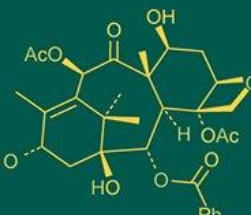
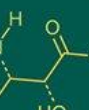
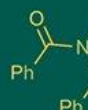
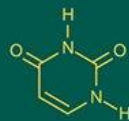


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Optimisation of the isolation process for ripe *Carica papaya*-derived exosome-like nanoparticles using density gradient ultracentrifugation: Physicochemical and morphological characterisation

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Abstract

Plant-derived exosome-like nanoparticles are nanoscale, lipid-bounded vesicles that carry proteins, lipids, RNA, and secondary metabolites out of plant tissue into the intercellular and cross-kingdom environment. These nanoparticles are non-immunogenic, inexpensive to produce at scale, and are being explored as anti-inflammatory agents, antioxidants, and drug-delivery vehicles. Ripe *Carica papaya* fruit is an attractive source given its high biomass yield, established food-safety profile, and documented anti-inflammatory bioactivity. Sucrose density gradient ultracentrifugation is widely regarded as the gold-standard separation method for vesicle purity, yet no ripe-papaya-specific protocol using this method had previously been reported, partly because papaya's high pectin content complicates clean gradient banding. Here we isolated papaya-derived exosome-like nanoparticles from ripe fruit mesocarp using a three-step differential centrifugation cascade (3,000, 10,000, and 16,500 × g) to clear pectin-rich debris, followed by a sucrose cushion (27%/68%, 110,000 × g) and a discontinuous sucrose density gradient (8/30/45/60%, 150,000 × g) to recover the particle band at the 8-30% interface. The isolated preparation was characterised by nano-flow cytometry and dynamic light scattering for particle size distribution, and by scanning electron microscopy for morphology. Nano-flow cytometry showed 99.6% of detected particle events below 100 nanometres in diameter, and dynamic light scattering showed a single, narrow, unimodal intensity peak, together indicating a predominantly homogeneous sub-100 nanometre particle population. Scanning electron microscopy showed dense fields of globular nanostructures below 150 nanometres, consistent with the size distribution data. Hydrodynamic diameter was 137.4 ± 5.05 nanometres, polydispersity index was 0.236 ± 0.07 , and zeta potential was -19.89 ± 0.74 millivolts. These results indicate that sucrose density gradient ultracentrifugation, without enzymatic pectin pretreatment, is achievable for ripe papaya using an intensified pre-clearing centrifugation cascade, yielding a smaller, more homogeneous particle population than other reports in the literature.

Keywords: Plant-derived exosome-like nanoparticles, *Carica papaya*, sucrose density gradient ultracentrifugation, extracellular vesicles, nanoparticle characterisation

1. Introduction

Extracellular vesicles (EVs) are membrane-bound nanoparticles secreted by cells of essentially every kingdom of life. In mammalian systems, exosomes and related EVs are established mediators of intercellular communication and are under active development as diagnostic biomarkers and therapeutic delivery vehicles. Over the past decade, an analogous population of nanovesicles has been isolated from the apoplastic fluid, juice, and homogenates of edible plants, including ginger, grape, grapefruit, lemon, broccoli, ginseng, and *Solanum nigrum* berries [1, 2]. These plant-derived exosome-like nanoparticles (PELNs) share the general size range (30-300 nm), lipid-bilayer architecture, and cargo classes (proteins, microRNAs, lipids, and secondary metabolites) of mammalian exosomes, but arise through a distinct, still incompletely defined biogenesis pathway rather than the canonical ESCRT-dependent route [1, 3].

PELNs are of particular translational interest because they can be produced from food-grade source material at low cost and large scale, are generally well tolerated in cell and animal models, and appear to retain bioactivities associated with their parent plant, including anti-inflammatory, antioxidant, and antitumour effects [3]. *Carica papaya* is a plausible PELNs

source on several counts: the ripe fruit is inexpensive and available year-round in tropical and subtropical production regions, and its extracts have long-documented anti-inflammatory and wound-healing activity [4].

Differential ultracentrifugation improves on precipitation but still co-isolates similarly sized non-vesicular particulates. Sucrose density gradient ultracentrifugation (SDG-UC), by contrast, separates particles by buoyant density rather than size alone, and is widely treated in the EV and PELNs literature as the reference-standard purification method against which faster methods are benchmarked [5,6]. In the plant EV literature, SDG-UC (alone, or combined with a sucrose cushion pre-concentration step) has been used to isolate exosome-like vesicles at reproducible densities of approximately 1.13-1.19 g/mL, corresponding to the interface between 8% and 30% (or in related formulations, between 20% and 45%) sucrose layers [5].

A ripe-papaya-specific SDG-UC protocol has not, to our knowledge, been reported. This is a meaningful gap because ripe papaya presents a processing challenge that most previously gradient-purified source plants (ginseng root, grape, ginger rhizome) do not share to the same degree: as papaya fruit ripens, a substantial fraction of its cell-wall pectin is solubilised into the pulp and juice, sharply increasing juice viscosity and particulate load [7,8]. High juice viscosity is known, in the pectin extraction and fruit-processing literature, to slow filtration and centrifugal clearing and to reduce the sharpness of density-based separations unless the pectin is enzymatically or chemically degraded beforehand [9]. An SDG-UC protocol for ripe papaya therefore has to solve the same viscosity problem while additionally preserving clean gradient banding.

This paper reports an SDG-UC isolation protocol for ripe papaya-derived exosome-like nanoparticles (papaya-derived ELNs) that adapts the sucrose cushion/gradient ultracentrifugation workflow developed for ginseng-derived exosome-like nanoparticles and other plant exosome-like vesicles [5] to ripe papaya mesocarp, using an intensified three-step differential centrifugation cascade in place of enzymatic pectin pretreatment to manage the high particulate and pectin load of ripe papaya juice. It presents the executed isolation workflow, preliminary particle-size and morphological characterisation of the resulting preparation (nano-flow cytometry, dynamic light scattering, and scanning electron microscopy), and benchmarks these findings against sucrose-gradient data reported for other plant sources [10]. We also outline the physicochemical assays needed to complete a MISEV2023-consistent characterisation panel [11] and the parameter space most likely to merit further optimisation.

2. Materials and Methods

2.1 Plant material and study area

Fully ripe *Carica papaya* L. var. Pusa Delicious fruits were obtained from the Agricultural Farm, Banaras Hindu University (BHU), Varanasi, India. Fruit meeting the target ripeness criterion (predominantly yellow-orange peel colouration with residual green patches) was selected for processing.

2.2 Chemicals and reagents

All chemicals and reagents used in the study were of analytical grade. Phosphate-buffered saline (PBS) pellets (P4417-50TAB), sodium potassium tartrate (CAS 304-59-6),

sodium carbonate (Na_2CO_3 ; CAS 497-19-8), sodium hydroxide (NaOH ; $\geq 98\%$, CAS 1310-73-2), and copper sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; CAS 7758-99-8) were procured from Merck (India) and SRL Chemicals Pvt. Ltd. (Mumbai, India). Folin-Ciocalteu reagent (analytical grade), 2,2-diphenyl-1-picrylhydrazyl (DPPH; CAS 1898-66-4), and gallic acid (CAS 149-91-7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The protocol for antioxidant analysis was performed according to study given in the literature [18]. Additional analytical-grade chemicals were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Molecular-biology-grade sucrose, 0.45 μm and 0.22 μm PES syringe filters.

2.3 Equipment

A domestic-grade fruit juicer/blender; a refrigerated high-speed centrifuge capable of $\geq 10,000 \times g$ at 4 °C; an ultracentrifuge with a swinging-bucket rotor rated to $\geq 200,000 \times g$ with open-top tubes suitable for gradient fractionation; a refractometer for verifying sucrose solution concentration; a dynamic light scattering and zeta-potential analyser; a nano-flow cytometer; and a scanning electron microscope were used.

2.4 Isolation of papaya-derived exosome-like nanoparticles by sucrose density gradient ultracentrifugation

Ripe papaya mesocarp was homogenised in $1 \times \text{PBS}$ using a laboratory blender for approximately 2 min. No enzymatic pectin pretreatment was applied at this stage; pectin-related viscosity and particulate load were instead managed downstream through an intensified differential centrifugation cascade. The homogenate was cleared by three sequential centrifugation steps at 4 °C, discarding the pellet after each spin and retaining the supernatant: $3,000 \times g$ for 30 min, then $10,000 \times g$ for 60 min, then $16,500 \times g$ for 60 min. This cascade is more intensive, both in maximum g-force and cumulative spin time, than the two-step cascades typically reported for lower-pectin plant tissues such as ginseng root [5], reflecting the additional debris load presented by ripe papaya's soluble pectin fraction. The cleared supernatant was passed sequentially through 0.45 μm and then 0.22 μm filters to remove residual micro-particulates ahead of ultracentrifugation, yielding the working filtrate.

The filtrate was layered above a two-step sucrose cushion (27% sucrose over 68% sucrose, prepared in PBS) and ultracentrifuged at $110,000 \times g$ for 90 min at 4 °C in a swinging-bucket rotor, following the method described by Kim *et al.* [5]. Papaya-derived ELNs banded at the 27%/68% sucrose interface and were aspirated from this interface for gradient loading. The concentrated interface material was loaded on top of a discontinuous sucrose step gradient (8%, 30%, 45%, and 60% sucrose, layered top to bottom) and ultracentrifuged at $150,000 \times g$ for 90 min at 4 °C. Papaya-derived ELNs banded at the 8%/30% sucrose interface, consistent with the 1.12-1.19 g/mL buoyant density range reported for sucrose-gradient-purified plant exosome-like vesicles from other tissues [5], and were aspirated from the tube with a syringe. The isolated papaya-derived ELNs fraction was resuspended in PBS and stored at -20 °C for further characterisation. The complete workflow is summarised in Figure 1.

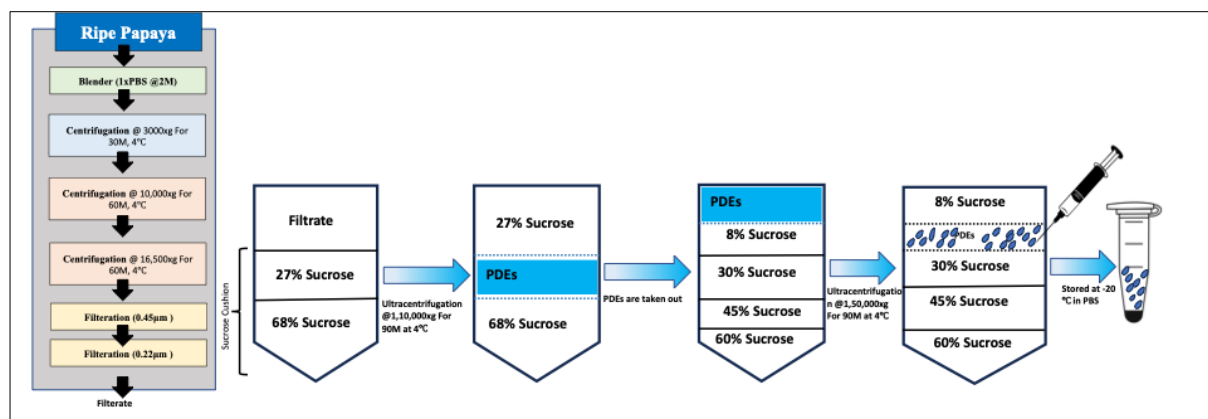


Fig 1: Schematic overview of the Ripe papaya-derived exosome-like nanoparticles isolation workflow

2.5 Physicochemical characterisation

Particle size distribution was assessed by dynamic light scattering, which gave hydrodynamic size and zeta potential, producing an intensity-weighted size distribution of the preparation. The population size of papaya-derived ELNs was estimated by nano-flow cytometry, in which individual particle events were gated by violet side-scatter against a fluorescence channel and binned by size threshold (below 100 nm, at approximately 100 nm, and above 100 nm).

2.6 Morphological characterisation

Morphology was studied by using field emission scanning electron microscopy (FE-SEM) on a Zeiss instrument using the InLens secondary electron detector at 5.00 kV accelerating voltage, 4.6 mm working distance, and 50,000× magnification.

3. Results and Discussion

3.1 Source material:

The characteristic deep-orange

mesocarp and central seed cavity, typical of fully ripe *Carica papaya*, were confirmed on cross-section before processing.

3.2 Particle size and physicochemical characteristics

Nano-flow cytometry of the isolated papaya-derived ELNs preparation showed that 99.60% of detected particle events fell below 100 nm in diameter, with 0.12% of events at approximately 100 nm and 0.18% above 100 nm. Dynamic light scattering corroborated this finding, showing a single, narrow, unimodal intensity-weighted size peak (Figure 2), with no evidence of a secondary large-particle or aggregate population. Together, these two independent sizing methods indicate that the SDG-UC-isolated preparation is dominated by a sub-100 nm particle population that is more homogeneous and smaller on average. The findings show coherence with other findings reported in the literature [12]. The hydrodynamic diameter, polydispersity index, and zeta potential of the isolated preparation are summarised in Table 1. 507 ± 8 µg gallic acid equivalents (GAE) per mL and a DPPH radical scavenging activity of $23 \pm 0.72\%$.

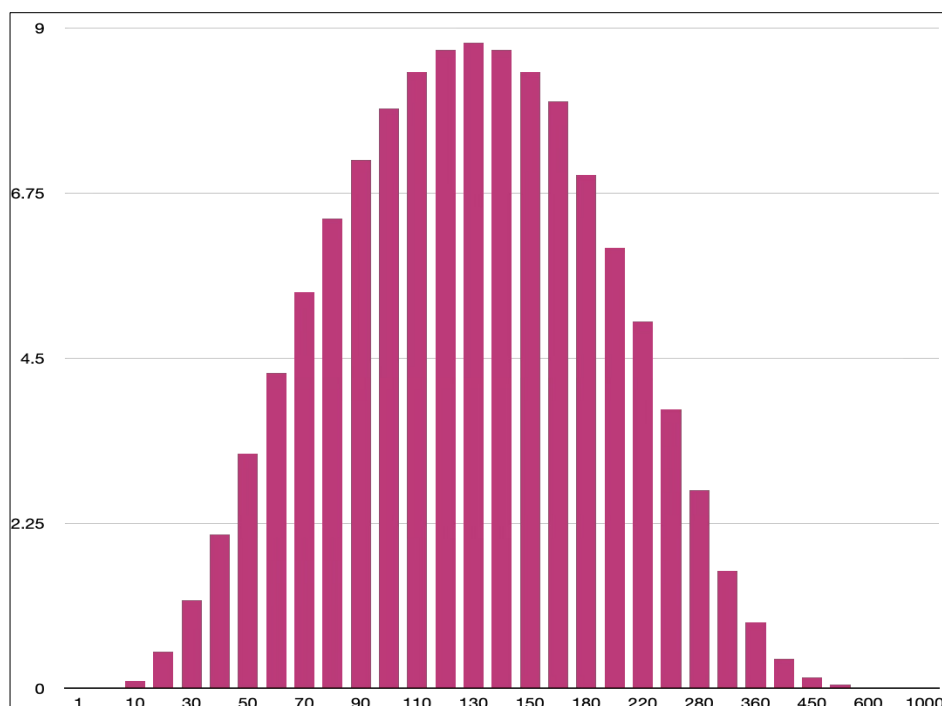


Fig 2: Size distribution (by intensity) of the isolated papaya-derived exosome-like nanoparticle preparation determined by dynamic light scattering

The present protocol demonstrates that SDG-UC is achievable for ripe papaya and yields a smaller, more homogeneous particle population by two independent sizing methods, consistent with the general purity/size advantage of density-gradient separation over precipitation reported across the plant EV literature ^[13, 14]. The three-step differential centrifugation cascade (3,000/10,000/16,500 × g) appears sufficient to manage ripe papaya's pectin-associated viscosity

and debris load ahead of gradient loading. This avoids introducing an exogenous fungal enzyme into the preparation, which may be advantageous for downstream applications sensitive to residual enzyme protein, though it comes at the cost of a longer, more centrifugation-intensive pre-clearing cascade compared with enzymatically assisted isolation approaches reported for other plant tissues ^[15].

Table 1: Physicochemical characteristics of isolated papaya-derived exosome-like nanoparticles

Parameter	Value
Hydrodynamic diameter (nm)	137.4 ± 5.05
Polydispersity index (PDI)	0.236 ± 0.07
Zeta potential (mV)	-19.89 ± 0.74

3.3 Morphology

Field Emission Scanning electron microscopy (FE-SEM) (Figure 3) showed dense fields of globular, sub-150 nm nanostructures across the imaged field. Individual particles were frequently observed in clusters rather than as fully dispersed discrete units; this clustering is a common feature of FE-SEM sample preparation, in which substrate drying

promotes particle aggregation, and is consistent with a preparation artefact rather than aggregation in suspension, given the single non-aggregated peak observed by dynamic light scattering. These results are consistent with the globular morphology reported by scanning electron microscopy for other plant-derived exosome-like nanoparticles ^[16, 17].

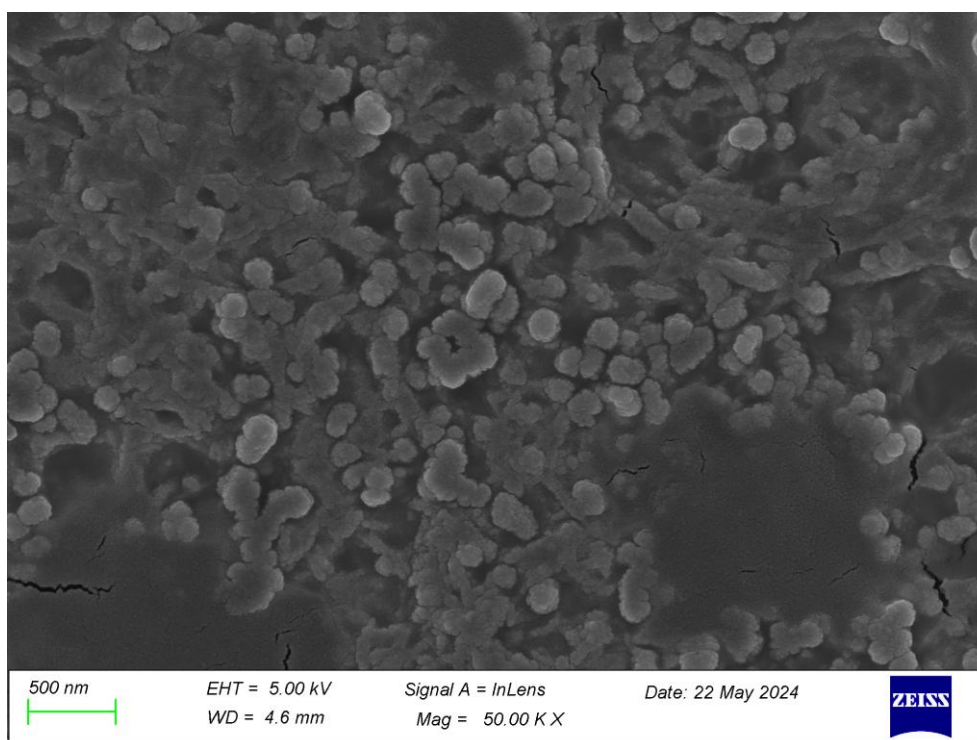


Fig 3: Scanning electron micrograph of the isolated papaya-derived exosome-like nanoparticle preparation (Zeiss, InLens detector, 5.00 kV, 4.6 mm working distance, 50,000x magnification; scale bar 500 nm)

4. Conclusion

Ripe *Carica papaya*-derived exosome-like nanoparticles were isolated using a sucrose density gradient ultracentrifugation protocol built around an intensified three-step differential centrifugation cascade, a sucrose cushion pre-concentration step, and a discontinuous sucrose density gradient, without enzymatic pectin pretreatment. Nano-flow cytometry and dynamic light scattering both indicated a predominantly sub-100 nm, homogeneous particle population, and scanning electron microscopy showed globular nanostructures consistent with this size range. These preliminary results support sucrose density gradient ultracentrifugation as a viable, higher-resolution alternative for ripe papaya-derived exosome-like nanoparticle isolation.

5. Acknowledgements and Conflict of Interest

The authors thank the Agricultural Farm, Banaras Hindu University (BHU), Varanasi, India, for providing the plant material used in this study. The authors affirm that any known competing financial interests or personal relationships did not influence the work described in this publication.

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