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Isolation of Native Plant-Derived Exosome-like Nanoparticles and Their Uptake by Human Cells

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ABSTRACT

The exosome is a nano-sized vesicular particle commonly secreted from eukaryotic cells to extracellular space. Exosome functions in cellular communication, nutrients or bioactive compounds delivery, and cellular immunity. It is believed that plant-derived exosome-like particle (PDEN) potential to deliver nano-bioactive compounds to the human body. As Indonesia is one of the herbal centers of the world, we are encouraged to develop natural richness for human welfare. The study aimed to verify various methods to isolate exosomes from the fresh ginger rhizome and quina cells and examine the ginger PDENs' capacity to internalize *in vitro* human cells. The PDENs isolation was carried out by centrifugation and precipitation. The particles were observed through a transmission electron microscope, dynamic light scattering, and particle size analysis. Differential ultracentrifugation combined with PEG6000 provided a better size range of particles. Nanoparticles tracking analysis performed the concentration of particles within a specific size range. Ginger and quina PDENs size ranged from 40-650 nm, and the concentration was 3.6×10^7 and 2.8×10^6, respectively. The PKH67-labelled ginger PDENs were taken up by human dermal fibroblast and human Wharton's jelly mesenchymal stem cells after 30 minutes and 21 hours. DPPH assays revealed that ginger PDENs have antioxidant activity, indicating the presence of some nano-molecules in those particles. This study would be the baseline for developing ginger PDENs as a functional food with nano-ingredients, or as an effective vehicle of nano-drug, for human health.

1. Introduction

The exosome is an extracellular membrane-bound vesicle generated in the endosomal compartment of eukaryotic cells. Plant-derived exosome-like nanoparticles (PDENs) have gained increasing interest. Their roles in plants are similar to those in mammalian or general animals, i.e., in cell communication, even inter-kingdom communication, signal transfer, and nutrients delivery (Akuma *et al.* 2019; Mu *et al.* 2016), despite their functions and biogenesis are still unclear. Animal cells readily secrete exosomes to extracellular spaces from multivesicular bodies (MVBs) through phagocytosis, pinocytosis, or receptor-mediated endocytosis (Pedrioli and Paganetti 2021; Verdera *et al.* 2017); the processes which are not yet understandable in plant cells with their cell wall. Therefore, nanoparticles isolated from plant tissues are called exosome-like nanoparticles. Indeed, some people have isolated PDENs from the apoplastic fluids (Regente *et al.* 2012; Rutter and Innes 2017; Stanly *et al.* 2016; Woith *et al.* 2021). PDENs size ranges between 50 and 500

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nm (Kim *et al.* 2022; Yu *et al.* 2020) instead of 30 to 100 nm for animal exosomes (Raposo and Stoorvogel 2013).

Plants are a golden source of the human diet. Based on this fact, PDEN is believed capable of delivering nano-nutrient and nano-drugs to the human body. Additionally, a plant can supply PDENs in large amounts, a superior raw material of choice. Recently, food-derived exosomes (FDEs) are also becoming popular to accommodate all the terms mentioned in food-related studies (Munir *et al.* 2020). Many researchers have attempted to extract and characterize PDENs from various edible plants, such as ginger (Kalarikkal *et al.* 2020; Mu *et al.* 2016; Suresh *et al.* 2021; Zhang *et al.* 2016a, 2016b), strawberry (Perut *et al.* 2021), grape (Mu *et al.* 2016), grapefruit (Garaeva *et al.* 2021; Mu *et al.* 2016), carrot (Mu *et al.* 2016), coconut water (Zhao *et al.* 2018), and cabbage (You *et al.* 2021). Biological fluids and *in vitro* cell/callus cultures could be used to get plant exosome-like vesicles by various techniques and strategies (Rutter and Innes 2017; Stanly *et al.* 2016; Woith *et al.* 2021; You *et al.* 2021).

Many studies have reported the biological activities of PDENs in human cells, such as reducing inflammation, preventing gingivitis, empowering the healing process of cancer, nervous system, and musculoskeletal disorder, supporting infant health, enhancing the beneficial intestinal microbiota, as well as preventing cancer and infection (Munir *et al.* 2020; Suharta *et al.* 2021). PDENs contain proteins, lipids, and genetic materials, mainly micro RNAs, which could exert multiple functionalities and health benefits when consumed in sufficient amounts (Garaeva *et al.* 2021; Suharta *et al.* 2021). Their stability modulates gene expression in a cross-kingdom fashion (Munir *et al.* 2020). As exosomes were biogenetically derived, their components and properties reflect the processes in their cells of origin (Hu *et al.* 2020; Kim *et al.* 2022). The type of lipids and proteins contained in the membrane of the PDENs would direct their fate upon reaching the recipient cells. It was suggested that PDENs were stable to withstand digestive conditions. Succeeding their absorption by intestinal cells and further traveling through the bloodstream, exosomes can reach distant organs and affect overall health. Therefore, PDENs could take part as potential functional food.

Indonesia is well-known as one of the herbal centers of the world with its mega biodiversity. We are encouraged to take part in the exploration of the potency brought by the natural richness of the country. Ginger rhizome has been well-known as a spice and herbal medicine for centuries. It contains over a hundred compounds, with the major classes gingerol, shogaols, zingiberene, and zingerone. It reduces nausea and vomiting in pregnancy, inflammation, and menstrual cramp, and has a digestive function (Anh *et al.* 2020). Quina plant produces quinoline alkaloids; among them is quinine, which is used to treat malaria and nocturnal leg cramps and has an anti-inflammatory effect (El-Tawil *et al.* 2010; Kabera *et al.* 2014; Maldonado *et al.* 2017), while quinidine is an anti-arrhythmia (Sheldon *et al.* 1995). The study applied several isolation methods on the fresh ginger rhizome and quina friable callus, aiming to compare their efficacy in obtaining the desirable PDENs. We also intended to examine the capacity of ginger PDENs to be taken in vitro by human cells. The findings would be the baseline for developing ginger PDENs as a functional food and nano drug vehicle for human health.

2. Materials and Methods

2.1. Plant Materials

Plant materials consisted of fresh ginger (*Zingiber officinalis*) rhizome var. Gajah and friable callus of *Cinchona ledgeriana* (quina plant) having been cultured on semi-solid media. The quina callus has been maintained for five years through several subcultures.

2.2. Methods

2.2.1. Extraction and Particles Isolation

Fresh ginger rhizome was peeled off and crushed in a blender for 30 seconds. As much as 2-3 g of the crushed rhizome and the quina callus were mixed with 10 ml ddH_2O by vortexing for about 1 min. The mixture was passed through a 100 µm nylon filter to get the filtrate. The extraction was carried out through differential centrifugation steps in ddH_2O. We followed two procedures: the first was Procedure A, described by Kalarikkal *et al.* (2020), and the second was Procedure B by Woith and Melzig (2019), with slight modifications. The two procedures differ in the centrifugation steps of isolation. The PDENs isolation was carried out using a refrigerated (4°C) centrifuge (Centurion Sci. K3 Series, UK) and refrigerated ultracentrifuge (Himac CS150NX, Japan). The supernatant collected from the last centrifugation step was mixed with Polyethylene Glycol (PEG6000, BDH, England. 1:1 v/v) or Total Exosome Isolation Reagent (TEIR, 1:0.5 v/v, Invitrogen-Thermo Fisher Sci.) as the precipitating agent. A brief vortexing homogenized the mixture; then, it was incubated overnight at 4°C. The following day, the mixture proceeded to

centrifugation; the upper liquid was removed very carefully, and the precipitated pellet was dissolved in ddH_2O or 1xPBS (1:1 v/v). The detailed methods of isolation are presented in Table 1.

2.2.2. Observation and Evaluation of PDENs

The isolated samples were observed using a Transmission Electron Microscope (TEM, Hitachi HT-7700, Japan) using a negative staining method to observe the particle morphology. Dynamic Light Scattering (DLS) and Particle Size Analysis (PSA) (Horiba SZ100 nanoparticle analyzer, Japan) were performed to get the size of the particles along with their distribution. The analysis was set at 25°C with a refractive index of 1.31 and viscosity of 0.89 cp. The analysis and observation were done in triplicate. Besides we also used NTA (nanoparticle tracking analysis) to reconfirm the PDENs size and concentration. Using a view sizer (Horiba Sci., Japan. Type 3000/0053; camera AVT Manta 319C), at 22°C, 1 ml of PDEN samples was diluted in ddH_2O. The average viscosity was 0.95 cp.

2.2.3. Analysis of Cell Uptake of the PDENs by Human Cells

The analysis of PDENs uptake by cells used human dermal fibroblast (HDF) and human Wharton's jelly mesenchymal (hWj MSC) stem cells. The PDEN was labeled using PKH67 green fluorescent cell linker mini kit (Sigma-Aldrich) and followed the protocol with slight modification. As much as 50 uL of the PDENs sample was mixed with 500 uL Diluent C; the same volume of Diluent C was added to 2 uL PKH67. The PDENs samples and PKH67 in Diluents were mixed, and the mixture was incubated at room temperature for 4 min. Subsequently, 1 ml 1% Bovine Serum Albumin (BSA) (Sigma) was added and then incubated for 1 min. The last mixture was uploaded into Amicon Ultra centrifugal filter units (Millipore) for centrifugation at 4,000 x g for 15 min. The pellet was washed with Phosphate Buffer Saline (PBS) three times, each time by passing through centrifugation. The solid sample (pellet) was dissolved in Dulbecco's Modified Eagle Medium (DMEM)-low glucose (Gibco) media equipped with 1% antibiotic-antimycotic solution (AbAm, Gibco) and ready for use.

The stem cells were grown on coverslips in a six-well plate with a density of 50,000 cells/well. The cells' medium comprised DMEM-low glucose, 10% FBS (Foetal Bovine Serum, Gibco), and 1% AbAm. The cells were incubated for two days at 37°C and 5% CO_2. Then the mounting medium was removed through three washing passages with PBS. The cells were subsequently mixed with the labeled PDENs solution and incubated at 37°C and 5% CO_2 for 30

Table 1. Various PDENs isolation procedures of ginger rhizome and quina callus

Sample sources	Centrifugation steps	Precipitation	Centrifugation steps
	Procedure A with PEG6000		
Fresh ginger rhizome and quina callus	• 2,000 x g – 10 min • 6,000 x g – 20 min • 10,000 x g – 40 min	In PEG 6000: 8, 10, 12, 15%. Incubated overnight at 4°C	8,000 x g – 30 min. Pellet dissolved in ddH_2O
	Procedure A with TEIR		
Fresh ginger rhizome and quina callus	• 2,000 x g – 10 min • 6,000 x g – 20 min • 10,000 x g – 40 min	In Invitrogen TEIR. Incubated overnight at 4°C	10,000 x g – 60 min. Pellet dissolved in 1xPBS
	Procedure B with PEG6000		
a. Fresh ginger rhizome	• 6,000 x g - 20 min • 15,000 x g - 20 min • 50,000 x g - 60 min	In PEG 6000 10%, and incubated overnight at 4°C	10,000 x g – 60 min. Pellet dissolved in ddH_2O
b. Quina callus	• 2,000 x g - 20 min • 6,000 x g - 20 min • 15,000 x g - 40 min	In PEG 6000 10%, and incubated overnight at 4°C	10,000 x g – 60 min. Pellet dissolved in ddH_2O
	Procedure B with TEIR		
a. Fresh ginger rhizome	• 6,000 x g - 20 min • 15,000 x g - 20 min	In Invitrogen TEIR. Incubated overnight at 4°C.	10,000 x g – 60 min. Pellet dissolved in 1xPBS
b. Quina callus	• 2,000 x g - 20 min • 6,000 x g - 20 min	In Invitrogen TEIR. Incubated overnight at 4°C.	10,000 x g – 60 min. Pellet dissolved in 1xPBS

min and 21 hours. At the end of incubation, the uptaking process was stopped by fixing the PDEN-treated cells with 4% paraformaldehyde for 15 min. The cells were washed with PBS prior to the DAPI (4′,6-diamidino-2-phenylindole, Thermo Fisher, USA) staining for 10 min. After the last wash with PBS, the cells were observed and imaged with a Confocal Laser Scanning Microscope (Olympus Fv1200).

2.2.4. Antioxidant Activity Analysis

The antioxidant activity in terms of ascorbic acid equivalent antioxidant capacity (AEAC), was determined using the method of Meda *et al.* (2005) with modifications. As much as 2.5 µL was added with 100 µL sodium acetate buffer (pH 5.5) and 187.5 µL methanol. A 10 µL aliquot of 3 M DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was put into the sample and the positive control mixture. For negative control, we added 10 µL ethanol. All the mixtures were incubated for 30 min at room temperature and in darkness. The antioxidant content was measured by a UV-Vis spectrophotometer (EPOCH type BioTech, USA) at 517 nm.

3. Results

3.1. Size, Distribution, and Concentration of the Isolated PDENs

By applying procedure A, we found that all the samples produced much better particle structure in 10% of the precipitation solution (PEG6000) than in 8, 12, or 15%. Therefore, we used only 10% PEG6000 in further isolations. Some examples of PDENs derived from fresh ginger rhizome and quina callus isolated by 10% PEG6000 and by Invitrogen TEIR are presented in Figure 1. They were mostly spherical with a distinct envelope.

DLS and PSA analysis demonstrated a broad PDENs size distribution. Table 2 shows that the particle

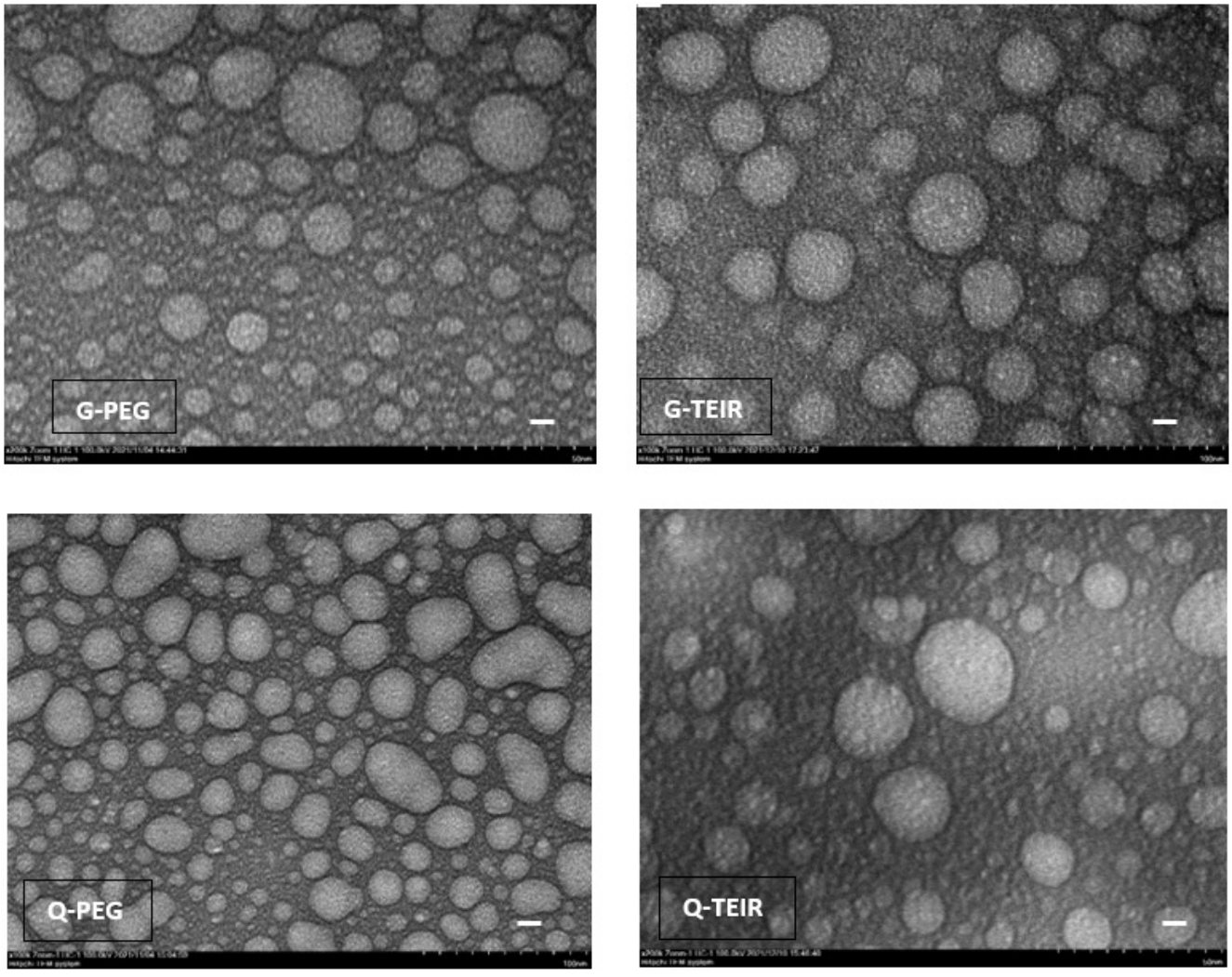


Figure 1. Transmission electron microscopy images of PDENs isolated from ginger rhizome (G) and quina callus (Q) using PEG6000 or Invitrogen TEIR precipitating solution. Bar scale = 100 nm

Table 2. PDEN sizes and their frequency in the samples isolated with PEG6000 or Invitrogen TEIR

Isolated samples	Average size (nm)	The smallest particles		Particles sizes with the highest frequency	
		Size (nm)	Frequency (%)	Size (nm)	Frequency (%)
		Procedure A			
G-PEG	1664.20±272.96	278.29±150.97	3.82	3795.60±1612.93	33.97
G-TEIR	1327.87±39.14	349.67±75.72	2.64	1967.14±0.00	15.81
Q-PEG	445.93±47.78	82.39±46.44	0.30	345.89±50.90	6.31
Q-TEIR	787.43±134.69	142.46±45.83	3.16	1308.95±776.49	44.65
		Procedure B			
G-PEG	528.93±44.96	132.81±31.95	0.96	379.50±142.07	14.85
G-TEIR	104906±28.64	371.61±21.80	5.03	2318.69±136.02	23.47
Q-PEG	431.97±16.14	79.95±11.76	6.27	630.64±35.52	45.80
Q-TEIR	1080.37±66.53	220.06±62.13	8.87	1295.61±1351.33	14.53

G = ginger, Q = quina. Data presented are means ± SE, based on three replicates

sizes with the highest frequency were still 5000s nm (33.97%) for ginger and 2000s nm (44.65%) for quina, particularly those produced by procedure A of isolation. From ginger rhizome, we obtained above 1000s nm particles on average, with the smallest sizes around 300s nm, while from quina cells, 446 to 900 nm on average, with the smallest sizes around 100 nm. At this stage, we could not say which precipitating solution (PEG6000 or TEIR) was more favorable for isolating PDENs from both plant materials. However, quina friable callus generally gave smaller particle sizes on average with PEG6000, either in Procedure A or B. By applying procedure B, we noticed that the ultracentrifugation steps combined with the PEG6000 precipitating solution produced particles with the average size and the smallest size better than the Invitrogen TEIR precipitating solution (Table 2). We obtained ginger PDENs size ranged 100-600 nm, the average size was 525 nm, and the highest frequency was between 230-580 nm. Meanwhile, the quina PDENs size ranged from 70-650 nm, the average size was 432 nm, and the greatest frequency was between 600-670 nm.

The NTA results demonstrated that the particle size of ginger and quina PDENs started from about 40 nm, and the average size was 100 to 200 nm, as shown in Figure 2. The PDEN samples resulted from procedure B with PEG6000 precipitating agent. However, the concentration of quina PDENs was lower than that obtained from ginger. Within 30-150 nm size ranges, the ginger and quina PDENs concentrations were 3.6×10^7 and 2.8×10^6 particles/ml, respectively (Table 3). The modal size presents the particle size with the highest frequency to appear. Figure 2A represents the particle size distribution of ginger PDENs and Figure 2B of quina PDENs within that size range.

Table 3. PThe concentration and size of ginger and quina PDENs resulted from procedure B with PEG6000 by NTA analysis

Parameter	Ginger PDEN	Quina PDEN
Concentration (particles/ml)	3.6×10^7	2.8×10^6
Size average (nm)	111±24	108±27
Modal size (nm)	103	202

3.2. PDEN Uptake Into Human Cells

Trials on the penetration capacity of the PDENs into human cells were carried out only on ginger. Ginger PDENs were taken since they are safer for the human body. Quina cells contain quinoline alkaloids that could give various adverse effects, such as severe allergic reactions or bleeding on certain people (Hogan 2015). It was observed that the internalization of ginger PDENs into the HDF cells took place only after 21 hours of incubation (Figure 3A). Meanwhile, with the mesenchymal (hWj MSC) cells, the ginger PDENs were taken up by the cells as earlier as 30 min of incubation (Figure 3B). They are located around the cell nucleus.

3.3. Antioxidant Activity of PDENs

As ginger is already well-known as healthy food and commonly used in herbal medication, we examined whether its extracted particles (PDENs) still have the potential as antioxidants.

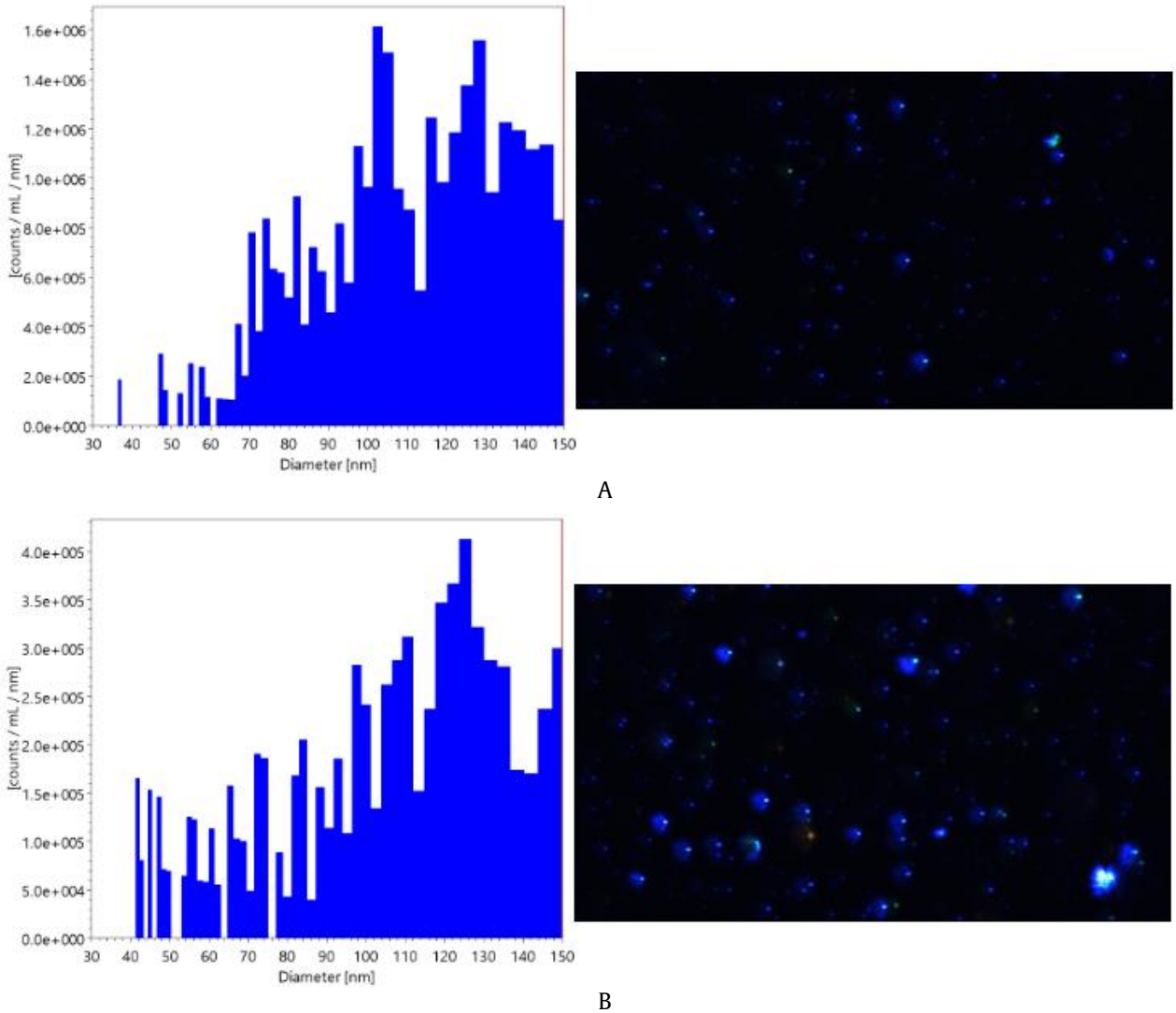
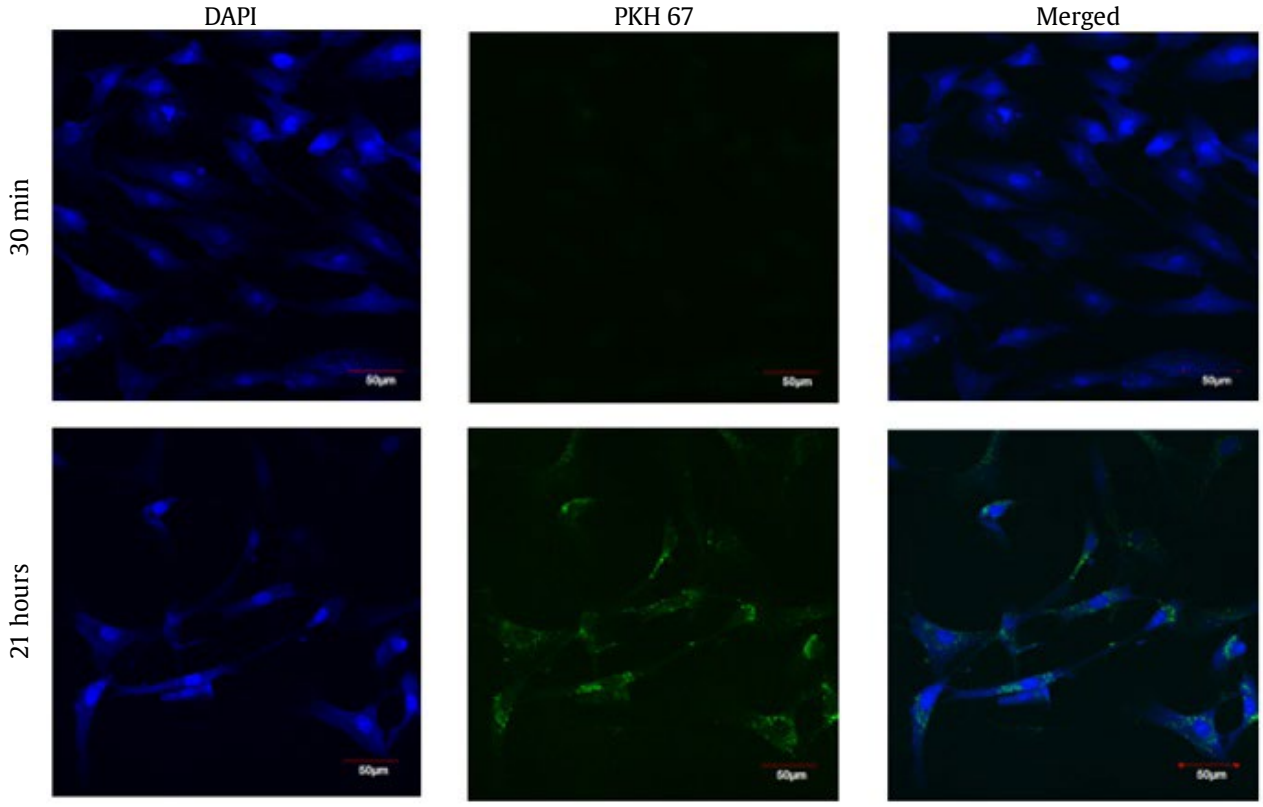


Figure 2. NTA analysis showing the concentration and distribution of ginger (A) and quina (B) PDENs by size

The antioxidant content in the particle samples was represented by ascorbic acid (AEAC) (Figure 4). Water extracts manually collected from fresh rhizomes were taken as the control. The data show that ginger PDENs still possessed high antioxidant capacity. Interestingly, particle samples from the Invitrogen TEIR precipitation solution demonstrated much more substantial capacity than those from PEG6000.

4. Discussion

The extraction and isolation method are determinants for the size, distribution, and yield of the particles obtained. After being isolated, their size was determined by DLS/PSA. The morphological analysis through transmission electron microscopy (TEM) revealed that ginger and quina PDENs substantially exhibit a uniform structure and have spherical morphology, corroborated with Garaeva *et al.* (2021) with grapefruit PDENs, while Woith *et al.* (2021) reported cup-shaped vesicles from *Craterostigma plantagineum* and *Nicotiana tabacum*. From the isolation methods we applied, the results might be the vesicle bodies or extracellular vesicles (EVs) in the majority, those with sizes above 1,000 nm. However, they were also mixed with the PDENs or nanovesicles, as we found the particle



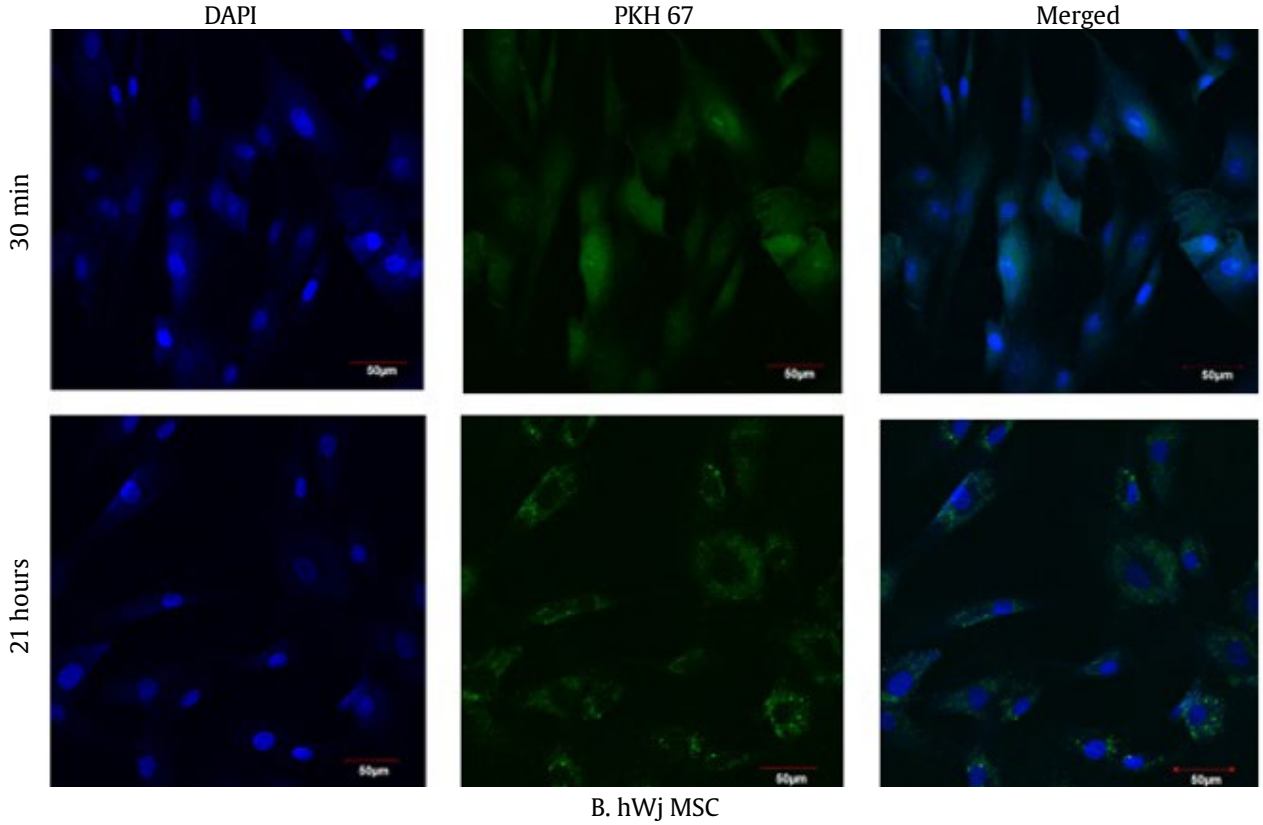


Figure 3. The capacity of ginger PDENs to penetrate human cells in timely observation through confocal image analysis. HDF: human dermal fibroblast; hWj MSC: human Wharton's jelly mesenchymal stem cells; PKH67: label marker for exosome (green); DAPI: nucleus coloration (blue)

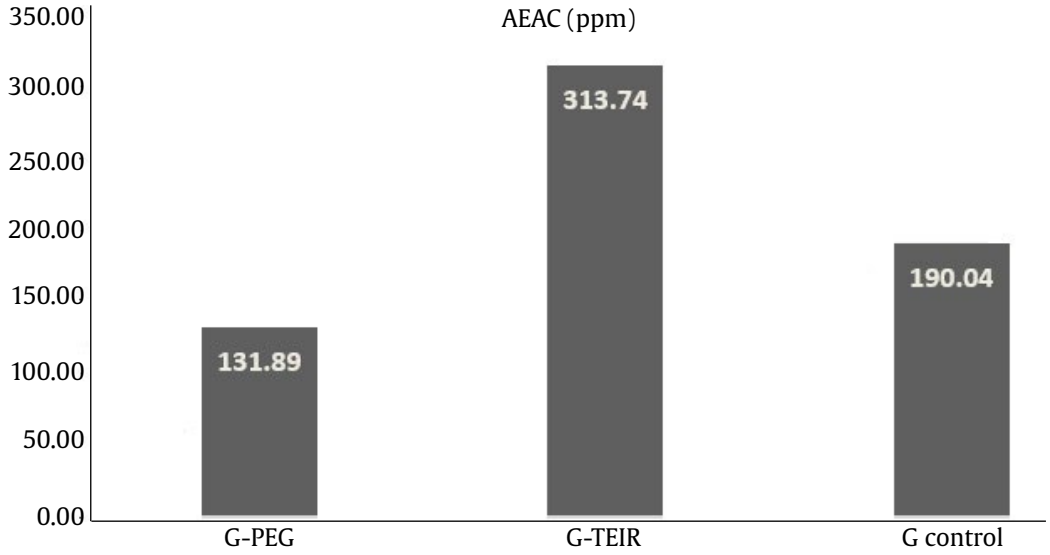


Figure 4. Antioxidant content of PDENs derived from fresh ginger rhizomes expressed by AEAC. *From procedure A of isolation. G = ginger

sizes range between 40 and 650 nm. Differential ultracentrifugation (procedure B) combined with a PEG6000 precipitation agent provided a better size range of particles. With a size range between 100 and 500 nm, PDEN particle morphology is similar to mammalian exosomes (Kim *et al.* 2022; Yu *et al.* 2020). Rutter and Innes (2017) categorized eukaryotic's EVs into three classes, i.e., apoptotic bodies, which have the largest size (1,000–5,000 nm); and the other two classes are microvesicles (100–1,000 nm) and exosomes (30–150 nm). Woith *et al.* (2021) recommended using the term nanovesicles for particle sizes range of 1–999 nm. Pocsfalvi *et al.* (2018) detected citrus's size range of 20 to 500 nm. The size range of 280 to 520 nm in grapes was reported by Woith and Melzig (2019). In contrast, Zhao *et al.* (2018) found PDENs from coconut water sized 13 to 60 nm, smaller than those of mammalian exosome, while Garaeva *et al.* (2021) isolated PDENs from grapefruit in a size range of 28 to 270 nm, with 90% of which sized 30 nm. In this study, the NTA reconfirmed that the concentration of the isolated particles within the size ranges of 30-150 nm was still low for ginger and quina (3.6×10^7 and 2.8×10^6 particles/ml, respectively), compared to that obtained by Garaeva *et al.* (2021) with 5.7×10^{13} particles/ml.

Quina-cultured cells were taken as the PDENs source because an exosome is an extracellular vesicle, that would be more easily secreted by loosened cells or biological fluids than solid plant tissues. It was true that quina friable callus provided smaller particle sizes (Table 2), although the concentration was also lower than ginger PDENs (Table 3). Thereby, several researchers isolated PDENs from, among others, coconut water (Zhao *et al.* 2018), citrus fruit (Pocsfalvi *et al.* 2018) and grapefruit juice (Garaeva *et al.* 2021), apoplastic fluid of sunflower (Regente *et al.* 2012). They all reported the presence of exosomes. *In vitro* quina cells commonly contain mostly quinine alkaloids (Ratnadewi *et al.* 2021). To anticipate any reluctance to use quina PDENs due to the possible adverse effects, we did not elaborate on the quina PDENs. Once its exosome content and membrane composition are determined, the functionality of this species' PDENs will be predictable.

Various techniques of PDENs isolation are available, from simple centrifugation to differential ultracentrifugation, density gradient steps, and immunoaffinity capture (Huang *et al.* 2021; Mu *et al.* 2016; Zhang *et al.* 2016a, 2016b) or by gel electrophoresis (Woith and Melzig 2019). In this study, the precipitating solution PEG6000 resulted in a higher yield of small-sized PDENs than Invitrogen TEIR. It might be due to the use of solid biomass (ground ginger rhizome and friable quina cells) as the plant material sources for the extraction, while the Invitrogen TEIR directed the use of culture media. The method of Kalarikkal *et al.* (2020) on ginger has been improved by Suresh *et al.* (2021) by applying low pH (4-5) in the PEG6000 precipitation.

To develop a cost-effective method without ultracentrifugation, they obtained 4 to 5-fold higher ginger PDENs yield recovery with a smaller size than by normal pH. Besides the different isolation methods, PDEN size is also species-specific.

Plant exosomes are known to function in cell communication and signaling. Proteomic analyses of PDENs revealed that they are highly rich in proteins involved in biotic and abiotic stress responses (Garaeva *et al.* 2021; Rutter and Innes 2017; Woith *et al.* 2021). PDEN secretion was enhanced in plants infected with pathogens, suggesting that PDENs may be an essential factor in the plant immune system (Garaeva *et al.* 2021; Rutter and Innes 2017). Further studies indicated that PDENs are essential for sRNA transport to regulate host-pathogen, even inter-kingdom communication (Alfieri *et al.* 2021; Cai *et al.* 2019; He *et al.* 2021). Some studies revealed that the PDEN membrane is rich in lipophilic compounds (Woith *et al.* 2021), including phosphatidic acid, phosphatidylcholines, digalactosyldiacylglycerol, and monogalactosyldiacylglycerol (Teng *et al.* 2018), while PDENs contain proteins, various amino acids, lipids, nucleotides, and polysaccharides, lipid signals related to their role in plant defense (Alfieri *et al.* 2021; Garaeva *et al.* 2021). This report enriched the proteomic data that de la Canal and Pinedo (2018) stated that PDENs contribute to releasing cell-wall-related proteins into the extracellular space. They comprised many proteins implicated in cell wall reorganization, such as glycosyl hydrolases, expansins, arabinogalactan proteins, lipases, protease, germin-like proteins, and lipid-transfer proteins. Recently, Woith *et al.* (2021) also found that plant cell wall's protein enzymes, such as 1,3-β-glucosidases, pectinesterases, polygalacturonases, β-galactosidases, were present in the PDENs. The presence of those enzymes in the PDENs, which are hydrolytic to the cell wall, could promote a transient destabilization of a cell wall structure and may explain the mechanism of transcellular passages and release of PDENs or extracellular vesicles through unconventional pathways (Alfieri *et al.* 2021; de la Canal and Pinedo 2018; Regente *et al.* 2012). Therefore, it is strongly believed that PDENs are extracellular vesicles secreted by plant cells and, at the same time, carry nutrients, certain secondary metabolites, signaling compounds, and many other nano-substances. The result on antioxidant activities in this study indicates that PDENs carry some nano-molecules with antioxidant function. A piece of accurate information on the content carried by the PDENs is indispensable for evaluating their biological effects on human health.

In the last few years, various research on the potential of PDENs on human health has been of interesting focus. They examined the possible function of PDENs as natural carriers to bring and release uploaded nutraceutical and pharmaceutical compounds. Our study on the internalization of ginger PDENs showed that human skin cells and mesenchymal stem cells can uptake the particles at different rates; the mesenchymal cells took them within 30 min, and the HDF cells after 21 hours of incubation. However, the exact time for ginger PDEN internalization in both human cells should be further studied using a better observation time series.

Zhang *et al.* (2016b) and Garaeva *et al.* (2021) compared the uptake of ginger and grapefruit PDENs by various *in vitro* stem cells and mice organs through oral modulation. They found comparable rates of uptake and its effects. Additionally, Garaeva *et al.* (2021) found that loading the PDENs with BSA and HSP70 proteins into both human peripheral blood mononuclear cells and colon cancer cells remarkably increased the uptake by the human cell in comparison with the same proteins without PDENs. Uptake of the protein-loaded grapefruit PDENs by those recipient human cells was detected within 30 min after incubation, and the accumulation of the fluorescence signal occurred along the increasing incubation time. Ginger PDENs took about 5 hours to be detected in A431 keratinocytes cells (Suresh *et al.* 2021). Song *et al.* (2020) reported the role of the CD98 receptor, a transmembrane glycoprotein heterodimer, and II lectins in the internalization process of garlic PDENs into the liver cells (HepG2). Specific surface proteins in the garlic PDENs also determined the endocytosis process. The type of lipids contained in the membrane of the PDENs also drives its rate of reaching the recipient cells (Munir *et al.* 2020). Many investigations focused on the lipid composition of the PDEN membrane that could determine the internalization capacity of PDENs into a recipient cell. The lipid composition is species-specific or family-specific (Woith *et al.* 2021).

The uptake mechanism of PDENs in mammalian cells was described by Zhuang *et al.* (2015), which includes phagocytosis, macropinocytosis, clathrin-mediated endocytosis, and plasma or endosomal membrane fusion. By applying specific inhibitors targeting cell entry pathways, Wang *et al.* (2014) found that grapefruit PDENs were taken up through macro pinocytosis and clathrin-dependent endocytosis. Similarly, ginger PDENs were internalized by macro pinocytosis in hepatocytes (Zhuang *et al.* 2015), whereas the vesicle uptake occurs prevalently through phagocytosis in colon cancer cells (Zhang *et al.* 2016b). To get better efficacy of PDENs uptake into specific recipient cells, analysis of PDEN membrane composition is highly encouraged.

This study using isolation procedure B with PEG6000 obtained particle sizes range of 40-650 nm, which can be defined as PDENs, but it still needs a technical improvement to get better size and yield of particles. The ginger PDENs demonstrated antioxidant activity, indicating that they contained some functional nano-substances that need to be determined. Preliminary trials to examine the internalization capacity of ginger PDENs revealed that those PDENs could be taken up by human cells in different periods, suggesting that PDENs could potentially supply nano-nutrients or nano-drugs by themselves or carry certain substances uploaded into them to the human body. Time series of incubation of PDENs-recipient cells in the internalization process are still encouraged.

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