

# Supplementary information for Extraction and Isolation of Extracellular Vesicles From *Piper betle* Leaves Using the Apoplastic Fluid Washing and Size Exclusion Chromatography Method

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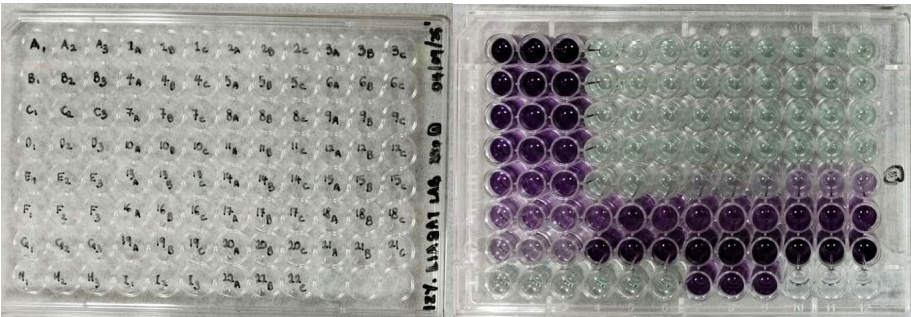
## Supplementary Data

**Supplementary Table 1.** BCA was conducted after SEC to find the protein concentration in each fraction using a BCA kit (ThermoFisher, Catalog number: 23227). The BCA was conducted in triplicates for each fraction, where Fraction 1 (F1) starts at well A4 to well A6, Fraction 2 (F2) starts at well A7 to well A9 and continues in that manner for other fractions. These are photographs captured of individual plates for three PBdEVs replicates as noted. The results across are replicates are similar visually, where F1 until Fraction 13 (F13) are generally clear of protein or have low protein concentration when compared to the standard. Protein concentration started to become gradually visible with purple colour intensity gradually increasing beginning from Fraction 14 (F14) to Fraction 22 (F22).

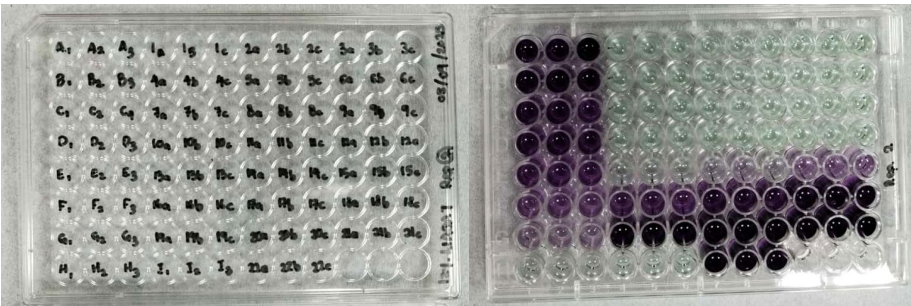
Replicates

BCA Plates

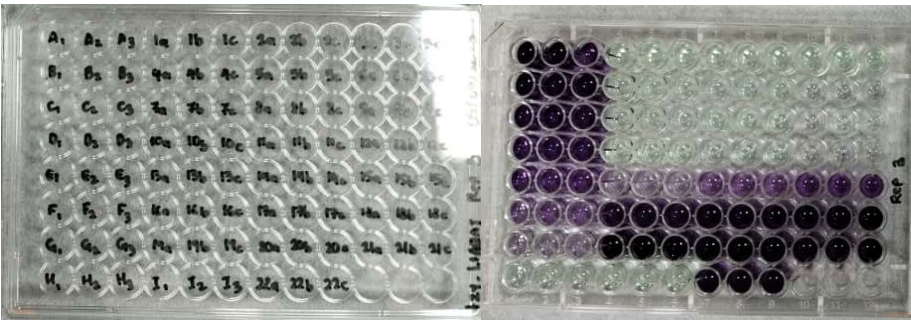
Rep. 1

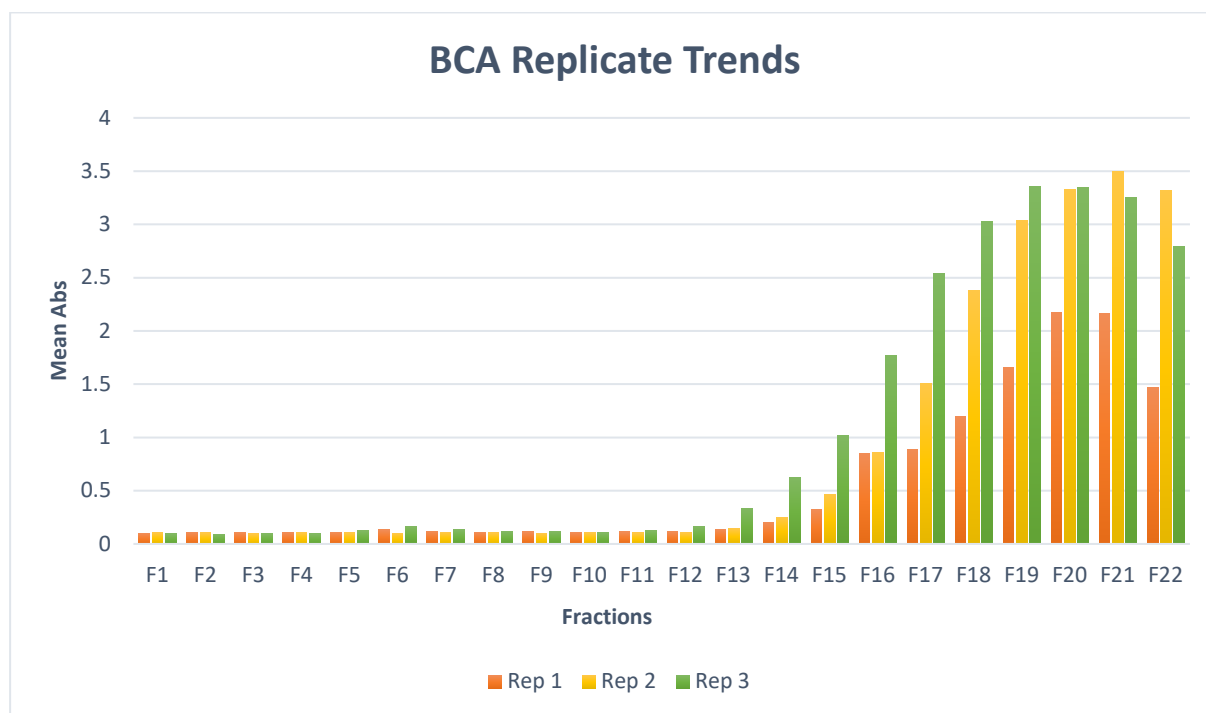


Rep. 2

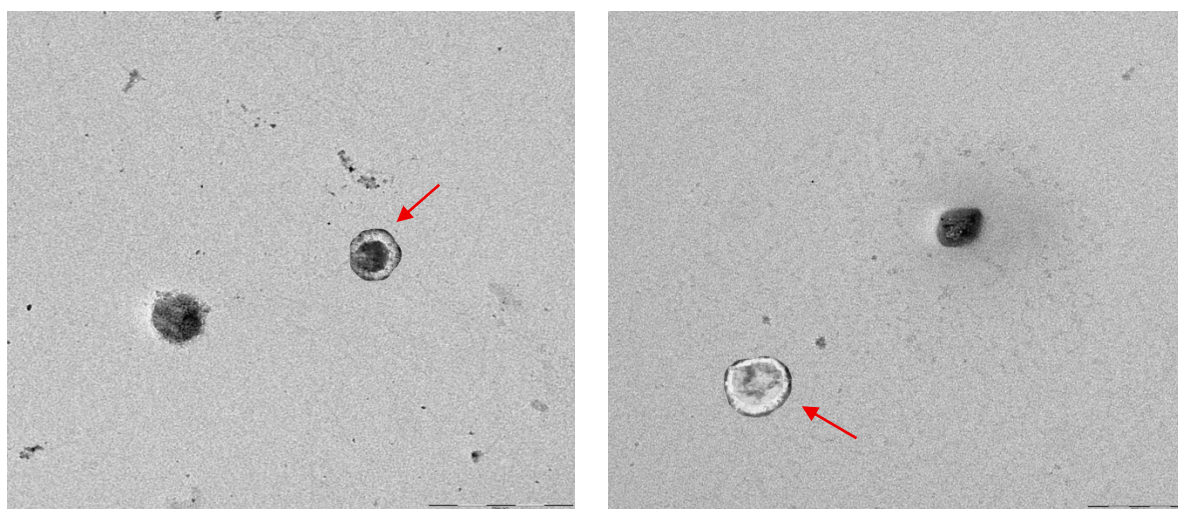


Rep. 3



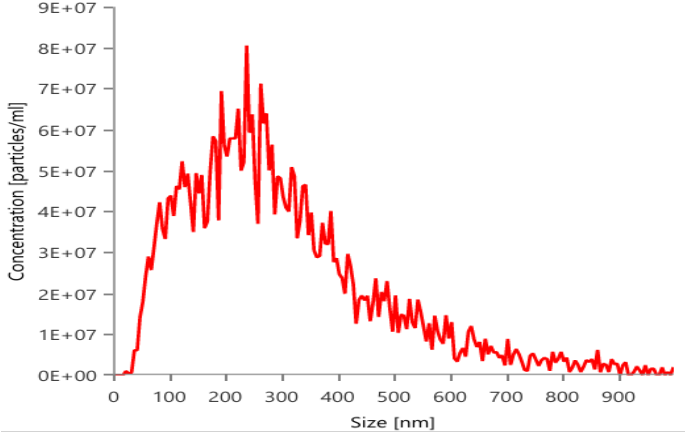
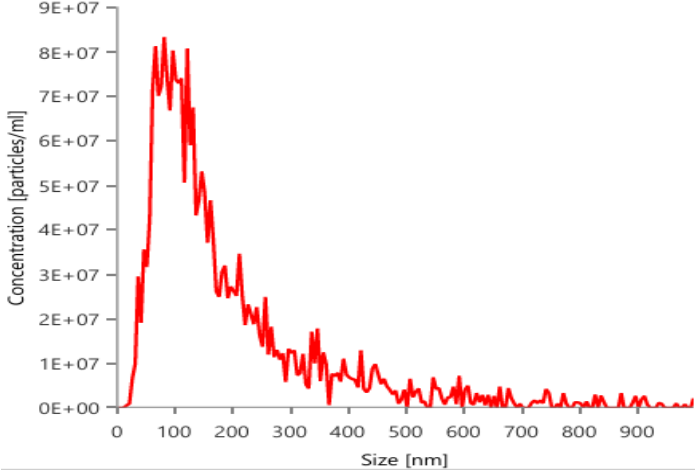
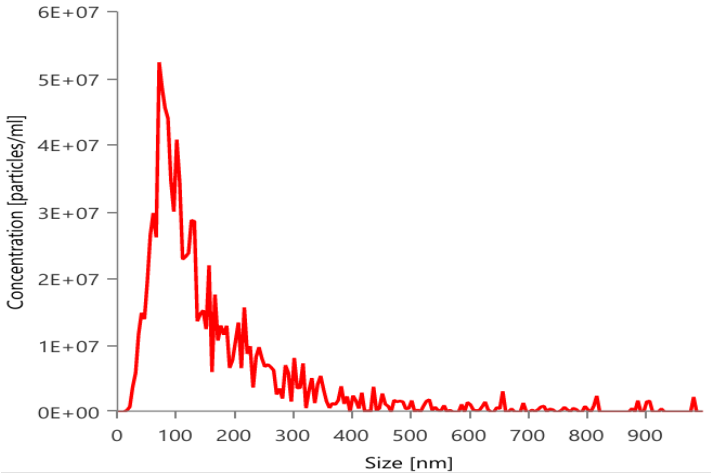


**Supplementary Figure 1.** BCA absorbance data analysis of each fraction yielded across all three replicates. This histogram showed that F1 to Fraction 12 (F12) has negligible protein concentration, where the absorbance reading is less than the blank standard ( $< 0.13$ ). Protein concentration is observed to be increasing starting from Fraction 13 (F13) to F22.



**Supplementary Figure 2.** Photographs captured using a TEM (Leica, Germany) showing the lipid bilayer and the cup shaped morphology of the PBdEVs (pointed with the red arrow). The sizes of the PBdEVs captured are within the range of 150 nm to 300 nm. In these images, the bilayer phospholipid membrane of the EVs (white outline) is visible against the grey background. The scalebar are automatically provided by the imaging software and overlaid into the photographs captured.

**Supplementary Table 2.** NTA data report for all three replicates obtained from NS XPLOER software (Malvern Panalytical). The mean size range across all replicates are similar, which ranges from 100 to 200 nm.

| Replicates | Average Size Distribution Graph                                                      |
|------------|--------------------------------------------------------------------------------------|
| Rep. 1     |    |
| Rep. 2     |   |
| Rep. 3     |  |

**Supplementary Table 3.** The differences between young betel leaf and old betel leaf. This comparison of young and old betel leaf provide a guide to choosing the correct leaf age.

| Characteristic                                            | <div>   </div> |                                                                           |
|-----------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------|
|                                                           | Young betel leaf                                                                                                                                                                   | Old betel leaf                                                            |
| <b>Colour</b>                                             | Light green, sometimes with a yellowish tint                                                                                                                                       | Deep green                                                                |
| <b>Texture</b>                                            | Softer, more tender                                                                                                                                                                | Thicker, leathery                                                         |
| <b>Surface</b>                                            | Smooth and less glossy                                                                                                                                                             | Glossier and waxier due to thicker cuticle                                |
| <b>Veins</b>                                              | Veins are more visible due to thin lamina                                                                                                                                          | Veins less prominent because of thicker mesophyll                         |
| <b>Cell wall &amp; cuticle</b>                            | Thinner cell walls, less lignification                                                                                                                                             | More lignified cell walls, thicker cuticle layer                          |
| <b>Flexibility</b>                                        | More pliable, easy to bend without cracking                                                                                                                                        | Stiffer, less flexible                                                    |
| <b>Metabolic activity</b>                                 | Active growth, high protein synthesis, active vesicle movement                                                                                                                     | Cells are more differentiated and structural                              |
| <b>Secondary metabolite content</b>                       | Lower total phenolic and flavonoid content                                                                                                                                         | Higher concentration of phenolics, tannins, alkaloids, and essential oils |
| <b>Aroma &amp; taste</b>                                  | Milder aroma and taste                                                                                                                                                             | Stronger aroma, more pungent or bitter taste                              |
| <b>Suitability for vacuum infiltration EVs extraction</b> | Easier infiltration, less damage, lower contamination from secondary metabolites                                                                                                   | Harder to infiltrate, more debris, higher risk of vesicle damage          |

## AWF Extraction Results & Plan

### 1<sup>st</sup> replicate

|                     |                        |                   |               |                       |
|---------------------|------------------------|-------------------|---------------|-----------------------|
| <b>Dry weight:</b>  | 229.37 g               | (Batch 1 – 28/07) | <b>Yield:</b> | 12.5 ml               |
|                     | 250.45 g               | (Batch 2 – 04/08) |               | 17.5 ml               |
|                     | 22.11 g                | (Batch 3 – 21/08) |               | 3.5 ml                |
| <b>Total weight</b> | <b><u>501.93 g</u></b> |                   |               | <b><u>33.5 ml</u></b> |

### 2<sup>nd</sup> replicate

|                     |                        |                   |               |                       |
|---------------------|------------------------|-------------------|---------------|-----------------------|
| <b>Dry weight:</b>  | 262.94 g               | (Batch 1 – 07/08) | <b>Yield:</b> | 35.0 ml               |
|                     | 248.14 g               | (Batch 3 – 18/08) |               | 16.5 ml               |
| <b>Total weight</b> | <b><u>511.08 g</u></b> |                   |               | <b><u>51.5 ml</u></b> |

### 3<sup>rd</sup> replicate

|                     |                        |                   |               |                       |
|---------------------|------------------------|-------------------|---------------|-----------------------|
| <b>Dry weight:</b>  | 239.79 g               | (Batch 1 – 21/08) | <b>Yield:</b> | 24.5 ml               |
|                     | 55.02 g                | (Batch 2 – 14/08) |               | 7.5 ml                |
|                     | 164.96 g               | (Batch 3 – 14/08) |               | 16.5 ml               |
|                     | 49.84 g                | (Batch 4 – 25/08) |               | 5.5 ml                |
| <b>Total weight</b> | <b><u>509.61 g</u></b> |                   |               | <b><u>44.0 ml</u></b> |

**Supplementary Figure 3.** The triplicates data of the total dry weight, the estimation of buffer infiltrated and the actual volume obtained. These data were excerpted from the scientist's logbook. Dry weight of the leaves were measured and totalled to approximately 500g before proceeding with the isolation. This is to ensure reproducibility of the experiment and minimizes variables. The number of yields obtained from each isolation were also noted to determine the relationship between the number of AF yield and the number of EVs obtained. These data is useful for further exploration and optimization of the protocol.