrostatic binding and conformational recognition (BelBruno, 2019). They are biomimetic materials which can be considered as replacement for antibodies and possess high specificity and shelf life (Liu et al. 2022). Hybrids of molecular imprinted polymers and aptamers (MIP-aptamer) have also been used for superior specificity (Zhou et al. 2022). MIPs have been successfully used for the isolation of exosomes from tear samples (Mori et al. 2019). The method provides the liberty to purify exosomes for which markers and specificities are unknown. The main disadvantage of the method is that the macromolecules present in the sample can block the binding sites for the exosomes. However, development of MIPs is still in its infancy and ongoing researches in the field would yield more interesting options for exosome separation.

Microfluidic techniques

Microfluidics, the technology to control and manipulate the behavior of small amounts of fluids that are geometrically constrained to channels measuring in micrometers, has been applied to devise methods for the purification of exosomes. Immuno-microfluidics based exosome separation systems make use of the specific recognition of molecular markers on the surface of the exosomes by antibodies immobilized on chips (Contreras-Naranjo et al. 2017). Microfluidic chips with antibodies, aptamers or peptides modified into the channels have been designed to improve the specificity of exosome separation (Xu et al. 2018; Yu et al. 2021). A variety of microchannel designs such as nanowires (Sun et al. 2020), Y-shaped micropillars (Xu et al. 2018) and trapping microchannels (Tayebi et al. 2020) have been developed. The main advantages with using microfluidics for exosome isolation are reduced sample requirements and independency from labels, as it relies mainly on physical properties of the exosomes for separation. It is a promising technology currently receiving considerable research attention, which could open avenues for cheaper and reliable exosome separation.

Asymmetric-flow field-flow fractionation (AF4)

AF4 is an analytical technique to separate nanoparticles from larger molecules in a sample, based on their size. The separation is obtained by a difference in mobility in the flow field caused by the liquid flow over membrane and across the channel. The separation is solely based on their inherent diffusion coefficients. The method has been used successfully for separation of extracellular vesicles from plasma (Wu et al. 2020). The technique has the unique capabilities to separate exosomes gently in the absence of known markers with high resolution for size. The only limitation with this method is that particles of same hydrodynamic size as the exosomes might contaminate the preparation (Zhang and Lyden, 2019; Marioli and Kok 2020).

Liposome fusion technique

Liposomes have significant structural similarity with exosomes and the fluidity of the bilayer membranes allow their fusion. Based on this principle, recently, antibody conjugated lipid patch microarray have been developed for the separation of extracellular vesicles from cancer samples (Liu et al. 2021b).

A growing interest in the exosomes for various research purposes have led to a noticeable increase in the advanced isolation and purification techniques (Liangsupree et al. 2021; Xu et al. 2022). The field is rapidly evolving, that even exosomes secreted exclusively by a “single cell” can be captured through advanced microwell array chips (Zhu et al. 2021). It should be noted that there is no common protocol for the extraction of exosomes as different methods offer varied advantages and limitations, the selection of isolation technique depends on the nature of the samples and the downstream purposes. The increased research interest in exosomes has also led to the introduction of commercial kits for their extraction. The Exosome isolation and analysis kit (Abcam) and Exosome isolation kit CD81/CD63 (Miltenyi Biotech) make use of the presence of specific markers on the surface of exosomes. “ ExoMir Exosome isolation” kits recently developed by BioScientific Corporation is based on sequential ultrafiltration. Exosome isolation kits based on size exclusion chromatography, such as qEV (izon) and pure EVs (Hansa-Biomed) are also available in the market. The miRCURY exosome isolation kits based on the principle of precipitation are manufactured by Qiagene (Shirejini and Inci, 2022).

The methods for the isolation of exosomes is rapidly progressing and is reviewed in depth elsewhere (Chen et al. 2022a).

Characterization of plant derived exosomes

Characterization of the isolated exosomes in terms of its size, concentration, surface charge, porosity and density is very important to understand the biological nature and the possible interactions of the exosome. Many bio-physical methodologies are applied to characterize the extracellular vesicles and new methods are being devised as the interest in exosome research is mounting.

Electron microscopy is the most commonly used method to obtain data on the physical properties of the exosomes. Electron microscopy helps in visualization of the exosomes and the images obtained via electron scattering data are used for diameter determination. Additional processing of samples, such as immunogold labelling can provide information on bio-chemical properties of the exosomes as well. The limitation of the method is that it requires extensive sample preparation methods involving multiple steps that can sometimes alter the nature of the particles. This limitation can be bypassed by using cryo-EM in which the image processing is done in freezing temperatures in liquid nitrogen, skipping dehydration and fixation steps. Atomic Force Microscopy (AFM) is an advanced microscopy technique that provides data based on interactions between a probing tip and exosome surface. The data is translated into information on particle morphology, abundance and make up. The advantages of the method include minimized sample preparation steps and measurement of the properties in native conditions. AFM has been successfully used to study the properties of the grapefruit derived exosomes (Szatanek et al. 2017; Chuo et al. 2018).

It has been identified recently that flow cytometry can also be used to physically characterize exosomes. This method is based on the measurement of difference in the degree of light scattering by fluorescently labelled particles in a sample mixture. It can provide valuable information on size, concentration and distribution of the exosomes in a sample. In addition, fluorescent staining used in the method can

provide details regarding the content of the extracellular vesicles. The main advantage of the method is the ability to multiplex with reagents and antibodies. However, the fact that flow cytometry is not effective in detecting particles less than 500 nm, reduces its application in exosome studies (Mastoridis et al. 2018).

Dynamic light scattering or photon correlation spectroscopy is often used to measure the size of exosomes. A laser beam is passed through the suspension of exosomes and the diffraction caused due to the Brownian movement of the particles is measured. The data is then correlated with the size and density of the particles in the sample. This method can be used to characterize extracellular vesicles in a broad range of sizes. But, the technique does not visualize the exosomes and cannot provide any information on biochemical nature of the particles analyzed.

Nanoparticle tracking analysis (NTA) is a biophysical approach that involves optical particle tracking, based on light scattering and Brownian motion, to decode the size distribution and concentration of nanoparticles in a liquid sample. NTA analyses each particle, and uses Stokes Einstein equation to calculate the hydrodynamic radius of the particles. The advantage of the method is that it can be used with wide range of sizes (10 nm – 2 μm) and the technique works with low sample volume with minimal sample preparation steps. The method is gaining popularity among exosome researchers, as it allows the visualization of the suspension and can also provide a detailed data profile of the particles (Gardiner et al. 2013). Resistive Pulse Sensing (RPS) is a recent method that measures the size and surface charge simultaneously based on Coulter's principle. The high resolution size measurement and zeta potential calculation on particle by particle basis helps in a better understanding of the physical and biological nature of the particles (van der Pol et al. 2014).

Many other methods such as X-ray microscopy, Simulated emission depletion microscopy (STED) and microfluidics based flow cytometry techniques are also infrequently used to characterize exosomes (Contreras-Naranjo et al. 2017). Additional methods are being rapidly devised and the existing methods are often modified to suit the customary needs in the field of exosome study.

Natural therapeutic potential of plant derived exosomes

Exosomes have been derived and purified from various plant sources. Due to their unique structure they have the ability to be taken up by cells and deliver their distinctive biochemical contents to the target cells. Many *in vitro* and *in vivo* studies manifest the therapeutic ability of exosomes in various biological aspects (Fig. 2; Table 1).

Antioxidative property of plant derived exosomes

Oxidative stress occurs within the cells when the balance between the free radical concentration and the anti-oxidant defenses is altered. It is implicated in the etiology of many common diseases including diabetes and neurodegenerative disorders. Increased oxidative stress triggers apoptosis and ultimately leads to the death of the cell. Unlike humans, plants are rich in antioxidant compounds which are often exploited for preparation of anti-ageing formulations and preservation of food. Current reports on the anti-oxidative property of plant derived exosomes has set off a new interest on them among researchers.

Carex, a preparation of nanovesicles and exosomes from carrots extracted by using size-exclusion chromatography in combination with ultrafiltration, was tested for its antioxidative property in cardio myoblast and neuroblastoma cell lines. Oxidative stress plays a crucial role in the pathogenesis of cardiac hypertrophy and Parkinson's disease. It was found that treatment of cell lines with Carex, prevents ROS spike in H_2O_2 treated cardio myoblast cell lines H9C2 and inhibits apoptosis in a time and dose dependent manner. The Nrf2-antioxidant response element signaling pathway is the major line of defense against ROS in humans, as it controls the genes involved in ROS scavenging (Nguyen et al. 2009). Treatment with carrot exosomes prevents the suppression of the Nrf2 pathway by H_2O_2. Carex has a significant antioxidative effect on neuroblastoma cells in an *in vitro* Parkinson's disease model as demonstrated by reduced caspase activity and suppression of Nrf2. Carex prevents oxidative stress in cells by reducing the decrease in levels of antioxidant proteins and arresting apoptosis (Kim and Rhee, 2021).

Blueberry derived exosome-like nanoparticles are found to be highly hepatoprotective due to their anti-oxidant property. When HepG2 cells were treated with these nanoparticles, significantly reduced ROS, increased mitochondrial membrane potential and reduced apoptosis were observed. The nanoparticles also improved liver dysfunction in high fat diet fed mice by decreasing the levels of AST and ALT. It reduces lipid droplet formation through attenuating the enzymes fattyacid synthase

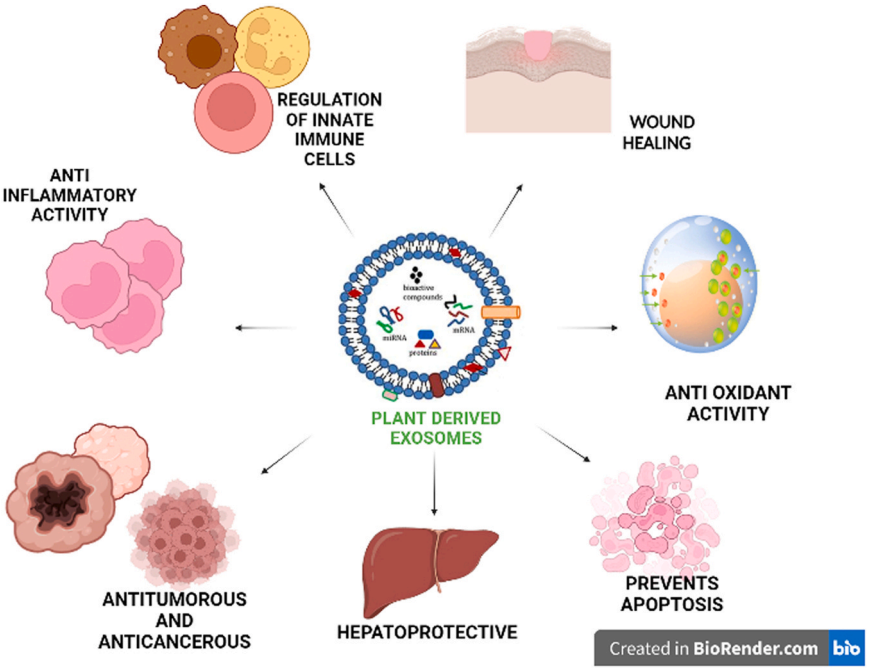

Fig. 2. Schematic representation of different therapeutic potential of plant exosomes.

Table 1
Therapeutic potential of plant derived nanovesicles from different sources.

SL NO	SOURCE	THERAPEUTIC POTENTIAL	ISOLATION METHOD	SIZE OF EXOSOME	INVITRO/INVIVO STUDIES	REFERENCE
1	Apple	Anti-inflammatory activity	Ultracentrifugation	157 nm (radius)	Cancer cell line, fibroblasts and macrophages	(Trentini et al. 2022)
2	Grapes	Anti-inflammatory activity	Sucrose gradient centrifugation	380.5 ± 37.37 nm (diameter)	Animal model (mouse)	(Ju et al. 2013)
3	Cabbage	Anti-inflammatory activity and inhibition of apoptosis	Ultracentrifugation and PEG based precipitation	148.2 nm, 134.2 nm, 98.8 nm	human and mouse cell line	(You et al. 2021)
4	Strawberry	Anti-oxidant	Ultracentrifugation	30–191 nm	Human mesenchymal stromal cells	(Perut et al. 2021)
5	Grapefruit	Wound healing	PEG based precipitation	30–100 nm	HaCaT cells (aneuploid immortal keratinocyte cell line)	(Savcı et al. 2021)
6	Grapefruit	Anti-oxidative, Anti- inflammatory and anti-cancerous	Differential ultracentrifugation	50–80 nm	A375 Human melanoma cell line	(Stanly et al. 2020)
7	Aloe vera	Antioxidant effect for wound healing	Ultracentrifugation and tangential flow filtration	50–200 nm (diameter)	Human keratinocytes and fibroblasts	(Kim et al. 2020b)
8	*Citrus limon*	Anti-cancerous	Ultracentrifugation	50–70 nm	Human carcinoma cell line and chronic myeloid leukemia cell line	(Raimondo et al. 2015)
9	*Citrus limon*	Antioxidant effect	Differential centrifugation			(Baldini et al. 2018)
10	Lemon	Anti-cancerous effect	Ultracentrifugation, electrophoresis was combined with dialysis	450 nm	The human gastric cancer cell line SGC-7901 and animal model (tumour bearing mice)	(Yang et al. 2020b)
11	Ginger (Rhizome)	NLRP3 Inflammasome inhibition	Sucrose gradient centrifugation	120–150 nm (diameter)	Mice Bone marrow-derived macrophages	(Chen et al. 2019)
12	Ginger	Antioxidant and hepatoprotective against alcohol induced liver damage	Sucrose gradient centrifugation	102.3–998.3 nm (Diameter)	Animal model (mouse)	(Zhuang et al. 2015)
13	Ginger	Anti-inflammatory activity	Centrifugation	200 nm	Animal model (mice)	(Mu et al. 2014)
14	Ginger	Anti-inflammatory property	Sucrose gradient ultracentrifugation	220–290 nm	Animal model (mice)	(Zhang et al. 2016a)
15	Blueberry	Anti-oxidant	PEG based centrifugation	189.62 nm (Diameter)	Animal model (mouse)	(Zhao et al. 2022)
16	Blueberry	Antitumour activity	Ultracentrifugation	114 ± 36 nm	Human stabilized endothelial EA. hy926 cell line	(De Robertis et al. 2020)
17	Broccoli	Anti-inflammatory effect	sequential centrifugation method	32.4 nm	Animal model (Mice with colitis)	(Deng et al. 2017)
18	Wheat	Wound healing activity	Exo-spin™ Exosome Purification Kit	40–100 nm	human dermal fibroblast cell line (HDF)	(Şahin et al. 2019)
19	Bitter melon	Antitumour and induce OSCC cell apoptosis	Centrifugation, electrophoresis and dialysis based method	100–300 nm	Animal model (mice)	(Yang et al. 2021)
20	*Momordica charantia* (Bitter melon)	Anti-oxidant activity	Density gradient centrifugation	106.0 nm	Rat cardiomyocyte cell line (H9C2)	(Cui et al. 2022)
21	Ginseng	Antitumour activity	Sucrose gradient centrifugation	344.8 nm	Murine melanoma skin cell line	(Cao et al. 2019)
22	Garlic chive	Anti NLRP3 inflammasome activity	Ultracentrifugation	113–153 nm	Bone marrow cell line of mice	(Liu et al. 2021a)
23	Tea flowers	Anti-cancerous (breast cancer)	Sucrose gradient ultracentrifugation	131 nm	Lung metastasis mice model	(Chen et al. 2022b)
24	*Petasites japonicus*	Immunostimulatory potential	Filtration and differential centrifugation	100–140 nm	Animal model (mice)	(Han et al. 2021)
25	Fingerroot *Boesenbergia rotunda* (L.)	Anticancer activity	Centrifugation	71.1 ± 1.4–106.7 ± 2.4 nm	Colorectal cancer (HT-29 and HCT116) and normal human colon epithelial (CCD 841 CoN) cell lines	(Wongkaewkhiaw et al. 2022)
25	Carrot	Anti-oxidant activity	Ultracentrifugation	143.9 nm	H9C2 embryonic rat heart-derived cardiomyoblasts and human neuroblastoma SH-SY5Y cells	(Kim and Rhee, 2021)
26	Garlic	Anti-inflammatory activity (brain) and anti-obesity	Sequential centrifugation		Animal model (mice)	(Sundaram et al. 2022)
27	Garlic	Anti-cancerous	Centrifugation and PEG based precipitation	50–150 nm	Human kidney carcinoma cell line A498, Human lung carcinoma cell line A549	(Özkan et al. 2021)
28	Garlic	Anti-inflammatory	Centrifugation	70–200 nm	Human cell line (HepG2 cell line)	(Song et al. 2020)

and acetyl-coA carboxylase. These findings have led to the consideration of the blueberry derived exosomes for the treatment of non-alcoholic fatty liver disease as a therapeutic agent (Zhao et al. 2022). Internalization of exosome-like nanovesicles from Strawberry, by human mesenchymal stromal cells reduced oxidative stress significantly without negatively affecting their viability, in a dose dependent manner (Perut et al. 2021).

Exosomes extracted from the juice of *Citrus limon* are taken up by human mesenchymal stromal cells with ease and do not affect their survival. When these pretreated cells are subjected to H_2O_2 they show a significantly improved survival in a dose dependent manner. ROS production was also reduced drastically in these cells (Baldini et al. 2018).

One of the main reasons behind the anti-oxidative property of these exosomes from diet sources is their ability to deliver a high concentration of vitamin C to the target cells. Vitamin C is a well-known ROS scavenger, which prevents apoptosis due to oxidative damage and also a known cofactor for many important enzymatic reactions. However, vitamin C has a limited bioavailability and bio-accessibility due to its unstable nature. The exosomal vesicles from the fruits and berries are heavily concentrated with vitamin C and they are capable of resisting gastric environment with enhanced intestinal absorption. This ensures the delivery of a high concentration of vitamin C to the target cells. For instance, 50 μg/ml of *Citrus limon* exosomes, which showed significant anti-oxidant property in MSC cells contain about 6.47 μM vitamin C. It is about forty times less than the concentration of other forms of vitamin C (such as ascorbic acid 2 phosphate) required to experimentally protect cells from H_2O_2 induced cell death (Baldini et al. 2018). This ability to effectively deliver vitamin C might be the physiological reason behind the anti-oxidant property of these exosomes. In addition to Vitamin C, several small RNAs, micronutrients and biomolecules are enclosed within the exosomes. The contribution of these factors to the anti-oxidative property cannot be disregarded and additional research is required to understand their role.

Anti-tumorigenic properties of plant derived exosomes

Tumors and malignancy have always been a challenge to modern medicine. Non-toxic, selective anti tumorigenic agents are always on demand. Recently, it has been shown in several cases that the plant-derived exosomes have anti-neoplastic capacity which can be exploited to develop more safer, natural substance mediated treatment plans for cancer. For instance,

Natural exosome like particles extracted from tea flowers (*Camellia sinensis*) showed strong cytotoxicity in breast tumor cells. They caused mitochondrial damage and cell-cycle arrest through stimulation of oxidative stress in cancer cells. This capacity to induce ROS amplification imparts anti-proliferation, anti-invasion and anti-migration effects on the breast cancer cells *in vitro*. Xenograft mouse models have been used to assess the effect *in vivo* and was observed that the mice that received the exosomes intravenously or orally had an increased survival rate. When administered orally, the tea-flower derived exosomes had significant moving capacity in the mucosa and were absorbed into blood via the intestinal epithelial cells. They get accumulated in the breast cancer cells, as demonstrated by DIR labelled exosomes and also prevent metastasis to lungs by affecting their proliferation, apoptosis and mobility. In addition, the oral administration had some beneficial effects due to the ability of the exosomes to modulate the abundance and diversity of microbiota (Chen et al. 2022b).

Extracellular vesicles, in the size range of exosomes, isolated from the sap of *Dendropanax morbifera* and *Pinus densiflora* had been shown to exhibit strong cytotoxic effect on tumor cells. The intake of these exosomes occurs through phagocytosis and caveolae mediated endocytosis, which is predominant in tumor lines. As a result they get accumulated more robustly in the tumor cells than the normal cells. The exosomes have significant cytotoxic effect on breast and skin cancer cells and the therapeutic efficacy is mainly due to their growth inhibition effect. The exosomes from *D.morbifera* and *P.densiflora* had a significant synergistic effect on the inhibition of cancer cells when administered together and are considered as a potential drug regimen to treat breast and skin malignancies with limited side effects (Kim et al. 2020b). The anti-cancer effects of *D.morbifera* sap derived exosomes was further investigated using a 3D microfluidic model that mimics the tumor microenvironment. It was demonstrated that the exosomes had a concentration dependent suppressive effect on the Cancer associated fibroblasts (CAF). Genotypic analysis revealed that the treatment with exosomes altered the expression levels of certain growth factors and ECM related proteins such as collagen. The study clearly demonstrates the time and concentration dependent anti-metastatic effect of *D.morbifera* derived exosomes (Kim et al. 2020a).

Nanovesicles extracted from grapefruit, which are majorly composed of exosomes, have been demonstrated to show a reduction of about 40 % viability in multiple tumor cell lines. The interaction studies with melanoma cell lines revealed that the therapeutic potential is due to the arrest of cell cycle in G2/M check point, leading to reduction in cyclins such as cycB1 and cycB2 with concomitant increase in the levels of cell cycle inhibitor P21. The down regulation of cycB2 impairs the invasiveness and prevents metastasis. The grapefruit exosomes also impair the viability of cancer cells through the inhibition of AKT and ERK signaling pathways and also demonstrate pro-apoptotic activity through the activation of PARP-1 thereby limiting cancer proliferation. The metabolome profile of the grapefruit derived nanovesicles revealed that they are enriched with alpha–hydroxy acids and leucine/isoleucine, myo-inositol in addition to doconexent, which is a compound already demonstrated to possess anti-proliferative effect on cancer cells. This bio-active metabolomic cargo is believed to be responsible for the well-demonstrated anti-tumorigenic effect of the grape fruit derived exosomes (Stanly et al. 2020).

Exosome like nanovesicles from *Citrus limon* is known to suppress the growth of lung carcinoma and chronic myeloid leukemia cell lines by 50 %. Further analysis into the mechanism revealed that the treatment with exosomes upregulates pro-apoptotic genes such as Bad and Bax while anti-apoptotic genes such as Survivin and Bclx-1 are downregulated. TNF-related apoptosis inducing ligand (TRAIL) is a natural mechanism for immunity against viruses and immunosurveillance of cancer cells (Shepard and Badley, 2009). TRAIL and its receptor Dr5 were both upregulated at mRNA level within 48 h of treatment of the cell lines with lemon nanovesicles. Using xenograft tumor models, it has been shown that lemon exosomes can reduce tumor growth *in vivo* by activating TRAIL-mediated apoptosis and by inhibition of secretion of cytokines that induce angiogenesis (Raimondo et al. 2015).

In chemically induced colorectal cancer models, oral administration of ginger derived nanoparticles, led to significant reduction in tumor size. The decrease in tumor size is caused by inhibition of apoptosis, IEC proliferation and by reduction of pro-inflammatory cytokines. It was found that the treatment with ginger exosomes can potentially upregulate 14 proteins and downregulate 3 proteins. These molecular targets include proteins such as PKG and transgelin which have already found to be associated with colon cancer (Zhang et al. 2016a).

Most of the conventionally available anti-cancer therapeutic agents affect the normal cells with same intensity, leading to complex side effects. In the above mentioned evidences where nanoparticles from natural plant sources were used against cancers, the normal cells remained unaffected. These plant exosomes with anti-neoplastic potential which are selective towards malignant tumor cells can play a significant role in cancer treatment regimens in the future. It should be noted that the exosomes from different sources have distinct interaction mechanisms with the cells and their therapeutic efficacy is due to their effect on unique molecular targets. This is caused by the non-identical, diverse bio-active cargos that they hold inside them. Further studies are required to decipher the contents of the exosomes for a better understanding of the molecular mechanism behind their therapeutic efficacy.

Restorative property of plant derived exosomes

Treatment with nanovesicles from *Citrus limon* enhances their ability to synthesize type I Collagen. Citrate, one of the micronutrients in the citrus juice is an important mediator of collagen structure of bone matrix and regulator of bone homeostasis. The effect of the nanovesicles might be due to the citrate and a combination of other bioactive molecules present in the exosomes (Baldini et al. 2018).

Extracellular vesicles, in the range covering exosomes, extracted from *Aloe vera* peels were internalized significantly into human keratinocytes (HaCaT) cells via clathrin and caveolae-mediated endocytosis. The *Aloe vera* vesicles (A-EVs) activated the antioxidant defense through *Nrf2* regulation and prevented oxidative damage in a dose dependent manner. The *in vitro* scratch wound assay showed that A-EVs promote the migration of keratinocytes and fibroblasts to the wound site, thereby promoting wound-healing. This shows that A-EVs have significant skin regeneration potential that can be therapeutically explicated (Kim et al. 2021).

Wheat grass juice derived exosomes have been shown to have significant proliferative and migratory effects on endothelial (HUVEC), epithelial (HaCaT) and dermal fibroblast (HDF) cells (Şahin et al. 2019). These observations show that further research on exosomes from selected plant sources can lead to products with clinical wound healing and cosmetic uses.

Anti-inflammatory property of plant derived exosomes

Inflammation is an essential, biologically conserved response of the body cells to external stimuli such as pathogen or irritant. Though it is a part of the healing mechanism, chronic inflammation is the reason behind many common diseases. Surprisingly, several reports show that plant derived nanoparticles can counteract inflammatory symptoms. Oral administration of ginger derived nanoparticles to colitis mouse models showed that they are readily taken up by the epithelial cells and macrophages without any local or systemic side effects. Both *in vitro* and *in vivo* studies indicated significant mucosal wound healing property to these nanoparticles. They increased the survival and proliferation of intestinal epithelial cells by inhibiting pro-inflammatory cytokines such as TNF- α, IL-6 and IL-1β and increase anti-inflammatory cytokines like IL-10 and IL-22 (Zhang et al. 2016a).

Exosome like nanoparticles from grapes promote self-renewal of intestinal epithelium by inducing the proliferation of stem cells. This ability to regenerate intestinal epithelium protects mice against colitis (Ju et al. 2013). Exosomes isolated from *Citrus sinensis* fruit juice reduced the expression of many inflammatory genes and upregulated permeability associated genes. They not only counteract inflammation but also improve the intactness of the intestinal mucosa by modulating the distribution of the tight junction OCLN protein (Bruno et al. 2021). Blueberry derived exosomes modulate the expression of around 29 genes involved in inflammatory pathways in the human endothelial cell lines in response to TNF- α. A target search on the miRNAs present in the blueberry exosomes unveiled probable mRNA targets which are involved in inflammatory response, cytokine release and oxidative stress. This confirms that blueberry exosomes act as therapeutic carriers of bioactive components with significant anti-inflammatory properties (De Robertis et al. 2020).

In mouse colitis models, broccoli-derived nanoparticles reduce inflammation considerably by enhancing the tolerance of dendritic cells through activation of adenosine monophosphate activated protein kinase (AMPK). It prevents the migration of monocytes and maintains the gut immune homeostasis by improving the tolerance of the immune cells (Deng et al. 2017). Recently it has been demonstrated that exosome like nanoparticles from the edible barks of mulberry promotes the activation of AhR signaling pathway, which in turn leads to the induction of COPS8 essential for anti-inflammatory effect (Sriwastva et al. 2022). Surprisingly, when intratracheally administered, ginger derived nanoparticles can ameliorate lung inflammation caused by the SARS-CoV-2 infection. The therapeutic factors responsible for the effect were recognized as miRNAs, aly-miR396a-5p and rlcv-miR-rL1–28–3p, capable of targeting *Nsp12* and spike genes of the virus (Teng et al. 2021). Exosome preparation from apples have been shown to have anti-inflammatory property mediated mainly by the production of miRNA146 by M2 macrophages (Trentini et al. 2022).

The anti-inflammatory property of exosomes from different sources is manifested by modulating different aspects of the inflammatory pathway from changing the expression levels of cytokines to activating counter pathways. The immune regulatory property of the bioactive contents of the exosomes is clearly established from the above findings.

Anti-microbial property of plant derived exosomes

Apart from the well-known function of plant derived exosomes in protecting plants from pathogens, recent findings have revealed that they can protect mammalian hosts from infections. The exosome like nanoparticles from ginger are shown to be selectively up-taken by pathogenic bacteria *Porphyromonas gingivalis* and reduce their virulence considerably. On oral delivery in mouse periodontitis models, they are selectively taken up in a dose dependent manner through a phosphatidic acid mediated pathway. The miRNAs, anti-microbial peptides and the lipids present in the ginger exosomes target multiple virulence factors of the *P.gingivalis* and reduces its pathogenicity. In addition to reducing the viability of the pathogen through increased membrane depolarization, the ability of the organism to attach and invade oral epithelial cells is also affected thereby diminishing its infectivity (Sundaram et al. 2019). This study opens a new avenue for the inclusion of plant derived exosomes in the treatment of periodontitis.

Exosome like nanoparticles extracted from coconut water were able to enter the *Escherichia coli K-12 MG1655* and *Lactobacillus plantarum WCFS1*cells on co-incubation for one hour. It was found that the miRNAs present in the coconut water exosomes such as miR-395 had potential targets in bacteria (Yu et al. 2019). Exogenous extracellular vesicles such as exosomes can alter the bacterial gene expression as a result of this cross kingdom transfer of miRNAs (Lefebvre and Lécuyer 2017). The study clearly indicates that bacterial survival and invasion could be modulated by the intake of these exosomes.

Plant derived exosomes are taken up by the gut microbiota and often contain biologically active constituents that can alter the microbiome itself. *Lactobacillaceae* preferentially take up ginger derived exosomes, which promote their growth. These exosomes have a preventive effect on mouse colitis not only through this alteration of microbiome but also through their miRNAs targeting LGG genes, indirectly leading to improvement of intestinal barrier function. Unlike ginger exosomes, grapefruit derived exosomes are preferentially taken up by *Runococcaceae* family members, which indicate that the diet derived exosomes are unique in their composition and the gut microbiota gets altered significantly based on the respective diet (Teng et al. 2018).

Recent studies on mono-floral honey derived from *Eucryphia cardifolia* show that they contain antibacterial properties. Bacterial growth and biofilm inhibition properties were observed with *Streptococcus mutans*, which is a leading cause for dental caries and abscesses (Schuh et al. 2019; Leiva-Sabadini et al. 2021; Deglovic et al. 2022). Lemon exosome like nanoparticles increase the survivability of *Lactobacillus rhamnosus* GG (LGG) and *Streptococcus thermophilus* ST-21 in the gut by increasing their bile resistance. These lemon derived exosomes are capable of preventing the *Clostridioides difficile* induced colitis through their ability to increase probiotic survivability and alter intestinal metabolite composition. Though not directly bactericidal, the lemon derived exosomes modulate the gut microenvironment to inhibit the growth of *C.difficile* (Lei et al. 2020).

The above studies clearly indicate that a crucial crosstalk exists between the dietary exosomes, their bioactive cargo including small

RNAs and the gut microbiota. The harmony among these entities alters host physiology and maintains the gut health. The composition of dietary exosome like particles varies considerably and targets specific group of microorganisms, which in turn can be manipulated to specifically modify the microbiome to prevent certain diseases. However it is clear that, the plant derived exosomes will soon find application as pretreatment for probiotics, as anti-microbial agent and as modifiers of microenvironment to prevent certain diseases.

Current challenges and future perspectives

Plant exosomes, which are membrane enclosed signalosomes with potentially active biological cargo have been implicated in multiple functions. The growing literature about their innate therapeutic properties imply that they are potent candidates for development into bionanomedicines. However, the various interactions and effect observed between the plant exosomes and other hosts need to be analyzed carefully to understand whether the effect is specific or stochastic. More extensive research on the basic biology of exosomes is required as the cellular and molecular aspects of the biogenesis, functions and uptake are not yet fully known. The biological characterization of the exosomes needs to be focused, as the surface markers and other identifying features of the plant exosomes still remain obscure. More robust analytical methods to identify the contents of the exosomes is a need of the hour. The isolation of exosomes still remains an expensive exploit requiring complicated equipment and labor. Translation of the therapeutic potential of these exosomes will not be possible without developing efficient isolation and scale up methodologies. Though clinical trials on a few plant derived exosomes is underway, regulatory affairs for their use as therapeutic agents are not set out with clarity.

Despite the challenges in the field, the exosomes show many advantages in biocompatibility, therapeutic ability, targeting capability and cellular uptake. Natural therapeutic agents without toxicity and side effects are always sought after. When focused and developed with multi-disciplinary expertise, plant exosomes can be developed into reliable therapeutic agents for many common ailments.

Data Availability

No data was used for the research described in the article.

Declaration of Competing Interest

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

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