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Isolation of Extracellular Vesicles Derived from Dried Leaves of Kinkéliba (*Combretum micranthum* G. Don) Using Tangential Flow Filtration

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Abstract

Extracellular vesicles (EVs) are particles released from all cells across kingdoms of life, delimited by a lipid bilayer, which cannot replicate on their own. They have high biological relevance, influencing various physiological and pathological processes. However, knowledge about their isolation and characterization remains a key challenge for certain sources, particularly in the plant kingdom, despite their emerging biomedical application potential. In addition, the studied plant is recognized for its traditional medicinal use and its potential therapeutic properties, which further supports the relevance of investigating its extracellular vesicles. The objective of this study was to isolate and characterize EV-enriched preparations from the dried leaves of *Combretum micranthum* G. Don using tangential flow filtration, optimizing different isolation protocols to increase the yield and purity of the obtained EVs. After isolation, the concentration and the size distribution of particles in the final sample were measured by NTA; FPLC-SEC was used to analyze the presence of additional particle subpopulations and non-EV-associated proteins, while TEM was used to evaluate the morphology of vesicle-like structures. The total lipid content was determined by the Sulfo-Phospho-Vanillin Assay (SPVA), and the total protein content was determined by means of the BCA assay. This approach allowed to isolate 2–4 $\times$ 10^{11} vesicles per gram of plant material with relatively high reproducibility in particle yields and lipid and protein content. In conclusion, these results suggest that TFF is a suitable approach for the isolation of plant-derived EVs from dried leaves, while providing a scalable and cost-effective production method for scientific and industrial applications.

Keywords: extracellular vesicle; *Combretum micranthum* G. Don; TFF isolation; plant EVs; characterization

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1. Introduction

Combretum micranthum G. Don is a traditional African medicinal plant [1]. It is primarily recognized for its beneficial effects on various ailments, including respiratory diseases

(such as cough and bronchitis), hypertension, cholera-like diarrhea, anemia, and digestive disorders [2]. The Combretaceae family includes about 600 species of trees, shrubs, and vines, distributed across 18 to 20 genera. These plants are mainly found in tropical and subtropical regions, particularly in Africa and India [3]. The *Combretum* genus comprises nearly 250 species, widely distributed in tropical and subtropical regions [4].

Extracellular vesicles (EVs) are membranous particles released by all cells across kingdoms of life and play an important role in intercellular communication [5]. In recent years, EVs have attracted increasing interest within the scientific community, becoming a multidisciplinary research field due to their unique physicochemical properties, resulting from their nanometric dimensions and bioactive content [6]. Plant-derived EVs possess a lipid membrane enriched in phospholipids, plant sterols, and sphingolipids, which provides them with exceptional stability and enables them to withstand a variety of physiological environments [5,7]. These vesicles not only carry signaling proteins and enzymes but also nucleic acids, such as microRNAs and messenger RNAs, which can modulate gene expression in target cells [8]. This ability to transport bioactive molecules makes them valuable tools for regulating cellular processes and interacting with human or animal cells. Due to these characteristics, EVs hold promising applications in areas such as drug delivery [9], therapeutic agents [10], and biomarkers [11,12].

One of the main challenges in using EVs for scientific and medical applications is the isolation process, as EVs can be present in various complex biological matrices composed of many nanometric biomaterials that overlap in size and density [13]. Several techniques are used, such as ultracentrifugation, density gradients, and size-exclusion chromatography [14], and these techniques are often combined. The combination of these techniques can lead to damage to the EV morphology and can cause difficulties in separating the different particles, etc. Tangential flow filtration (TFF) remains scarcely used. However, it offers capabilities and advantages that are not present, or are less present, in other techniques, such as the ability to process larger volumes more quickly and efficiently [15–17]. Unlike ultracentrifugation, which is a lengthy process requiring high rotational speeds that can damage EVs, TFF is gentler and preserves their integrity. Therefore, it is important to conduct studies to better understand these limitations and explore the potential of other techniques more thoroughly.

The characterization of plant-derived extracellular vesicles (PDEVs) is essential for elucidating their physicochemical, morphological, and molecular properties, which are fundamental to understanding their biological activity and mechanisms of action. A comprehensive characterization requires the integration of several complementary approaches. Nanoparticle tracking analysis (NTA) enables the determination of particle size distribution and concentration, while transmission electron microscopy (TEM) provides information on the morphology and ultrastructural characteristics of isolated vesicles. In addition, fast protein liquid chromatography coupled with size-exclusion chromatography (FPLC-SEC) can be used to assess particle separation and purification according to size and to evaluate the quality of the isolated vesicle fractions. Complementary biochemical analyses of proteins and lipids provide further insights into the molecular composition of PDEVs. Plant-derived EVs are often enriched in phospholipids and sterols, which contribute to membrane stability and the maintenance of structural integrity during the transport of bioactive molecules. These components include sterols such as sitosterol and campesterol, as well as phospholipids such as phosphatidylcholine and phosphatidylethanolamine. By contributing to membrane stability under varying extracellular conditions, these lipids may facilitate interactions with specific target cells and the controlled delivery of bioactive or therapeutic molecules [18]. In parallel, proteins identified in plant EVs include annexins, heat shock proteins (HSPs), lipoxygenases, ATPases, and other functional proteins, which

may contribute to their biodistribution, cellular interactions, and potential therapeutic effects [19,20]. The integration of NTA, TEM, FPLC-SEC, and biochemical quantification of proteins and lipids therefore provides a preliminary multidimensional characterization of PDEVs, facilitates the comparison of different isolation and purification protocols, and establishes a foundation for subsequent molecular and functional validation. However, despite these advances, the biological activity and precise mechanisms of action of plant-derived extracellular vesicles remain incompletely understood, largely due to the complexity and heterogeneity of their molecular composition and the technical challenges associated with their isolation and characterization. Therefore, this study aimed to isolate and characterize PDEVs from *Combretum micranthum* G. Don leaves using TFF. Four isolation conditions were compared by varying the dispersant volume, temperature/pretreatment, and incubation time. The resulting PDEV preparations were characterized using NTA, FPLC-SEC, and TEM, together with the quantification of their total protein and lipid content, to identify the conditions providing the best balance between EV yield, purity, and physicochemical characteristics.

2. Results and Discussions

2.1. NTA Measurement of Concentration and Size of Extracellular Vesicles

To evaluate the different extracellular vesicle (EV) isolation approaches, we measured the mean particle size and concentration by NTA from four independent *Combretum micranthum* G. Don-derived biological samples (PDEV01, PDEV02, PDEV03, and PDEV04), with the aim of optimizing sample purity and yield. All four samples were prepared using the TFF isolation method. NTA revealed particle yields for the four samples, all expressed as particles per gram of plant material: PDEV01 ($3.78 \pm 0.07 \times 10^{11}$), PDEV02 ($3.22 \pm 0.08 \times 10^{11}$), PDEV03 ($2.30 \pm 0.09 \times 10^{11}$), and PDEV04 ($2.94 \pm 0.07 \times 10^{11}$). NTA revealed that PDEV01, incubated for 24 h at room temperature, showed the highest particle concentration, followed by PDEV03 (24 h at 4 °C), PDEV04 (containing 500 mL PBS, incubated for 3 days at room temperature), and PDEV02, as shown in Figure 1.

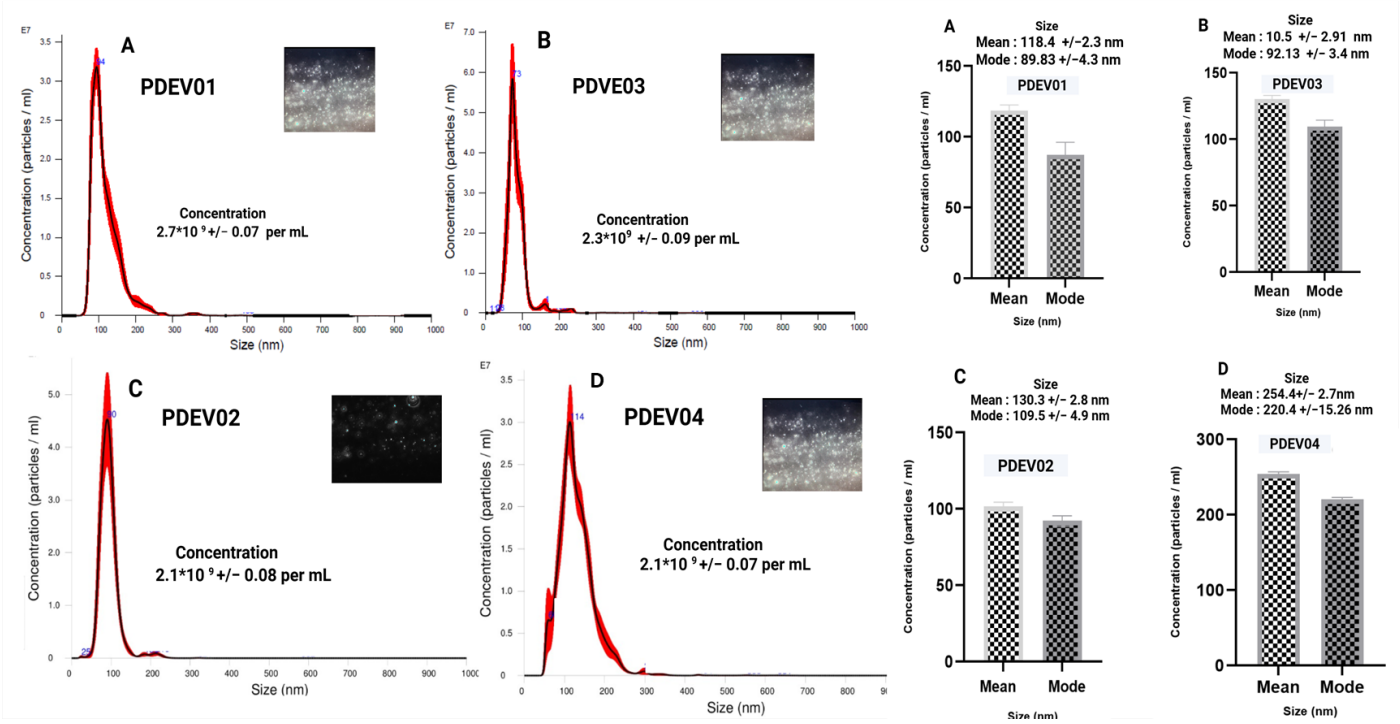


Figure 1. NTA size-distribution histograms for the 4 isolation approaches employed.

A pronounced difference between the mean and mode particle sizes was observed for the PDEV04 sample (Figure 1D) when compared with PDEV01, PDEV02, and PDEV03 (Figure 1A–C). While the mean and mode values were relatively close for PDEV01, PDEV02, and PDEV03, indicating a more homogeneous size distribution, PDEV04 exhibited a marked discrepancy between these parameters, suggesting an asymmetric distribution skewed toward larger particle sizes. This divergence is typically associated with increased polydispersity or the presence of a subpopulation of larger particles, which can elevate the mean size without substantially affecting the mode. Similar observations have been reported in extracellular vesicle characterization studies, where aggregation, protein co-isolation, or contamination with non-vesicular particles contribute to broader size distributions [21,22]. In plant-derived extracellular vesicles (PDEVs), heterogeneity in size distribution has also been widely documented and is often attributed to differences in isolation, purification protocols, and sample handling [23]. Therefore, the mean–mode discrepancy observed for PDEV04 likely reflects increased size heterogeneity and higher polydispersity relative to the other samples.

The results of extracellular vesicle concentrations measured by NTA show variations depending on the incubation conditions, extraction volume, and storage duration.

PDEV01 exhibits a high concentration ($3.78 \pm 0.07 \times 10^{11}$ particles per gram of plant material) after incubation at room temperature for 24 h with an extraction volume of 700 mL. This elevated value may be explained by the combined effect of a short incubation period and a relatively large suspension volume, which promotes better particle dispersion and limits significant degradation or aggregation phenomena in Table 1.

Table 1. Comparison of extraction conditions and EV concentrations from *Combretum micranthum* G. Don using different protocols (PDEV01–04).

Sample Identification	Quantity of *Combretum micranthum* G. Don *Dry* Leaves	Dispersant Volume	Temperature	Incubation Period	Concentration of EVs by NTA (Particles per Gram of Plant Material)
PDEV01	5 g	700 mL	room temperature	24 h	$3.78 \pm 0.07 \times 10^{11}$
PDEV02	5 g	700 mL	snap-frozen in LN2 followed by incubation at RT	24 h	$3.22 \pm 0.08 \times 10^{11}$
PDEV03	5 g	500 mL	4 °C	24 h	$2.30 \pm 0.09 \times 10^{11}$
PDEV04	5 g	700 mL	room temperature	3 days	$2.94 \pm 0.07 \times 10^{11}$

PDEV02, frozen at −196 °C (*Snap-freezing in liquid nitrogen followed by incubation at room temperature)* for 24 h, also shows a high concentration ($3.22 \pm 0.08 \times 10^{11}$ particles per gram of plant material), suggesting that ultra-low-temperature freezing effectively preserves the integrity and detectable quantity of EVs. Cryopreservation thus appears to maintain particle structural stability and limit degradation in Table 1.

In comparison, PDEV03, incubated at 4 °C with a volume of 500 mL, shows a lower concentration ($2.30 \pm 0.09 \times 10^{11}$ particles per gram of plant material). This relative decrease may be related to a dilution effect (lower PBS volume) as well as potential differences in extraction efficiency. However, despite the slightly reduced concentration, a temperature of 4 °C still appears to ensure acceptable particle preservation, confirming its stabilizing role [24].

PDEV04, incubated at room temperature for 3 days, shows a concentration of $2.94 \pm 0.07 \times 10^{11}$ particles per gram of plant material, slightly lower than the samples incubated for 24 h. This decrease may be attributed to prolonged incubation, which can promote aggregation, degradation, or structural modifications of EVs. Extended exposure at room temperature, in the absence of stabilizing agents, may reduce particle stability and affect their quantification by NTA [25] in Table 1.

2.2. Fast Protein Liquid Chromatography–Size-Exclusion Chromatography (FPLC-SEC)

In this study, fluorescence detection (Figure 2A) was employed to monitor the distribution of fluorescent material during size-exclusion chromatography (SEC). The fluorescence detector was configured at 488/510 nm, allowing the detection of fluorescent signals across the chromatographic fractions and the assessment of their distribution during elution. However, this fluorescence signal cannot be directly attributed to extracellular vesicles (EVs) or to intrinsic protein autofluorescence, as intrinsic protein fluorescence typically occurs at excitation/emission wavelengths around 280/340 nm, primarily due to aromatic amino acids such as tryptophan and tyrosine. Therefore, the observed fluorescence peak (Figure 2A) should be considered a signal associated with fluorescent biomolecules present in *Combretum micranthum* G. Don samples processed via tangential flow filtration (TFF), rather than a specific EV marker. These fluorescent signals may originate from various biological components potentially co-isolated with EVs, including plant-derived secondary metabolites or other chromophoric compounds. Thus, fluorescence detection during FPLC-SEC provides complementary qualitative information regarding the distribution of fluorescent components across the chromatographic fractions, but does not allow the definitive identification of EVs, determination of their purity, or identification of the molecular species responsible for the detected fluorescence [26].

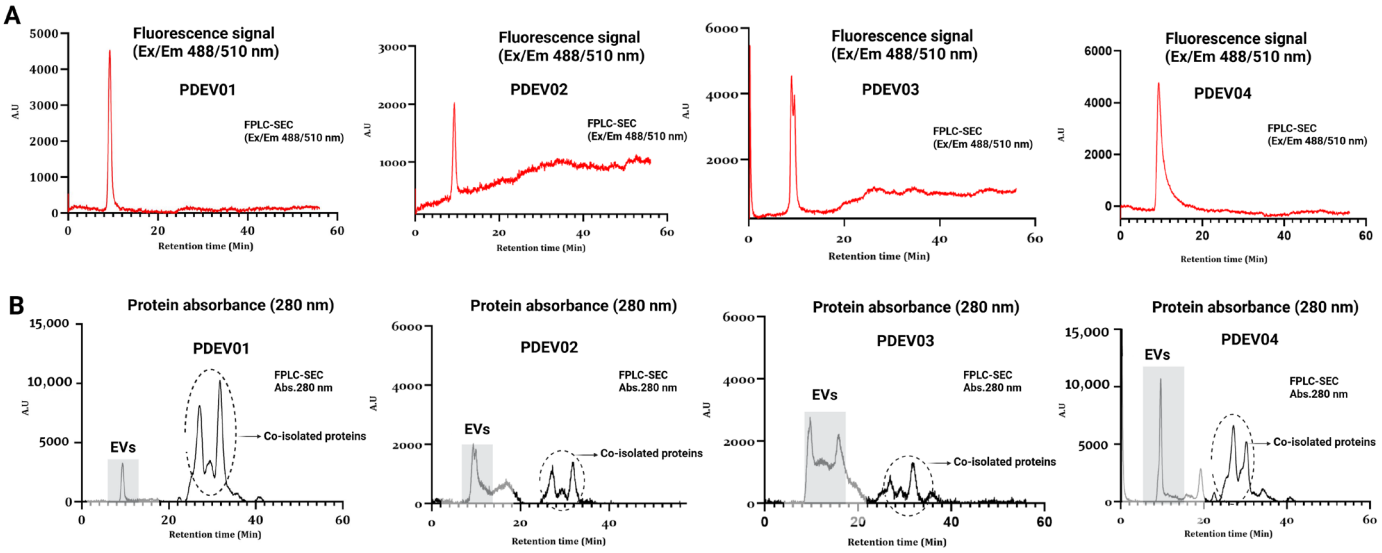


Figure 2. The detection of EV autofluorescence (**A**) enables the real-time tracking of their presence and concentration during elution, offering valuable insights into their purity. Protein absorbance (**B**) offers the direct quantification of the protein load in the fractions, which can be used to identify co-isolated proteins.

Protein absorbance, measured at 280 nm (Figure 2B), was used to monitor protein-containing fractions in *Combretum micranthum* G. Don samples following TFF extraction. This measurement is based on the intrinsic ability of proteins to absorb ultraviolet light at this wavelength, mainly due to the presence of aromatic amino acids such as tryptophan and tyrosine. During chromatographic separation techniques such as FPLC or SEC, absorbance detection at 280 nm is commonly used to monitor the distribution of protein-containing material across the elution profile. In the present study, the 280 nm absorbance profile was therefore used to evaluate the distribution of protein-associated material and to identify fractions containing co-isolated proteins.

The combination of fluorescence detection and UV absorbance measurements provides complementary information regarding the composition and distribution of different biomolecular components across the SEC fractions. However, neither fluorescence at 488/510 nm nor

absorbance at 280 nm is specific for EVs. Therefore, these measurements were interpreted as complementary information on the chromatographic fractionation profile rather than as independent evidence of EV identity or purity. Nevertheless, further analytical characterization would be required to precisely determine the molecular origin of the detected fluorescence and to distinguish EV-associated components from soluble proteins, plant-derived metabolites, and other potentially co-isolated non-vesicular materials [27].

The fluorescence profile detected at 488/510 nm may be particularly relevant for monitoring the distribution of fluorescent compounds in complex plant-derived preparations. Nevertheless, because the exact fluorophore responsible for this signal was not determined in the present study, its molecular origin remains undetermined. It may potentially reflect fluorescent plant-derived biomolecules, including secondary metabolites such as polyphenolic or flavonoid-related compounds, although this possibility requires confirmation by dedicated analytical approaches. Accordingly, the fluorescence signal was not used as a specific marker of EVs in the present study. Instead, the FPLC-SEC profiles were considered together with complementary characterization techniques, including nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and biochemical analyses, to provide an initial assessment of the isolated preparations. Further molecular characterization, including proteomic and lipidomic analyses and the identification of EV-associated markers, will be necessary to establish the molecular identity and purity of the isolated vesicular preparations [28].

2.3. Determination of Protein Content in Extracellular Vesicles

The bicinchoninic acid (BCA) assay is a widely used colorimetric method for the quantification of total protein concentration in biological samples, including extracellular vesicles. This method is widely appreciated for its sensitivity, reproducibility, and compatibility with complex biological matrices [29].

The principle of the BCA assay is based on the reduction of Cu^{2+} ions to Cu^{+} by proteins under alkaline conditions. The generated Cu^{+} ions subsequently react with bicinchoninic acid to form a purple-colored complex whose intensity is proportional to the protein concentration. The absorbance of this complex is measured at 562 nm, enabling the indirect quantification of proteins using a calibration curve [30,31].

In this study, total protein quantification was performed on different EV preparations (PDEV01, PDEV02, PDEV03, and PDEV04) using the colorimetric BCA assay. The standard protocol, valid for a concentration range between 20 and 2000 µg/mL, was applied. A calibration curve was established using bovine serum albumin (BSA) as the protein standard. BSA solutions at different concentrations (20 to 2000 µg/mL) were prepared by serial dilution in PBS from a 2 mg/mL stock solution. For each sample, 25 µL of the preparation was mixed with 200 µL of BCA working reagent (prepared at a 50:1 ratio of reagents A and B) in a 96-well plate. The samples were incubated at 37 °C for 30 min, and the absorbance was measured at 562 nm using a photometer. Three replicates were performed for each sample, and the mean corrected absorbance values were calculated. The absorbance values of the BSA standards were plotted against their corresponding concentrations to generate the calibration curve used to determine the protein concentrations of the samples [32].

The total protein content measured by the BCA assay varied among the different PDEV preparations (PDEV01–PDEV04), indicating differences in the amount of protein associated with the isolated preparations. The obtained values were PDEV01 = 21.96 mg/g, PDEV02 = 22.01 mg/g, PDEV03 = 15.72 mg/g, and PDEV04 = 21.95 mg/g (Figure 3). Although the BCA assay is reliable and widely used, measurements may be influenced by the presence of free proteins or protein complexes co-isolated with EVs, which may affect quantification

accuracy. Therefore, despite the robustness of this approach, interpretation of the results should consider possible protein contamination during EV isolation [33–35].

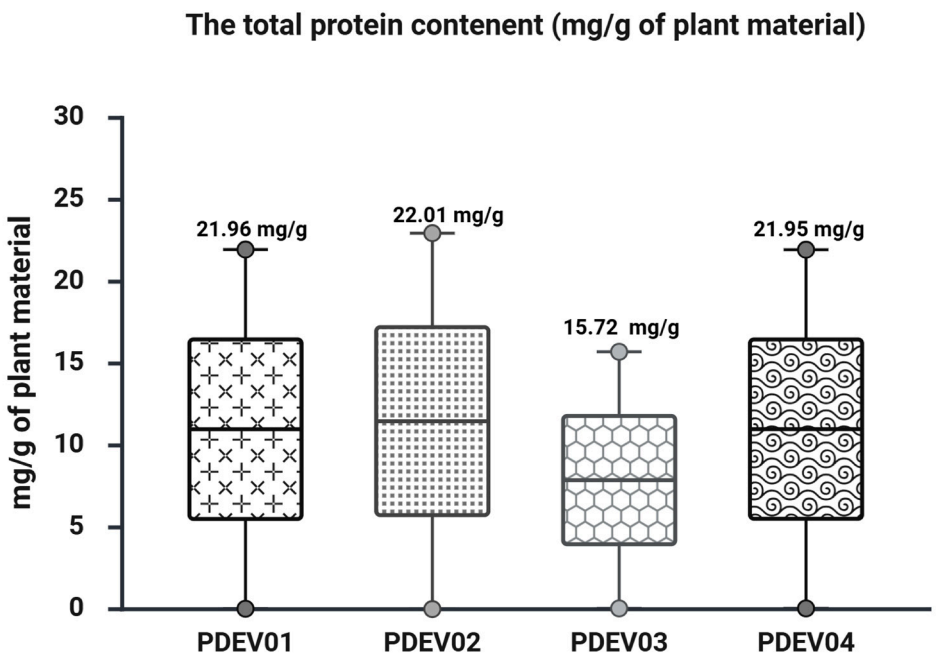


Figure 3. Protein content of *Combretum micranthum* G. Don samples obtained by TFF isolation for all samples (PDEV01 to PDEV04), normalized to the same number of particles.

The protein composition of PDEVs varies depending on their origin, secretion pathways, and matrices, although certain protein families have been identified in PDEVs from different species, despite ongoing debates about their origin. Among the most commonly found protein families in PDEVs, annexins are particularly prominent. Annexin A1 and annexin A2 play a crucial role in the biogenesis of EVs in mammals and in the formation of multi-vesicular bodies [36,37]. These proteins have been detected in plant-derived extracellular vesicles, including those present in citrus juice [38] and in the apoplastic fluid of sunflower seeds [33], as well as in PDEVs from *Arabidopsis thaliana* leaves, where they are associated with proteins involved in biotic and abiotic stress responses [8]. Another frequently found family of proteins in plant-derived EVs is the heat shock proteins (HSPs). HSP60 is present in sunflower PDEVs, while HSP70 has also been observed in PDEVs from several citrus species [20], where HSP80 and HSP90 have also been identified [19]. In a recent study on EVs isolated from tomato using size-exclusion chromatography, high levels of HSPs were detected, along with lipoxygenase and ATPases [39]. Finally, proteins from the aquaporin family have been found in PDEVs from citrus fruits [40] and grapes [41], suggesting that it is possible to identify the protein families present in extracellular vesicles from *Combretum micranthum* G. Don, and that further studies of these proteins would be necessary to learn more. This suggests that it is possible to identify the protein families present in *Combretum micranthum* G. Don extracellular vesicles, and that more in-depth study of these proteins would be necessary to gain further insights.

2.4. Determination of Lipid Content in Extracellular Vesicles

The sulfophosphovanillin (SPVA) assay was used to determine the total lipid content of extracellular vesicle samples derived from *Combretum micranthum* G. Don following tangential flow filtration extraction. This method is widely employed for the overall quantification of lipids, particularly phospholipids, which are major structural components of EV membranes [42]. The SPVA is based on a chemical reaction between lipids and

sulfuric acid, followed by a reaction with vanillin, producing a colored complex whose intensity is proportional to the lipid concentration [43]. The absorbance of this complex is measured spectrophotometrically, providing the indirect quantification of the total lipid content. While the SPVA does not allow the detailed analysis of specific lipid classes, it provides a reliable estimation of total lipid content in samples. It is particularly suitable for rapid analyses and laboratories lacking advanced analytical instrumentation. However, more precise techniques such as gas chromatography or mass spectrometry allow detailed lipidomic characterization, although they are more expensive and technically complex [44]. In this study, the SPVA was applied to different EV preparations (PDEV01, PDEV02, PDEV03, and PDEV04). The standards and EV samples were treated with sulfuric acid, incubated, and then mixed with the phospho-vanillin reagent. Absorbance was measured at 540 nm, and EV lipid concentrations were determined based on the liposome standard calibration curve.

The total lipid content measured by the SPVA varied among the different PDEV preparations (PDEV01–PDEV04). The obtained values were PDEV01 = 2.52 mg/g, PDEV02 = 2.64 mg/g, PDEV03 = 1.86 mg/g, and PDEV04 = 2.74 mg/g (Figure 4), indicating variability in the total lipid content among the isolated preparations. The lipid analysis of plant-derived EVs is gaining increasing attention due to the role of lipids in EV interactions with mammalian cells, as well as their biological and functional effects. The major lipid classes identified in plant-derived EVs include phosphatidic acid (PA), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). Phosphatidic acid has recently been detected in nanovesicles from *Uvaeursi folium*, *Craterostigma plantagineum*, and *Zingiberis rhizoma* [45]. Phosphatidylcholine has been reported in grapefruit [46], while phosphatidylethanolamine has been identified in grape EVs [41] and in nanovesicles from *C. plantagineum*. A lipidomic study of EVs from *Arabidopsis thaliana* leaf rosettes revealed 23 lipid classes and 279 distinct lipid species, with a notable enrichment in sphingolipids, particularly glycosylinositolphosphoceramides, representing approximately 46% of the lipid content compared to leaf tissues [7]. These findings suggest that identifying and characterizing the lipid classes present in *Combretum micranthum* G. Don EVs is pertinent. A thorough lipidomic analysis would be necessary to gain deeper insights into their composition and functional roles.

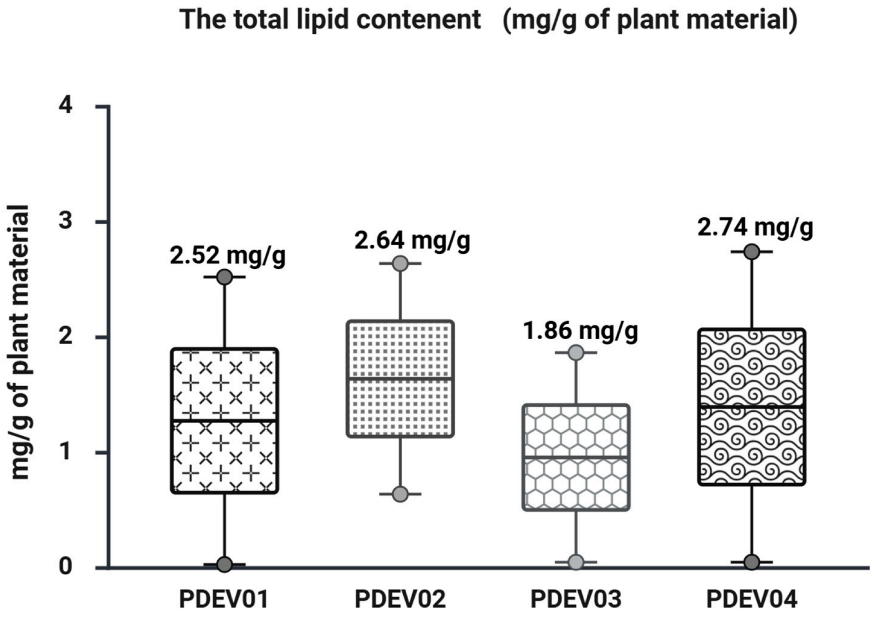


Figure 4. Lipid content of *Combretum micranthum* G. Don samples obtained from TFF extraction for all samples (PDEV01 to PDEV04), normalized to the same number of particles.

2.5. TEM Analysis of Negatively Stained Extracellular Vesicles

Transmission electron microscopy (TEM) is widely used for the morphological characterization of EVs in biological samples [47]. In this study, negative-stain TEM was used to examine the vesicular structures obtained from *Combretum micranthum* G. Don following isolation by tangential flow filtration using four different protocols (PDEV01–PDEV04). TEM revealed vesicular structures morphologically compatible with EVs in all four preparations (Figure 5). The vesicles displayed variable electron contrast and morphology across the different preparations. PDEV02 showed relatively well-defined and more strongly contrasted vesicular structures in the fields examined, whereas PDEV01, PDEV03, and PDEV04 displayed more heterogeneous contrast and morphology. Some vesicular structures exhibited a cup-shaped appearance, a morphology frequently reported in negatively stained EV preparations that may be associated, at least in part, with dehydration and staining-related preparation artifacts [48].

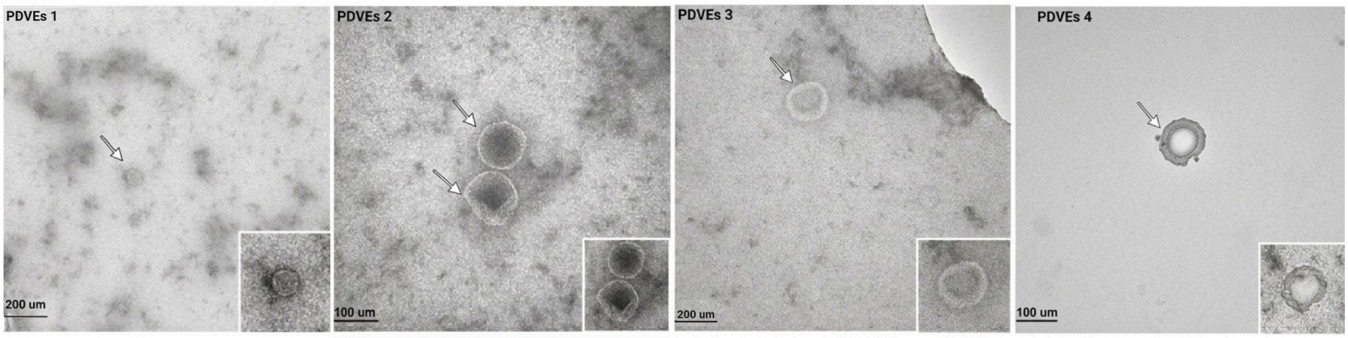


Figure 5. Representative negative-stain TEM images of vesicular structures obtained from *Combretum micranthum* G. Don following tangential flow filtration using four isolation protocols (PDEV01–PDEV04). Arrows indicate representative vesicular structures morphologically compatible with extracellular vesicles. Insets show enlarged views of representative vesicular structures. Scale bars: 200 nm for PDEV01 and 100 nm for PDEV02–PDEV04.

In PDEV01, vesicular structures were less frequently observed and less clearly defined in the main TEM field, although a structure morphologically compatible with an EV was visible in the inset. This observation should be interpreted cautiously, as TEM provides information on the morphology of particles deposited on the grid and may be influenced by sample preparation, including fixation, negative staining, dehydration, grid deposition, particle distribution, and electron contrast. Although NTA indicated a relatively high particle concentration for PDEV01, this does not necessarily translate into a higher number of clearly visible vesicles in a given TEM field. Overall, the observed differences in vesicle morphology and visibility may reflect, at least in part, differences in isolation and sample preparation conditions. These findings highlight the influence of the isolation procedure on the characteristics of the vesicular preparations obtained.

2.6. Tangential Flow Filtration (TFF)

TFF offers several advantages for the isolation and concentration of plant-derived extracellular vesicle (PDEV)-enriched preparations, including relatively gentle processing, size-based separation, reduced reliance on chemical precipitation reagents, and scalability to larger volumes [49–51]. However, its efficiency strongly depends on the characteristics of the starting material and the conditions applied prior to filtration.

In the present study, the pre-treatment conditions clearly influenced the recovery of particles detectable by NTA. PDEV01, obtained from 5 g of dried *Combretum micranthum* G. Don leaves in 700 mL of dispersant at room temperature for 24 h, showed the highest parti-

cle concentration ($3.78 \pm 0.07 \times 10^{11}$ particles/g). In comparison, snap-freezing followed by 24 h of incubation at room temperature (PDEV02) resulted in a lower concentration ($3.22 \pm 0.08 \times 10^{11}$ particles/g, approximately 15% lower than PDEV01). This suggests that snap-freezing did not improve particle recovery under the conditions tested and may have promoted some disruption or aggregation of particulate components. PDEV03 showed the lowest particle concentration ($2.30 \pm 0.09 \times 10^{11}$ particles/g), approximately 39% lower than PDEV01. However, PDEV03 differed from PDEV01 in both extraction temperature (4 °C versus room temperature) and dispersant volume (500 versus 700 mL). Therefore, the individual contribution of each parameter cannot be determined from the present experimental design. Nevertheless, this result indicates that the combination of these conditions was less favorable for particle recovery. Extending the incubation period from 24 h to 3 days while maintaining room temperature and 700 mL of dispersant (PDEV04) also reduced particle recovery to $2.94 \pm 0.07 \times 10^{11}$ particles/g, approximately 22% lower than PDEV01. This finding suggests that prolonged incubation does not necessarily improve particle recovery and may instead promote degradation, aggregation, or changes in the composition of the extracted material. These findings demonstrate that the optimization of the upstream extraction conditions is essential for TFF efficiency. Although TFF is a scalable and relatively gentle isolation strategy, membrane adsorption, concentration polarization, and membrane fouling may affect particle recovery, particularly when processing complex plant extracts containing proteins, polysaccharides, phenolic compounds, and other secondary metabolites [7]. Thus, the higher particle recovery obtained with PDEV01 suggests that 24 h of extraction at room temperature with 700 mL of dispersant represented the most favorable condition among those tested.

The morphological observations obtained by transmission electron microscopy (TEM) provide complementary information on the physical characteristics of the isolated preparations. The differences in particle recovery observed under the different pre-treatment conditions were also associated with variations in the morphological characteristics observed by TEM. The samples PDEV01, PDEV02, PDEV03, and PDEV04 all displayed vesicle-like structures, although differences in apparent electron density and morphology were observed among the preparations. Overall, these observations suggest that the pre-treatment conditions applied prior to TFF may influence both particle recovery and the apparent structural characteristics of the isolated preparations. Nevertheless, the morphology observed by TEM alone cannot definitively establish the molecular identity of the isolated particles such as EVs.

Biochemical analyses revealed variability in the protein and lipid composition among the different preparations. This variability did not directly follow the differences in particle concentration measured by NTA, suggesting that the total amounts of proteins and lipids do not necessarily reflect the number of detected particles. This observation may be explained by differences in the composition of the preparations obtained under the different pre-treatment conditions, as well as by the possible presence of co-isolated non-vesicular components. In particular, the BCA and SPVA measure the total protein and lipid content present in the preparations, respectively, and cannot determine which fraction of these components is specifically associated with EVs. Therefore, these results should be considered complementary parameters for characterizing the overall composition of the preparations rather than direct indicators of EV yield or purity.

Compared with other approaches commonly used for EV isolation, TFF offers several practical advantages. Ultracentrifugation is widely used for EV isolation, but it involves high centrifugal forces and is less readily adaptable to the processing of large volumes [50]. PEG precipitation can provide substantial particle recovery, but it may also result in the co-isolation of considerable amounts of soluble proteins and other contaminants [52]. Im-

munocapture offers greater specificity but depends on the availability of appropriate surface markers, which remain insufficiently established for plant-derived EVs. In contrast, TFF provides a continuous and potentially scalable separation approach that does not require chemical precipitation reagents [51]. Nevertheless, the results obtained in the present study also demonstrate that TFF performance strongly depends on the prior preparation of the samples. Membrane adsorption, concentration polarization, and membrane fouling may affect particle recovery and filtration efficiency, particularly when processing complex plant extracts containing proteins, polysaccharides, phenolic compounds, and other secondary metabolites [7]. Therefore, the optimization of the pre-treatment conditions, dispersant volume, temperature, incubation time, membrane characteristics, and filtration parameters is essential for obtaining reproducible preparations.

Overall, the results obtained indicate that 24 h of extraction at room temperature with 700 mL of dispersant (PDEV01) resulted in the highest concentration of NTA-detectable nanoparticles among the conditions tested. Importantly, this finding should not be interpreted as demonstrating higher EV purity, but rather as identifying a condition associated with higher particle recovery. The systematic comparison of these different pre-treatment conditions represents an important aspect of the present study, as it demonstrates that relatively limited changes in upstream extraction conditions can substantially influence the quantity and biochemical characteristics of the resulting preparations. Further molecular characterization, including proteomic and lipidomic analyses and the identification of EV-associated markers, will be necessary to determine whether the observed differences in particle recovery are related to variations in EV abundance, vesicle integrity, or the co-isolation of non-vesicular components.

3. Materials and Methods

3.1. Plant Material and Drying of Combretum micranthum G. Don Leaves

The *Combretum micranthum* G. Don plant was collected from the Sélibabi region in Mauritania. The samples, including leaves, flowers, fruits, and stems, were identified at the Herbarium of the Department of Botany at the University of Agronomical and Veterinary Sciences of Cluj-Napoca, Romania (Voucher No. 30431/31.10.2024). The leaves were air-dried in the shade at room temperature (25 ± 3 °C) and then ground into powder using an electric grinder before being transported to Salzburg, Austria. The isolation of extracellular vesicles was carried out at the Laboratory of Chemical Biology and Biological Therapies, Department of Biosciences, University of Salzburg, Austria. Several leaf rehydration protocols for *Combretum micranthum* G. Don were applied to assess the optimal yield of extracellular plant-derived vesicles (PDEV01, PDEV02, PDEV03, and PDEV04), as shown in Table 2 and Figure 7A.

Table 2. Preprocessing parameters used prior to the TFF step for the isolation of EVs from the samples PDEV01 to PDEV04.

Identification	Protocol
PDEV01	A 5 g quantity of *Combretum micranthum* G. Don leaves was weighed and added to a beaker containing 700 mL of phosphate-buffered saline (PBS) at pH 7.4. The mixture was placed on a magnetic stirrer for 24 h (Figure 6A). Afterwards, the solution was collected, filtered using Whatman paper (Figure 6B), and then filtered again through a 0.45 µm mPES membrane (Figure 6C).
PDEV02	A 5 g quantity of *Combretum micranthum* G. Don leaves was weighed, then immersed in liquid nitrogen at −196 °C for a few minutes before being ground in a mortar and pestle. The ground material was transferred to a beaker containing 700 mL of phosphate-buffered saline (PBS), pH 7.4, and stirred on a magnetic stirrer for 24 h at room temperature (Figure 6A). The obtained solution was then collected, filtered through Whatman paper (Figure 6B), and subsequently filtered again through a 0.45 µm mPES membrane (Figure 6C).

Table 2. *Cont.*

Identification	Protocol
PDEV03	A 5 g quantity of *Combretum micranthum* G. Don leaves was weighed and added to a beaker containing 500 mL of phosphate-buffered saline (PBS), pH 7.4. The mixture was briefly stirred on a magnetic stirrer (Figure 6A), then placed in the refrigerator at 4 °C for 24 h. After this period, the obtained solution was collected, filtered through Whatman paper (Figure 6B), and subsequently filtered again through a 0.45 µm mPES membrane (Figure 6C).
PDEV04	A 5 g quantity of *Combretum micranthum* G. Don was weighed and added to a beaker containing 700 mL potassium phosphate saline buffer (PBS), pH 7.4. The mixture was stirred on a magnetic stirrer for 3 days at room temperature (Figure 6A). The solution was then collected, filtered through Whatman paper (Figure 6B) and filtered again through a 0.45 µm mPES membrane (Figure 6C).

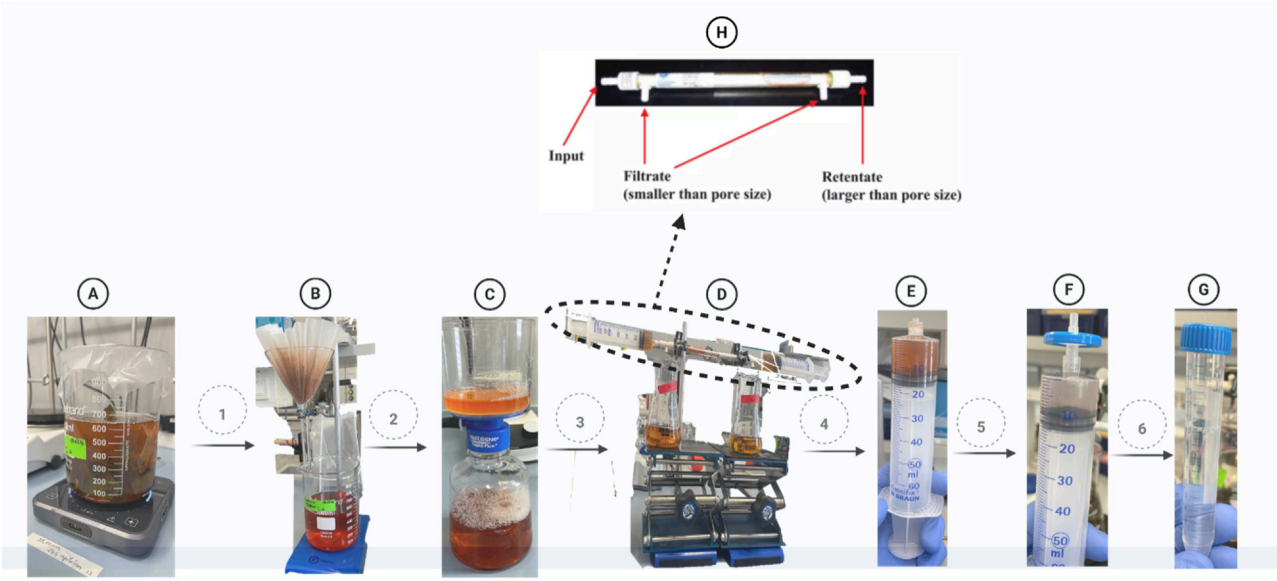


Figure 6. Rehydration (**A**); filter paper filtration (**B**); filtration through 0.45 µm mPES membrane (**C**); tangential flow filtration (**D**); final product after TFF and before filtration (**E**); final product after TFF subsequent filtration through 0.45 µm mPES membrane (**F**,**G**) and schematic representation of the TFF membrane, showing the input, filtrate, and retentate streams according to particle size (**H**).

3.2. Extracellular Vesicle Isolation by Tangential Flow Filtration

Extracellular vesicle-enriched preparations were obtained by manual tangential flow filtration (TFF) using a hollow-fiber membrane made of modified polyethersulfone (mPES) with a nominal molecular weight cut-off (MWCO) of 750 kDa, allowing the removal of small cellular debris and soluble components not associated with EVs. Before use, the membrane was rinsed three times with sterile phosphate-buffered saline (PBS, pH 7.4).

The plant extracts were loaded into a 50 mL syringe and manually circulated through the hollow-fiber filtration system. The retentate was collected in a second 50 mL syringe and recirculated through the membrane. During filtration, small soluble components, including free proteins, passed through the membrane and were collected as a permeate and subsequently discarded (Figure 6D), whereas larger particulate material was preferentially retained in the retentate. The 750 kDa MWCO was considered a molecular retention threshold and was not interpreted as a defined pore diameter. The retentate was initially concentrated to a final volume of approximately 10 mL (Figure 6E). This concentrate was subsequently diluted with PBS to a final volume of 450 mL and reconcentrated to approximately 10 mL (Figure 6F–G), providing an additional washing and concentration step. The final retentate was then filtered through a 0.45 µm Whatman syringe filter to remove larger

particulate debris before downstream characterization, including nanoparticle tracking analysis (NTA) and other analytical techniques (Figure 6H).

Because the TFF procedure was performed manually, the flow rate and transmembrane pressure were not continuously monitored or recorded during the experiments. Similarly, the dilution–reconcentration step was performed as a washing and concentration procedure rather than according to a predefined number of diafiltration volumes. Importantly, the 750 kDa membrane was used as a size-based enrichment step rather than as an EV-specific purification method. Therefore, the resulting preparations are referred to as PDEV-enriched preparations, and their characterization was performed using complementary physico-chemical and morphological approaches. A schematic representation of the overall TFF protocol is provided in Figures 6 and 7B–D.

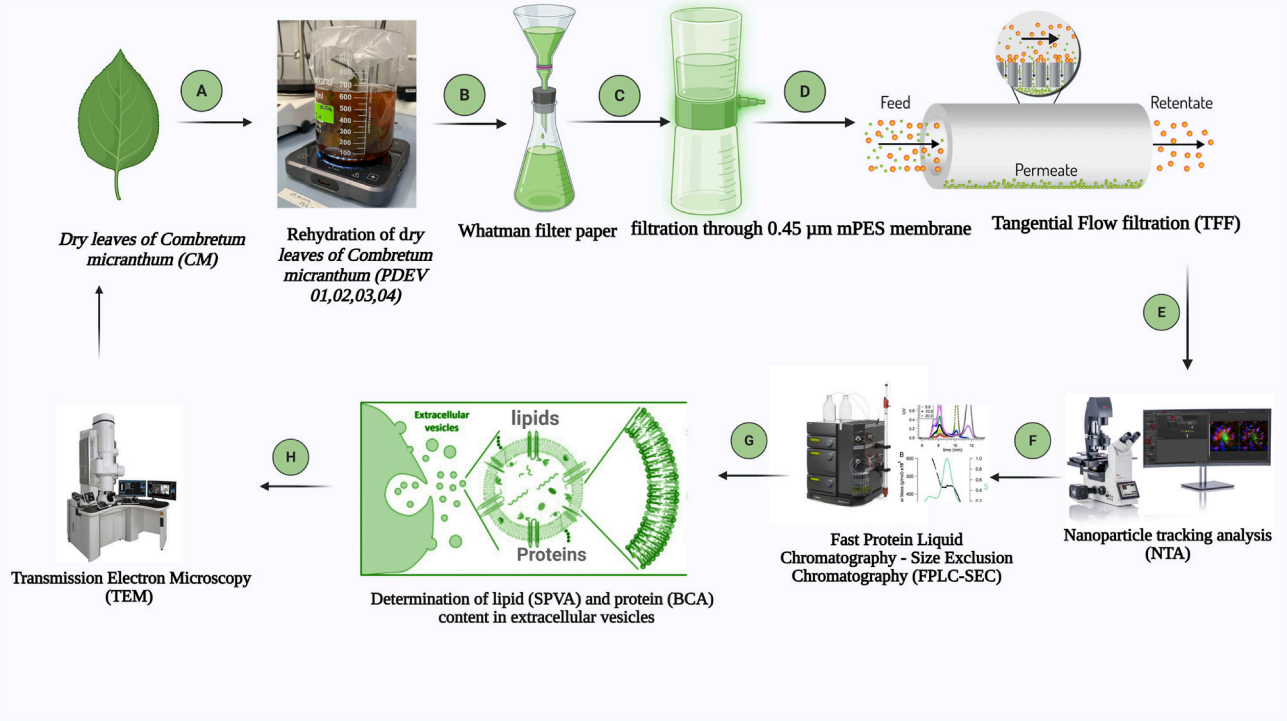


Figure 7. Overview of the isolation of extracellular vesicles from *Combretum micranthum* G. Don. After rehydration of the plant leaves (**A**), the samples were subsequently filtered through Whatman filter paper (**B**), followed by filtration through a 0.45 µm mPES membrane (**C**), and purified by tangential flow filtration (**D**). Nanoparticle tracking analysis (NTA) (**E**) was performed to determine the size and concentration of particles in the extracellular vesicle samples, followed by size-exclusion chromatography (**F**) to visualize the separation of diff erent extracellular vesicle populations based on their differential partitioning between the stationary and mobile phases. Lipid and protein content were determined (**G**). Transmission electron microscopy (TEM) (**H**) was used to assess the presence of extracellular vesicles in the samples.

3.3. Nanoparticle Tracking Analysis (NTA)

NTA is a commonly used method to determine the size and concentration of EV samples [53,54], based on the combination of two different physical principles: light scattering and Brownian motion. In this study, the concentration and size distribution of particles in the collected samples were measured using a NanoSight LM14C (NanoSight Ltd., Amesbury, UK). The measurements were performed at the Department of Biosciences and Medical Biology, Faculty of Natural and Life Sciences, Paris Lodron University of Salzburg, Austria, which was equipped with a 488 nm laser module and NTA 3.1 software. The samples were diluted (1:100–1:1000) in PBS and analyzed. After injecting the sample,

it was subjected to a laser beam for 60 s, and tracking of each particle was recorded at a constant camera level of 12, with a detection threshold of 5. To obtain reliable results, the sample was advanced, and another video was recorded. This procedure was repeated 5 times for each replicate (n = 3). In this study, the detected particles ranged from 80 to 120 nm, which corresponds to the expected size for extracellular vesicles and microvesicles. A schematic representation of the overall NTA protocol is provided in Figure 7E.

3.4. Fast Protein Liquid Chromatography–Size-Exclusion Chromatography (FPLC-SEC)

Size-exclusion chromatography is a technique used to separate different particle populations by the differential partitioning of analytes between a stationary phase and a mobile phase. The stationary phase is typically made up of a porous gel matrix that allows for differential elution; the larger the particle, the earlier it will be eluted [55]. The system can be used either as an isolation system in a single step in the sample collection mode or for size distribution analysis using the analytical mode. An analysis further indicates the purity of the sample after treatment by integrating the peak areas. Since the *Combretum micranthum* G. Don EV samples used in this study were already isolated by TFF, FPLC-SEC was used to study their size distribution and protein profile. In this study, 50 µL of *Combretum micranthum* G. Don EV sample was loaded onto a Superdex 200 Increase 10/300 GL column as the stationary phase, and an isocratic elution system of phosphate-buffered saline (PBS; pH 7.4) with a flow rate of 0.8 mL/min and a constant temperature of 4 °C for 60 min was used. The column was mounted on a custom-built Shimadzu LC-20AI FPLC instrument, equipped with a PDA SPD-M20A detector and an RF-20A fluorescence detector. A schematic representation of the overall FPLC-SEC protocol is provided in Figure 7F.

3.5. Determination of Protein Content in Extracellular Vesicles

To assess the protein content of the samples, total protein quantification is performed using the bicinchoninic acid (BCA) colorimetric assay [32]. Different EV preparations are tested to quantify the total protein content of various EV preparations. These preparations include PDEV01, PDEV02, PDEV03, and PDEV04. The standard BCA protocol is performed, which is valid for a protein concentration range of 20 to 2000 µg/mL. The BCA working reagent is prepared by mixing reagents A and B in a 50:1 ratio. First, a calibration curve is constructed using bovine serum albumin (BSA) as the protein standard. BSA solutions of different concentrations are obtained by serial dilution in PBS, ranging from 20 to 2000 µg/mL, starting from a 2 mg/mL BSA stock solution. For this, 25 µL of each sample and 200 µL of the reagent mixture are pipetted into a 96-well plate. The reactions are incubated at 37 °C for 30 min, and the linear absorbance at 562 nm is measured using a photometer. Three replicates are performed for each sample, and the average of the final absorbance value is calculated accordingly. The corrected absorbance values of the BSA standard solutions at different known concentrations are plotted against the concentration. A schematic representation of the determination of protein content protocol is provided in Figure 7G.

3.6. Determination of Lipid Content in Extracellular Vesicles

The method was adapted from Visnovitz et al. [42]. In brief, DOPC liposomes were prepared as standards for the SPVA. This was done by dissolving 2 mg of DOPC in 1 mL of chloroform in a 2 mL Eppendorf tube. The tube was placed with an open lid in a Thermoblock at 60 °C under a fume hood to evaporate the chloroform. After complete evaporation, 1 mL of PBS was added, and the mixture was vortexed for 2 min at maximum speed. The tube was then sonicated at 45 °C and 37 kilohertz (kHz) for 10 min. An additional powerful vortexing for 2 min followed. Before use, the liposomes were vortexed vigorously. A standard dilution series was then prepared using PBS with 10 dilution steps,

where step 10 was prepared as the blank. To prepare the phosphorus–vanillin reagent, 50 mg of vanillin was mixed in 50 mL of 17% phosphoric acid using a 50 mL volumetric flask. For the lipid content measurement of EVs, 1.5 mL Safe Lock tubes were filled with either 40 µL of EV solutions or 40 µL of the liposome standards, and 200 µL of 96% sulfuric acid was added. This was prepared in triplicate. The tubes were briefly vortexed and placed uncovered in the Thermoblock under the fume hood for 20 min at 90 °C. After cooling to room temperature, 120 µL of the phosphorus–vanillin reagent was added to each tube and vortexed. Finally, 280 µL from each tube was transferred to a 96-well plate and incubated at 37 °C for 1 h. Absorbance was measured at 540 nm using a Tecan plate reader. A calibration curve of the liposome standards was plotted, and the lipid concentration of the EV samples was determined. A schematic representation of the Determination of lipid content protocol is provided in Figure 7G.

3.7. Protocol for TEM Analysis of Negatively Stained Extracellular Vesicle

A 5 µL volume of the selected representative EV samples was dropped undiluted onto a holey carbon-coated grid (Quantifoil® carbon support grid R 1.2/1.3 Cu 400 mesh) and incubated at RT for 10 min in Petri dishes to promote the settling of the EVs onto the grids. Negative staining was performed with 2% (*w*/*v*) uranyl acetate (Honeywell Fluka, NC, USA, Cat. #72943) dissolved in ddH2O for 2 min, with excess stain removed using filter paper. Cryo-TEM imaging was performed by the TEM model JEOL JEM-F200 (Tokyo, Japan) equipped with a cold field emission gun, a TVIPS XF416 4k × 4k CMOS camera and a CEOS CEFID energy filter. A schematic representation of the overall TEM protocol is provided in Figure 7H.

4. Limitations and Future Perspectives

The main originality of the present study lies in the systematic comparison of different pre-treatment conditions, particularly temperature, treatment duration, and freeze–thaw cycles, applied to dried *Combretum micranthum* G. Don leaves prior to their isolation by tangential flow filtration (TFF). To the best of our knowledge, this study represents a first comparative approach aimed at optimizing the recovery and basic physicochemical characteristics of vesicle-like nanoparticles from dried medicinal plant material using a tangential flow filtration method with potential scalability. The integration of several complementary approaches, including NTA, FPLC-SEC, TEM, and biochemical quantification of proteins and lipids, to compare the different protocols provides a preliminary multidimensional characterization and establishes a basis for future molecular validation.

Despite the promising results obtained in this exploratory study, several limitations should be acknowledged. The characterization of particles isolated from *Combretum micranthum* G. Don leaves was mainly based on complementary biophysical and morphological approaches, including nanoparticle tracking analysis (NTA), FPLC-SEC, transmission electron microscopy (TEM), and the quantification of total proteins and lipids. Throughout this study, the term "PDEV-enriched preparations" is used to describe the isolated preparations, as the current characterization does not allow the definitive confirmation of their extracellular vesicle identity. These approaches provide evidence supporting the presence of nanoparticles displaying vesicular characteristics; however, they are not sufficient on their own to definitively establish the molecular identity of plant-derived extracellular vesicles (PDEVs) or completely exclude the presence of co-isolated non-vesicular components. In accordance with the recommendations of MISEV2018, the characterization of extracellular vesicles should ideally combine several complementary approaches, including the assessment of EV-associated molecular markers and the analysis of potential contaminants. It should also be noted that the currently available marker examples are mainly derived from studies of mammalian EVs, and

their applicability to non-mammalian sources, particularly plants, remains limited. Therefore, the lack of specific and universally accepted markers for plant-derived EVs represents an important methodological challenge in the present study [31].

Future research should build upon these findings by implementing a more comprehensive molecular and functional characterization of plant-derived EVs, combining advanced proteomic and lipidomic approaches with the targeted identification of candidate proteins and lipids associated with the vesicles. Particular attention should be given to the characterization of membrane and cytosolic proteins, the identification of potential non-vesicular contaminants, and the assessment of preparation purity. Analysis of the RNA cargo associated with EVs, particularly small RNAs, together with assessment of the association of these biomolecules with the vesicles, will also contribute to defining their molecular composition and strengthening their characterization. These approaches should be complemented by functional assays to establish a relationship between the molecular composition of EVs and their biological properties.

5. Conclusions

This study explored and optimized the isolation of plant-derived extracellular vesicle (PDEV)-enriched preparations from *Combretum micranthum* G. Don leaves using tangential flow filtration (TFF). The comparison of different pre-treatment conditions showed that extraction at room temperature for 24 h with 700 mL of dispersant (PDEV01) resulted in the highest particle concentration among the conditions tested. The combination of NTA, FPLC-SEC, TEM, and protein and lipid analyses provided a preliminary physicochemical characterization of the obtained preparations and highlighted the influence of pre-treatment conditions on particle recovery and characteristics.

However, these results should be considered as a preliminary characterization, as the approaches used cannot, on their own, definitively confirm the molecular identity of PDEVs or completely exclude the presence of co-isolated non-vesicular components. Further studies are therefore needed to confirm the identity and purity of PDEVs in accordance with the MISEV recommendations. This confirmation should be based on several complementary levels of evidence, including (i) the characterization of particle size, generally ranging from approximately 50 to 1000 nm; (ii) the analysis of individual particles or particle populations; and (iii) the identification of EV-associated markers, including transmembrane/external membrane proteins and cytosolic proteins. Further investigations, including the assessment of potential contaminants and advanced proteomic and lipidomic analyses, will also be necessary to strengthen the molecular characterization and provide greater confidence in the identity and purity of PDEVs.

Further optimization of the TFF process should also focus on membrane cut-off, flow rate, transmembrane pressure, and diafiltration steps to improve the recovery, purity, and reproducibility of the preparations. Complementary purification strategies, such as advanced size-exclusion chromatography or density-gradient centrifugation, could also be considered. Finally, functional studies will be necessary to establish the relationship between the molecular composition of PDEVs and their biological properties and therapeutic potential.

Overall, this study represents an initial step toward the standardization and optimization of PDEV isolation from *C. micranthum* and opens new perspectives for their potential biomedical and therapeutic applications.

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Visualization, C.-T.M., A.M., N.M.-K., and A.-F.T.; Supervision, C.-T.M., N.M.-K., and A.-F.T.; Project Administration, N.M.-K. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

EVs	Extracellular Vesicles
TFF	Tangential Flow Filtration
NTA	*Nanoparticle Tracking Analysis*
TEM	*Transmission Electron Microscopy*
SPVA	Sulfophosphovanillin
BCA	Bicinchoninic Acid
PDEV	Plant-Derived Extracellular Vesicle
FPLC-SEC	Fast Protein Liquid Chromatography–Size-Exclusion Chromatography

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