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Submission date: 18-Aug-2022 04:39PM (UTC+0800)
Submission ID: 1883876710
File name: artikel-Plant-derived.pdf (1.1M)
Word count: 10026
Character count: 54716

Received: 26 November 2020 | Revised: 24 February 2021 | Accepted: 28 April 2021

DOI: 10.1111/1750-3841.15787

CONCISE REVIEWS & HYPOTHESES IN FOOD SCIENCE

Journal of Food Science WILEY

Plant-derived exosome-like nanoparticles: A concise review on its extraction methods, content, bioactivities, and potential as functional food ingredient

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Funding information
Indonesian Collaboration Research, Grant/Award Number: 1761/IT3.L1/PN/2020

Abstract

Plant-derived exosome-like nanoparticles (PDENs) are small vesicles released by multivesicular bodies mainly to communicate between cells and regulate immunity against pathogen attack. Current studies have reported that PDENs could modulate gene expression in a cross-kingdom fashion. Therefore, PDENs could be a potential future functional food ingredient as their cross-kingdom communication abilities were reported to exert multiple health benefits. Macrophage and other cells have been reported to absorb PDENs in a manner regulated by the membrane lipid and protein profile and the intactness of the PDENs lipid bilayer. PDENs could be extracted from plant materials by various techniques such as ultracentrifugation, immunoaffinity, size-based isolation, and precipitation, though each method has its pros and cons. PDENs mainly contain lipid, protein, and genetic materials, mainly micro RNAs, which could exert multiple health benefits and functionalities when consumed in sufficient amounts. However, most studies on the health functionalities of PDENs were conducted through *in-vitro* and in-vivo studies, and its potency to be used as a functional ingredient remains a question as PDENs are sensitive to storage and processing condition and requires costly extraction method. This concise review features various exosome extraction methods, contents of PDENs and their roles, the health functionalities of PDENs, and its potency as a functional food ingredient.

KEYWORDS
bioactive compound, exosome, functional food, nanoparticles, plant-derived

1 | INTRODUCTION

Exosomes are small vesicles produced by multivesicular bodies (MVBs) of most cells into the extracellular area with a diameter of 40–100 nm (Tauro et al., 2012). It differs from other vesicles such as micro-vesicles and apoptotic blebs with a diameter of 100–350 nm and 500–1000 nm, respectively (P. Li et al., 2017). Exosomes are enclosed in a lipid bilayer and membrane proteins while containing various cytosol components, such as proteins and RNAs (P. Li et al., 2017; M. Zhang, Villenois, Xu et al., 2016).

Despite being historically thought as artifacts from trials or remnants of cells (Pan & Johnstone, 1983) and as a method for cells to dispose of its waste and unnecessary materials (Johnstone, 1992; Rashed et al., 2017), exosomes are currently known for its role in cell-to-cell communication through bioactive material transfer and gene/protein expression regulation (Ju et al., 2013; M. Zhang, Viennois, Prasad, et al., 2016). Recently it is known that plant MVBs excreted exosome-like vesicles as a method of cell-to-cell communication and immunity regulation to defend the plants from pathogen invasions (An et al., 2006; An et al., 2007; Ding et al., 2012; Nielsen et al., 2012; Wei et al., 2009).

On top of that, plant-derived exosome-like nanoparticles (PDENs) were shown to be absorbed by intestinal macrophages and were able to exhibit interspecies communications by inducing multiple cytokines (Mu et al., 2014). The extraction yield of PDENs was much higher than that from mammalian cell culture (Z. Li et al., 2018), indicating its economic potency as nano-factories (M. Zhang, Viennois, Prasad, et al., 2016). Exosome isolation remains a daunting task as biological samples are incredibly complicated, and its physicochemical and biochemical properties often overlap with other extracellular vesicles. To further complicate the problem, exosomes are quite heterogenous in themselves (P. Li et al., 2017).

PDENs have been shown to demonstrate various health benefits, as seen in Figure 1, through *in-vitro* studies or animal models, which make PDENs as potential functional food ingredients in the future to alleviate human illnesses. However, previous studies to investigate this are quite limited at the moment. The intact lipid bilayer of PDENs is necessary for their health functionalities. This requirement further complicates the problem since the integrity of PDENs is sensitive to change in temperature, storage, pH, and other processing condition that is necessary to create a functional food with a long shelf life.

This review attempts to highlight the various exosome extraction method and the contents of PDENs and their roles, the bioactivities of variously derived PDENs towards mammals' health, and their potency for application as a functional food ingredient.

2 | EXTRACTION METHODS

The extraction of PDENs follows the methods that have been established for mammalian sourced exosomes. Proper extraction and purification method are crucial for the analysis of exosomes as they are often present within an overly complex matrix. As studies on exosomes have been gaining much attention lately, many studies have been published to improve extraction specificity and efficiency. Each method would have its advantages and drawbacks; a more specific method such as immunoaffinity would typically result in a pure sample but suffer in efficiency and recovery, while methods with high efficiency and recovery

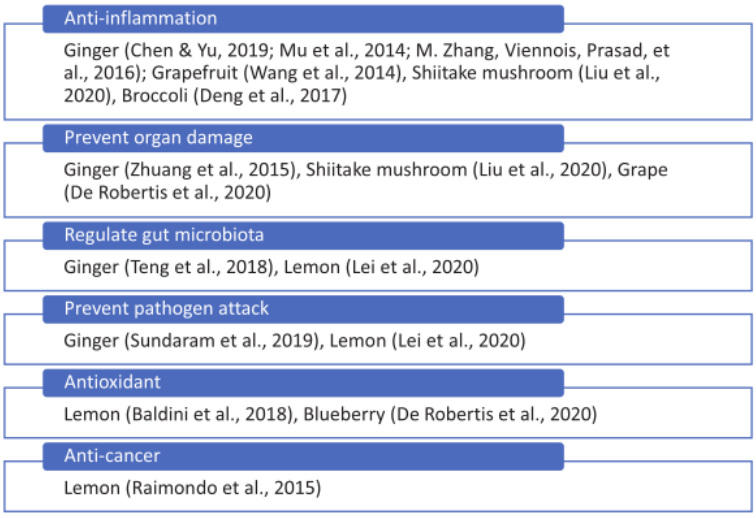

FIGURE 1 Previously reported health functionalities of PDENs and the sources

TABLE 1 Typical exosome extraction methods and examples of application to isolate PDENs

Method	Extraction condition[a]	Examples of application[b]
Ultracentrifugation	Series of high-speed centrifugation and gradient ultracentrifugation. Regarded as the gold standard in exosome extraction because of its ability to extract exosomes in a relatively high purity fashion.	Ginger, grapefruit, carrot, grape, lemon, blueberry, shiitake, broccoli
Size-based isolation	Separating similar-sized particles with ultrafiltration, size-exclusion chromatography, and flow field-flow fractionation, which typically resulted only in an exosome-enriched sample	–
Immunoaffinity	Utilize the interaction between the antigen proteins on the surface of exosomes and matching antibodies. Very specific in nature but risking leaving out other subpopulation of exosomes without the targeted protein.	–
Precipitation	Combination of centrifugation and crowding agent such as PEG6000 to trap exosomes which is a more cost-effective method but tend to result in an unpure sample.	–

[a] Reference: (Kalarikkal et al., 2020; Kang et al., 2008; Li et al., 2017).
[b] Reference: (Baldini et al., 2018; Chen & Yu, 2019; Deng et al., 2017; De Robertis et al., 2020; Ghiasi et al., 2018; Ju et al., 2013; Lei et al., 2020; Liu et al., 2020; Mu et al., 2014; Raimondo et al., 2015; Sundaram et al., 2019; Teng et al., 2018; Wang et al., 2014; Zhang, Viennois, Prasad, et al., 2016; Zhuang et al., 2015).

such as membrane filtration would suffer in its specificity and purity (Sidhom et al., 2020).

It is important to note that the activities of PDEN would depend on the intact bilayer membrane (Ju et al., 2013). Therefore, special care must be utilized during the extraction and storage to prevent its degradation, for example, by utilizing protease inhibitors, low-temperature storage, avoidance of freeze-thaw cycle, and maintaining neutral pH (Cheng et al., 2019; P. Li et al., 2017). Some of the extraction techniques such as ultracentrifugation, ultrafiltration, immunoaffinity capture, precipitation, or microfluidics typically made use of the unique characteristics of exosomes like their density, size, shape, and protein (P. Li et al., 2017). Interestingly, all the studies reviewed in this article utilized ultracentrifugation as the sole extraction method to isolate PDENs as seen in Table 1. Further studies should then be conducted to compare different extraction techniques to isolate PDENs while considering their simplicity and yield efficiency.

2.1 | Ultracentrifugation

Out of the previously mentioned techniques, ultracentrifugation (differential and density gradient) is the most commonly applied technique and remained the gold standard in exosome isolation because of its simplicity, ease of use, affordability in the long run, moderate time consumption, and the absence of complicated sample preparation (P. Li et al., 2017). Differential centrifugation relies on the separation of particles based on its density, size, and shape using a series of high-speed centrifugation in a homogenous medium such as phosphate buffer saline preceded with initial sample clean-up to remove larger cells and debris (P. Li et al., 2017).

Further purification is often necessary to remove unwanted debris such as proteins, RNAs, and membrane aggregates, which can be done by gradient ultracentrifugation. This is done by creating layers of different concentration of sucrose solution (10-90%) or other materials such as iodixanol, placing the samples on top of the lowest concentration layer, and exposing the tube in ultracentrifugation at 4°C (Alexander et al., 2016; Tauro et al., 2012). This technique managed to not only purify PDENs but also split the PDENs into separate layers with different bioactivities, size distribution, and even stability levels, thus increasing the purity of the exosomes extracted from the studies (M. Zhang, Viennois, Prasad, et al., 2016; Zhuang et al., 2015). However, ultracentrifugation is exceptionally costly to purchase and maintain. It also requires extensive training and a dedicated team to operate, which would hinder its application mainly in resource-poor countries or laboratories (Liga et al., 2015).

2.2 | Size-based isolation

Ultrafiltration is a very prevalent size-based isolation method with primary goals similar to regular membrane filtration, isolating the exosomes based on their size or molecular weights (P. Li et al., 2017). 100 ml of a sample could be prepared within 30 minutes using five simultaneous nanomembrane concentrators and short time centrifugations compared to 4 hr by ultracentrifugation method (Cheruvanky et al., 2007). However, many nanoparticles and some nonvesicular nanoparticles were in the same size range of exosomes; therefore, this technique would instead result in a sample enriched in exosomes rather than obtaining pure exosomes themselves (Zeringer et al., 2015).

Size-exclusion chromatography (SEC) is another method that could isolate exosomes by using a porous stationary phase. Having large hydrodynamic radii, exosomes could not enter the pores, resulting in early elution compared to components with smaller hydrodynamic radii that could enter the pores, resulting in late elution. As SEC depends on gravity to perform, this method could preserve the intact structures and bioactivity of exosomes (P. Li et al., 2017). Isolating a higher purity exosome could be obtained through HPLC (Lai et al., 2010); however, this method will face scaling up and equipment availability issues (Zeringer et al., 2015).

Flow field-flow fractionation (F|FFF) is a more recent method of exosome isolation based on the distinctions in hydrodynamic diameter using a porous rectangular channel where the smaller particles would flow further and elute earlier compared to the larger ones (Kang et al., 2008). This method is typically used to investigate the size distribution of starches and celluloses (Qureshi & Kok, 2011); however, it was successfully used to characterize the size distribution and isolate exosomes from various sources (Kang et al., 2008; Sitar et al., 2015; J. S. Yang et al., 2017; H. Zhang et al., 2018).

2.3 | Immunoaffinity

Within the membranes of exosomes lies numerous proteins and receptors, which could be utilized as a highly specific isolation technique based on the interactions between the antigen proteins and matching antibodies (P. Li et al., 2017). The protein target should be bound within the membrane, absent as a soluble component within the sample, and abundant or exclusively present in the exosomes to separate the exosomes from other vesicles or fragments within the sample (Zarovni et al., 2015). This method could be conducted by coating magnetic beads with matching antibodies targeting proteins within the membrane of the exosomes and incubating them with the target sample (Théry et al., 2006).

Releasing the bound exosomes from the beads would pose another challenge to overcome, especially in scaled-up condition (Akuma et al., 2019); however, a recent study managed to exploit the specific interaction between the phosphatidylserine along the surface of exosomes and Tim4 protein that was dependent on Ca^{2+}, thus allowing the easy release of the exosomes from the beads by using Ca^{2+} chelators (Nakai et al., 2016). Despite its high potential, the immunoaffinity method has not been studied extensively to isolate PDENs, probably due to a lack of extensive knowledge about the PDENs surface composition and the antibody–antigen interaction that could be utilized in isolating PDENs. One drawback of the immunoaffinity method comes from its high specificity because of the heterogenous nature of exosomes; only parts of the exosome within a sample would possess the antigen target, whereas the other subpopulation which did not have such antigen target would not be captured by the antibody used in the experiment (Théry et al., 2006).

2.4 | Precipitation

As purification through ultracentrifugation was deemed an expensive method for practical application, a more cost-effective method of exosome extraction was developed using a series of centrifugation and purification by polyethylene glycol-6000 (PEG6000). This crowding agent could create a net-like structure that could trap PDENs before being precipitated (Kalarikkal et al., 2020). This method has an extended utilization to precipitate small particles and even viruses (Adams, 1973; Lewis & Metcalf, 1988; Yamamoto et al., 1970). By adjusting the concentration of PEG6000 used in the purification, this method managed to result in different average nanoparticles with size ranging from 365 nm, 304 nm, 256 nm, and 252 nm at 8%, 10%, 12%, and 15% PEG6000, respectively compared to 403 nm obtained by ultracentrifugation. The PDENs obtained from this method had similar macrophage cell uptake efficiency and profiles of small RNAs, proteins, and lipid, despite lower content of bioactive polyphenol compounds as well as lower yield compared to that from ultracentrifugation (Kalarikkal et al., 2020). The precipitation method also tends to co-precipitate other non-exosome impurities, for example, various vesicles, aggregates, complexes of proteins, and large-sized proteins (Zarovni et al., 2015). A commercially available kit based on precipitation methods such as ExoQuick™ is also available for use (System Biosciences).

3 | CONTENT OF PDENS

PDENs would typically contain various components such as proteins, lipids, and genetic materials such as microRNAs (miRNAs) as could be seen in Figure 2. However, an extensive database on the components derived from variously sourced PDENs is not currently available as each plant would have its unique characteristics. Each component of the exosome has its functionalities in the bioactivities; for example, the protein profile will determine the uptake mechanism, the lipid is necessary for efficient absorption by the cells, while the miRNAs would alter the gene expression of the cells that absorb the PDENs.

Unfortunately, a specific marker of PDENs based on its content has not currently been established as each plant

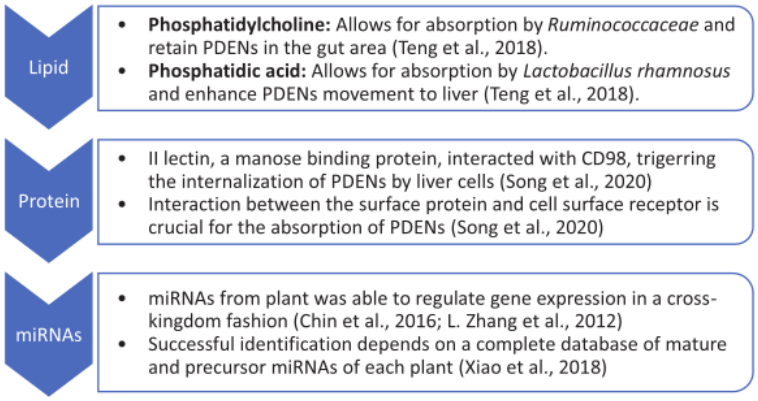

FIGURE 2 General contents of PDENs and their functions

has its characteristic miRNAs content. Furthermore, there has not been a study reporting the difference and similarities of miRNAs content of PDENs from the same fruits or vegetables but differing in their varieties. Although the surface protein marker of mammalian exosomes has been established, such as fusion and membrane transport proteins, heat shock proteins, or tetraspanins surface protein markers for exosomes such as CD63, CD9, and CD81 (X. X. Yang et al., 2019), the surface markers of PDENs has not been established at the moment. An extensive research should be conducted in the future to analyse which surface proteins present in all PDENs could be utilized as the surface marker of PDENs. The discovery of such a marker could allow researchers to better identify and characterize PDENs. The contents typically found in PDENs are highlighted as follows.

3.1 | Proteins

PDENs were found to contain a low concentration of protein, most of which were cytosolic proteins, namely actin, proteases, as well as membrane proteins that could function as a channel and transporter within the membrane itself, such as aquaporins and chloride channels (M. Zhang, Viennois, Prasad, et al., 2016). Complete profiling of proteins inside PDENs is quite challenging since different plants require different sets of protein databases for matching.

A study managed to characterize the proteins of lemon-derived nanoparticles, which identified 580 proteins; however, their health roles remain unanswered as no study has been conducted to analyse it. Interestingly, 56.7% of the identified proteins matched proteins from mammalian exosomes based on the ExoCarta protein database (Raimondo et al., 2015). Mammalian-derived exosomes typically comprise of more than 1000 proteins, while plant exosomes such as those derived from ginger would typically contain only 28 proteins (Ju et al., 2013; M. Zhang, Viennois, Xu, et al., 2016).

It was claimed that PDENs secretion sourced from Arabidopsis leaves was increased when the plants were facing pathogen infection or stress (salicylic acid). Based on the proteomic analysis, the PDENs were rich in proteins helping the plant, whether biotic or abiotic, cope against stress (Rutter & Innes, 2017).

Surface proteins of PDENs were reported to play a role in its uptake mechanism by human liver cancer cells (HepG2) as it was shown that when the PDENs were digested with trypsin to remove such proteins, the absorption was significantly reduced compared to the intact PDENs (Song et al., 2020). The membrane of garlic-derived exosome-like nanoparticles was rich in II lectin, a mannose-binding protein that was found to interact with the CD98 receptors on the target cells, which triggered the absorption of PDENs by the cells (Song et al., 2020). This finding provided a more in-depth understanding of the mechanisms of PDENs uptake by cells.

3.2 | Lipids

The structure and lipid content of PDENs were essential in contributing to its unique uptake by the intestinal cells. The mixture of lipid extracted from grape-derived exosome-like nanoparticles was not detected in the intestinal stem cells when orally administered into mice. In contrast, when the same mixture of lipids was assembled into liposome-like nanoparticles, they could be absorbed by the intestinal stem cells (Ju et al., 2013). PDENs tended to contain a high concentration of phospholipids, but mammalian-derived exosomes were abundant in cholesterol and sphingomyelin (M. Zhang, Viennois, Xu, et al., 2016).

Nanoparticles of grapefruit and garlic origin mostly consisted of phosphatidylcholine (PC) as their main lipid;

however, nanoparticles of ginger and turmeric origin mostly consisted of phosphatidic acid (PA) as their main lipid (Teng et al., 2018). This difference in lipid profiles affected each nanoparticle's target; PC was necessary for the uptake of nanoparticles by *Ruminococcaceae*. In contrast, PA was necessary for the uptake of nanoparticles by *Lactobacillus rhamnosus* (LGG) in the guts. The depletion of PA in ginger-derived exosome-like nanoparticles (GDENs) hinders its uptake by LGG, and the depletion of PC in grapefruit-derived exosome-like nanoparticles (GFDENs) hinders its uptake by *Ruminococcaceae* (Teng et al., 2018).

On top of that, PA lipids tended to retain the nanoparticles in the gut area. In contrast, PC tended to enhance nanoparticle movement into the liver cells, as shown by the imaging analysis of mouse intestinal cells and liver cells after 1 hr and 6 hr upon consumption of DiR-labeled GDENs and GFDENs (Teng et al., 2018). Grape-derived nanoparticles were also abundant in PA (53.2%). Its lipid bilayer assembly was crucial in the nanoparticles' activities as oral consumption of lipids from grape nanoparticles did not accumulate in the mice intestinal stem cells. In contrast, the liposomes made from similar lipids could be absorbed by the intestinal stem cells (Ju et al., 2013).

3.3 | miRNAs

miRNAs are a part of small RNA with a size of around 22 nt, lacking in coding properties whose main functions were to modulate gene expression by regulating cleavage or inhibiting translation of mRNAs (Bartel, 2004) or to induce the expression of specific target genes (Vasudevan et al., 2007). miRNAs could be found in various bodily fluids through passive leakage and active secretion of membrane vesicles such as exosomes or protein-miRNA complex (Redis et al., 2012). The old sayings of "you are what you eat" might bear some truth as numerous natural compounds, namely curcumin, genistein, EGCG, resveratrol, or quercetin, have been reported to regulate the expression of human miRNA and exert multiple health benefits (Otsuka et al., 2018)

Previous reports have shown that miRNAs could regulate the gene expression of species from the opposite kingdoms (Chin et al., 2016; L. Zhang et al., 2012). Another study has also attempted to extract and characterize different PDENs from 11 plant species (Xiao et al., 2018). It is important to note that although each PDENs had similarly sized miRNAs of around 20–22 nt, it was found that each species had different kinds of miRNAs ranging from 32 kinds in ginger to 127 kinds in soybeans. Unfortunately, the identification of miRNAs from plants was quite a difficult task to complete because pre-miRNAs sequences were not available for certain plants, lowering the matching ratios to a range of 43.88 % in grapefruit and 85.54% in blueberry. This huge variation in successful identification ratio indicated that many of the extracted genetic materials were still in their precursor form or the database was still not complete. When the potential pairing of those PDENs was compared with mammalian mRNAs, some of those miRNAs could hypothetically regulate mammalian genes, such as those that play a role in inflammation, immunity, and cancer signaling (Xiao et al., 2018).

4 | HEALTH FUNCTIONALITIES OF VARIOUSLY SOURCED PDENS

Many studies have been published to highlight the bioactivities of PDENs, such as reducing inflammation, encouraging the healing process, preventing gingivitis, supporting the growth of beneficial intestinal microbiota, as well as preventing cancer and infection as highlighted in Figure 1. However, the studies were *in-vitro* or utilizing animal models with no clinical trials on the efficacy of PDENs been published. Despite all that, two ongoing clinical trials in the USA have been reported. One clinical trial was investigating the effect of ginger and aloe PDENs to alleviate the resistance of insulin and inflammation on patients with a history of polycystic ovary syndrome (clinicaltrials.gov, NCT03493984), while another clinical trial was to look into the effect of grape PDENs in averting the oral mucositis due to chemoradiation on patients with head and neck cancer (clinicaltrials.gov, NCT01668849). Further clinical trials would be necessary to confirm the human bioactivities of PDENs and indicate the minimum dosage necessary to display health functionalities. The bioactivities and mechanisms of certain PDENs are highlighted in the following section.

4.1 | Ginger

Ginger (*Zingiber officinale*) is a very well-known spice and herbal medicine, especially in Asia that possesses many health functions such as an antioxidant (Nile & Park, 2015), reducing inflammation (Nile & Park, 2015; M. Zhang, Viennois, Prasad, et al., 2016), preventing microbial growth (Kumar et al., 2014), alleviating cancer (Morimoto et al., 2019), protecting nervous (Ho et al., 2013), cardiovascular (Akinyemi et al., 2015), and even respiratory system (Townsend et al., 2013). A complete review of ginger's health effects is available (Mao et al., 2019), while a thorough review of ginger's health effects on gastrointestinal cancer is also available (Prasad & Tyagi, 2015).

Many studies have also attempted to obtain ginger-derived exosome-like nanoparticles (GDENs) from ginger juice and study its bioactivities because of many health functionalities possessed by ginger. Ginger was shown to produce a similar yield of EVs compared with other plants such as grape, grapefruit, and carrot; however, ginger managed to produce a much higher RNA concentration (Mu et al., 2014). Interestingly, it was found that by altering the pH of the surrounding solution, the physical properties of the nanoparticles were changed as well. GDENs were shown to decrease in negative charge and generated a larger subgroup of GDENs by immersing them in two kinds of solutions mimicking the pH of the stomach and intestine at pH 2.0 and 6.5, respectively (Mu et al., 2014). Using confocal analysis of the mouse intestinal tissues, orally administered PDENs (ginger, grape, carrot, and grapefruit) were shown to be absorbed by macrophages and intestinal stem cells at a similar rate (Mu et al., 2014). A later study showed that the uptake of GDENs in the colon area was due to gut macrophages and intestinal stem cells (M. Zhang, Viennois, Prasad, et al., 2016). GDENs was able to increase the gene expression of both anti-inflammatory cytokines HO-1 and IL-10, as well as proinflammatory cytokines IL-6 and TNFα at a significantly higher rate, compared to other PDENs, indicating the potency of GDENs to maintain gut homeostasis (Mu et al., 2014).

Further research to study GDENs to alleviate the symptoms of inflammatory bowel disease found that three distinct bands of GDENs were generated from differential centrifugation and discontinuous sugar gradient purification, namely GDENs 1,2, and 3 with a total yield of 50 mg per 1 kg of fresh ginger where GDENs 1 and 2 were able to tolerate multiple freeze-thaw cycles and be stable for 7 days at room temperature whereas GDENs 3 could not (M. Zhang, Viennois, Prasad, et al., 2016). It was also found that GDENs contained a small number of proteins and miRNAs, 124 of which could potentially regulate human gene expression, various kinds of lipids, as well as bioactive compounds (6-gingerol and 6-shogaol) that are present mostly in GDENs 2. Intriguingly, when orally administered to mice, GDENs showed greater retention in the colon area by starved mice than non-starved ones, indicating the interaction of food digestion to the retention of GDENs in the colon area. GDENs 2 were also able to yield an anti-inflammatory effect by lowering lipocalin-2 level, a biomarker of gut inflammation, and did not pose both local and systemic side effects nor affect cell viability. GDENs 2 was also shown to increase the production of anti-inflammatory cytokine while decreasing the production of proinflammatory cytokine. It was also proven, through both in-vivo and *in-vitro* assays, to encourage the mucosal tissue healing of colitis induced mice (M. Zhang, Viennois, Prasad, et al., 2016).

GDENs were also shown to be absorbed by the guts and transported into the livers of mice to prevent liver damage induced by alcohol through inhibiting the reactive oxygen species (ROS) production created by the alcohol metabolism (Zhuang et al., 2015). This protective effect was due to the 6-shogaol content of GDENs that could be absorbed much more readily than its free form that passes through the guts, activating Nrf2 by TLR4/TRIF pathway (Zhuang et al., 2015). On top of that, GDENs were also reported to inhibit the assembling and activation of the NLRP3 inflammasome, whose abnormal activities are connected to various diseases such as type 2 diabetes, multiple sclerosis, and atherosclerosis (Chen & Yu, 2019).

Besides, phosphatidylcholine lipid content in GDENs caused *Lactobacillus rhamnosus*, one of the gut microbiota, to favorably absorb GDENs, causing a cascade of reactions to stimulate the expression of IL-22 cytokines and induce gut tissue healing of colitis induced mice through activating the AHR pathway by the miRNA content of GDENs as well as increasing the homeostasis between the immune system and gut microbiota, adjusting the microbiota composition, increasing the production of metabolites, supporting the microbiota growth, and regulating the gut microbiota localization (Teng et al., 2018).

Additionally, *Porphyromonas gingivalis*, a well-known gum disease pathogen, was reported to selectively absorb GDENs through interaction between the phosphatidic acid on the GDENs membrane and the hemin-binding protein 35 (HBP35) located at the exterior of *P. gingivalis*. Upon internalization, the content of GDENs was released inside the bacteria, reducing its pathogenicity, depolarizing its membrane, inhibiting its ability to attach and attack oral epithelial cells and preventing bone loss of the teeth induced by *P. gingivalis* (Sundaram et al., 2019).

4.2 | Citrus fruits

Lemon-derived exosome-like nanoparticles (LDENs) are currently one of the most studied citrus-based nanoparticles. It was shown that LDENs isolated by differential centrifugation from lemon (*Citrus limon L.*) acted as an antioxidant based on an *in-vitro* study using mesenchymal stromal cells (MSC) due to their content of small RNAs, vitamin C, and citrate preserved inside the LDENs, absorbed by the MSC to exert the potential of cross-species influence from food intake (Baldini et al., 2018).

On top of that, LDENs were shown to be absorbed by cancer cells at 37°C but not at 4°C, indicating that the uptake was regulated by a biological process that is active at normal body temperature. Through

Methyl-thiazol-tetrazolium (MTT) assay, LDENs was able to hinder three types of cancer cells (A549, SW480, and LAMA84) from growing after a 48-hr incubation at a concentration of 20 μg/ml LDENs by inducing the expression of pro-apoptotic genes of cancer and tumor cells (Raimondo et al., 2015). Interestingly, LDENs did not inhibit normal cells' growth, and the destruction of the nanovesicles impaired their anti-proliferative activities through boiling or sonication (Raimondo et al., 2015).

The death of cancer cells was mediated by TRAIL signaling, which was confirmed by the absence of death of cancer cells when the cells were incubated with TRAIL neutralizing antibodies (Raimondo et al., 2015). This anticancer activity was confirmed in-vivo by injecting LDENs into the tumor sites or intraperitoneally of LAMA84 inoculated mice that showed the reduction of tumor growth and size by both local and intraperitoneal injection of LDENs into the mice (Raimondo et al., 2015). As the LDENs were injected intraperitoneally into the mice, the LDENs were quickly distributed within 15 minutes and up to 24 hr into the its organs, such as liver, spleen, and kidneys, as well as into the tumor tissue (Raimondo et al., 2015)

LDENs were also reported to reduce the mortality and bacterial shedding on mice when infected with *Clostridioides difficil*. However, this protection was done by modulating two probiotics, namely *Lactobacillus rhamnosus* GG and *Streptococcus thermophilus* ST-21. LDENs increased the viability of the probiotics to withstand digestion which in turn, reduced the mortality of the mice when infected with such pathogen. This modulation induced a cascade of metabolic reactions, which increase the lactic acid production in the gut to inhibit the growth and shedding of *C. difficil*, reducing the mortality and chance of transmission (Lei et al., 2020).

Grapefruit-derived exosome-like nanoparticles (GFDENs) have been reported to contain much less total RNA content compared with GDENs and experienced a reduction in negative charge when submerged in a solution mimicking the stomach environment but not in a solution mimicking intestinal environment (Mu et al., 2014). In contrast with GDENs, GFDENs did not induce anti-inflammatory cytokines such as IL-10 and HO-1 but managed to induce the translocation of macrophage Nrf2 as well as the activation of Wnt/TCF (Mu et al., 2014). However, other studies managed to show that GFDENs were absorbed by the macrophages in the intestine to decrease the DSS (dextran sulfate sodium) induced colon inflammation by reducing the expression of proinflammatory cytokines such as IL-6 and TNF-α (Wang et al., 2014) and were selectively transported into the liver tissues (Teng et al., 2018). This anti-inflammatory activity might have been caused by naringin and naringenin content in grapefruit DENs; however, as the author explicitly mentioned, further research was needed to pinpoint the bioactive components that contributed to the anti-inflammatory activities of grapefruit DENs (Wang et al., 2014).

4.3 | Other fruits and vegetables

Shiitake mushroom-derived exosome-like nanoparticles (SDENs) were also found to inhibit the production of macrophage NLRP3 inflammasome and proinflammatory cytokines, namely IL-1β and IL-18 that were induced by lipopolysaccharide and free fatty acid (sodium palmitate). In contrast, six other mushroom DENs did not inhibit the production of NLRP3 (Liu et al., 2020). Intraperitoneal administration of SDENs into mice was also able to protect the liver from acute injury caused by an injection of GaIN/LPS as marked by the reduction in serum AST and ALT levels as well as proinflammatory cytokines (IL-1β and IL-18 but not TNFα), thus showing its potential in alleviating fulminant hepatic failure (FHF) disease in human (Liu et al., 2020). However, the responsible bioactive compounds that managed to induce such a protective effect were not identified in the study.

By an in-vivo and *in-vitro* study, orally administered grape DENs (GRDENs) was shown to be able to pass through the mouse intestinal mucus barrier, transported into the intestinal stem cells, stimulate the proliferation of $Lgr5^+$ stem cells via Wnt/β-catenin pathway, thus increasing the creation of intestinal epithelial cells to restore the mouse intestinal structure from the damage caused by DSS-induced colitis (Ju et al., 2013). Another study has also shown that Syrah GRDENs were able to pass through the intestinal tract and promote the proliferation of Lgr5+ stem cells without any toxic effect against the liver and kidney shown by not significantly different level of AST/ALT/BUN/Cr levels compared to control (Ghiasi et al., 2018). Broccoli-derived exosome-like nanoparticles (BDENs) could also prevent colitis on rats that have been induced by DSS through stimulating tolerogenic intestinal dendritic cells (DC) by activating adenosine monophosphate-activated protein kinase (AMPK) of DCs that were facilitated by the BDENs lipid profile, as well as to maintain homeostasis of proinflammatory and anti-inflammatory cytokines (Deng et al., 2017).

An *in-vitro* study reported that blueberry-derived exosome-like nanoparticles were absorbed by human endothelial cells in a dose-dependent manner to counteract the shift in the gene expression induced by TNF-α by decreasing the production of reactive oxygen species, increasing cell viability, and regulating the expression of 29 genes (De Robertis et al., 2020).

5 | APPLICATIONS OF PDENS AS AN INGREDIENT OF FUNCTIONAL FOODS

Multiple studies have shown that PDENs have the potential to display various health benefits; therefore, it would be of interest to apply them as a novel functional food ingredient. However, the stabilities of PDENs in digestion, food processing, and storage should be clearly demonstrated before PDENs could be utilized in functional foods. PDENs were able to withstand its structure when exposed to the ex-vivo digestion process with only change in size and charge (Ghiasi et al., 2018; M. Zhang, Viennois, Prasad, et al., 2016). When given orally, the PDENs also managed to survive the digestion process and enter the colon area (M. Zhang, Viennois, Prasad, et al., 2016) and, depending on the source of PDENs, stayed in the colon area to alleviate gut inflammation or traveled into the liver cells (Teng et al., 2018). This report showed the potency of PDENs as an ingredient in functional food to alleviate various illnesses. However, PDENs are only functional when the membrane is intact (Ju et al., 2013); therefore, its integrity must be maintained throughout the shelf life and processing.

Although the stability of PDENs has been reported in the digestive environment, its stability during food processing and storage has not been extensively studied. This lack of in-depth knowledge on the stability of PDENs could be a promising future research question to solve. One study has reported that exosomes were stable at −80°C for up to 6 months while storage at 4°C could result in partial loss of exosomes (Munagala et al., 2016; Théry et al., 2006). Since no studies on the stabilities of PDENs have been reported, we then resorted to other sourced exosomes. Based on the research on urine-derived exosomes, they were reported to be stable when stored at 4°C, followed by freezing at −80°C (Cheruvanky et al., 2007). The requirement of −80°C freezing to stabilize the exosome structure could thwart the application of exosomes in functional foods due to its prohibitive cost in storage and distribution. Therefore, future research should not only focus on analysing the stabilities of PDENs in various storage condition, but also attempt to increase the stabilities of PDENs so they could be refrigerated or stored at ambient temperatures.

However, recent research showed that human saliva-derived exosomes were stable at refrigeration temperature even after 20 months of storage. However, they showed a decrease in membrane integrity when subjected to multiple freeze-thaw cycles at −20°C indicated by the shift of dipeptidyl peptidase IV activity from the exosome pellets into the supernatant liquid in which the isolated exosomes were stored (Kumeda et al., 2017). Therefore, the author suggested that storage at 4°C was ideal for long-term storage of human saliva-derived exosomes. However, urine-derived exosomes suffered a significant loss when stored at −20°C, whereas storing at −80°C for up to 7 months showed no significant degradation of exosomes (Zhou et al., 2006).

Another critical challenge in PDENs application in functional foods is its stability towards processing, especially foods that require heat treatment in the process. Intact exosomes were found in commercially pasteurized milk. They were more stable than macrophage-derived exosomes, as milk-derived exosomes could withstand unfavorable conditions such as acidification to pH 2, 15-minute boiling, and freeze-thaw cycle. In contrast, macrophage-derived exosomes were degraded by 90%, 90%, and 70% due to acidification, boiling, and freeze-thaw cycles, respectively (Pieters et al., 2015). However, another study reported that miRNAs contents of raw milk decreased by 63% after pasteurization and homogenization, which was hypothesized due to the disruptions of exosome membrane during processing, but miRNAs content was not affected significantly during storage at 4°C (Howard et al., 2015).

All these findings indicate that different sources of exosomes would possess distinct stability towards storage and processing conditions. There is no one-size-fits-all solution in utilizing PDENs as a functional food ingredient as each PDENs could behave differently in each step of the formulation, processing, and storage of the foods. Unfortunately, the stability of plant-derived exosomes has not been studied extensively at the moment, and its application towards commercially feasible functional foods remains a question that should be worthy of further research as PDENs could bring numerous health benefits.

6 | CONCLUSION

Plant-derived exosome-like nanoparticles have the potential to be stable to withstand the digestive environment. The types of lipids contained in the membrane of the PDENs would dictate its fate upon reaching the colon area. PDENs were shown to provide multiple health functionalities to alleviate numerous types of illnesses potentially. Milk-derived exosomes were stable in harsh processing and long-term storage; however, PDENs stability has not been fully understood. As exosomes-derived from different sources possessed different levels of stability, one could only hypothesize that each PDENs would also possess distinct stability towards processing and storage. As the intactness of PDENs membrane was crucial in their bioactivities, PDENs application in functional food products remains a high-value research problem to be solved.

ACKNOWLEDGMENTS

Research funding: Indonesian Collaboration Research, Contract no. 1761/IT3.L1/PN/2020

AUTHOR CONTRIBUTIONS

S. Suharta gathered and interpreted necessary data and drafted the manuscript. A. Barlian, C. H. Wijaya, A. C. Hidajah, H. B. Notobroto, D. Ana, S. Indraiani, and T. D. Kencana conceptualized the study and contributed intellectually to this manuscript by interpreting data, critically revising the manuscript, and approving the final version of the article.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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How to cite this article: Suharta, S., Barlian, A., Hidajah, A. C., Notobroto, H. B., Ana, I. D., Indariani, S., Wungu, T. D. K., & Wijaya, C. H.. Plant-derived exosome-like nanoparticles: A concise review on its extraction methods, content, bioactivities, and potential as functional food ingredient. *J Food Sci*. 2021;*86*:2838–2850. https://doi.org/10.1111/1750-3841.15787

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