

# An optimized protocol for plant extracellular vesicle isolation from *Ophiopogon japonicus* root: a comparative evaluation based on miRNA cargo

**Yang Xiao**

College of Pharmacy, Chengdu University of Traditional Chinese Medicine

**Liqi Feng**

College of Pharmacy, Chengdu University of Traditional Chinese Medicine

**Xin Zhao**

School of Medical Technology, Chengdu University of Traditional Chinese Medicine

**Siyu Chen**

School of Medical Technology, Chengdu University of Traditional Chinese Medicine

**Fengqi Lv**

School of Medical Technology, Chengdu University of Traditional Chinese Medicine

**Zihan Li**

College of Pharmacy, Chengdu University of Traditional Chinese Medicine

**Qi Zheng**

College of Pharmacy, Chengdu University of Traditional Chinese Medicine

**Tao Zhou**

College of Pharmacy, Chengdu University of Traditional Chinese Medicine

**Yuntong Ma**

College of Pharmacy, Chengdu University of Traditional Chinese Medicine

**Binjie Xu**

**binjiexu@outlook.com**

Innovative Institute of Chinese Medicine and Pharmacy, Chengdu University of Traditional Chinese Medicine

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## Research Article

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# Abstract

**Background:** Plant extracellular vesicles (PEVs), hold significant therapeutic potential due to their roles in intercellular communication and cross-kingdom regulation, primarily mediated by their miRNA cargo. However, isolating high-purity PEVs from complex plant tissues, such as the tuberous roots of *Ophiopogon japonicus*, is challenging due to the dense cell wall matrix and high content of contaminants like polysaccharides. Existing isolation methods, including differential centrifugation and density gradient ultracentrifugation, involve trade-offs between yield, purity, and vesicle integrity, necessitating the development of optimized protocols.

**Results:** We developed and systematically optimized an integrated protocol for isolating high-purity EVs from *O. japonicus* roots. Key optimizations included: 1) refining the differential centrifugation protocol by incorporating a double ultracentrifugation step. 2) implementing a modified density gradient ultracentrifugation approach with a pre-clearing step for superior debris removal; and 3) evaluating enzymatic pre-treatment with cellulase and pectinase to enhance EV release. Comparative analysis demonstrated that the optimized method, particularly utilizing enzymatic pre-processing and double ultracentrifugation, significantly improved EV yield and purity. Small RNA sequencing of the resulting high-purity EVs successfully characterized their functional miRNA cargo profile, validating the efficacy of the isolation strategy.

**Conclusions:** This study establishes a robust and adaptable pipeline for isolating high-quality, functionally intact PEVs from challenging plant root tissues. The optimized protocol effectively addresses the critical methodological challenges of yield and purity, enabling reliable downstream functional characterization and advancing therapeutic investigations of plant-derived vesicles.

## Background

Extracellular vesicles (EVs) are lipid bilayer-enclosed nanoparticles that serve as crucial mediators of intercellular communication. They transport a diverse cargo of bioactive molecules—including proteins, lipids, nucleic acids (DNA, mRNA, miRNA), and metabolites—reflective of their cellular origin [1]. EVs are classified into subtypes such as exosomes (30–150 nm, derived from multivesicular bodies), microvesicles (50–1000 nm, budding from the plasma membrane), and apoptotic bodies (500–4000 nm, released during apoptosis) based on their biogenesis pathways and size [2–4]. While initially viewed as mechanisms for cellular waste disposal [5], EVs are now recognized as sophisticated signaling entities with pivotal roles in mammalian physiology and pathology, including immune regulation, tumor progression, and tissue repair [6]. Their significant diagnostic and therapeutic potential as disease biomarkers and drug delivery vehicles is well-established [7–10], driving intense research into their fundamental biology and functions [11].

Plant extracellular vesicles (PEVs), first observed in carrot cell cultures in 1967 [12], have garnered substantial research interest only in recent years. PEVs play essential roles in fundamental plant processes such as growth and development, defense against pathogens, and intercellular signaling [13–

15]. Particularly, note are plant-derived exosome-like nanovesicles (PDENs), which exhibit remarkable therapeutic potential, demonstrating effects including anti-inflammation [16], antioxidant activity [17], antitumor properties [18], gut microbiota modulation [19], and tissue repair [20]. The natural biocompatibility, stability, and inherent capacity of PDENs to carry both hydrophilic and lipophilic molecules make them highly promising candidates as nanocarriers for targeted drug delivery applications [21]. A key functional component underpinning the biological activity of PEVs is their microRNA (miRNA) cargo. miRNAs are small (~ 18–24 nucleotide (nt)) non-coding RNAs that silence target gene expression post-transcriptionally [22]. Importantly, PEVs can transport these regulatory plant miRNAs across kingdoms, influencing the physiology of recipient cells. For instance, *Arabidopsis thaliana* EVs deliver miRNAs capable of suppressing virulence genes in the fungal pathogen *Botrytis cinerea*, thereby inhibiting infection [23–25]. This cross-kingdom regulatory potential highlights the significant functional relevance of PEVs and their cargo.

Critically, the therapeutic efficacy and functional relevance of isolated PEVs are intrinsically linked to their purity and yield. However, isolating high-quality PEVs presents unique and substantial challenges due to the complex composition of plant tissues, which are rich in polysaccharides, secondary metabolites, and structural components like cellulose and pectin [1, 26]. Current isolation strategies involve significant trade-offs. Differential centrifugation (DC), often considered a standard method, frequently suffers from low resolution, significant sample loss, and the co-isolation of non-EV contaminants such as protein aggregates and organelle fragments, resulting in suboptimal purity [1, 26]. Density gradient ultracentrifugation (DG-UC), utilizing media like sucrose or iodixanol, significantly improves purity by separating EVs based on their characteristic buoyant density (typically 1.10–1.18 g/mL) [27, 28], but it can be time-consuming and may reduce overall yield. Alternative techniques such as ultrafiltration, immunoaffinity capture, polymer-based precipitation, and commercial kits offer different advantages but may introduce biases, risk damaging EVs, or lack scalability [29, 30]. Furthermore, optimal isolation protocols must be carefully tailored to specific plant organs (e.g., roots vs. leaves), considering factors like sap content, fiber abundance, and cell wall composition [26, 31]. A particularly persistent challenge is efficiently liberating EVs trapped within the dense plant cell wall matrix without introducing contaminants or degrading the vesicles themselves. Enzymatic pre-treatment (e.g., with cellulase and pectinase) offers a potential solution by degrading structural polysaccharides [3], but its impact on final EV yield and the integrity of their molecular cargo requires careful evaluation.

In this study, we address these critical methodological gaps by developing and systematically optimizing an integrated protocol for isolating high-purity EVs from the tuberous roots of *O. japonicus*, a valuable medicinal plant. We specifically evaluated and optimized enzymatic digestion using cellulase and pectinase to maximize EV release while minimizing co-extracted impurities. We refined DC protocols, incorporating a crucial post-ultracentrifugation wash step to enhance purity. Furthermore, we implemented a modified DG-UC approach that included a pre-clearing centrifugation step to maximize debris removal prior to gradient separation. We rigorously compared the yield, purity, and integrity of EVs isolated via single versus double ultracentrifugation and enzymatic versus non-enzymatic pre-processing methods. Finally, to validate the functionality of the isolated vesicles, we utilized the high-purity EVs

obtained through our optimized density gradient method for comprehensive small RNA sequencing to characterize their miRNA cargo profile. This work establishes a robust and adaptable pipeline for obtaining high-quality, functionally relevant PEVs from challenging root tissues, enabling reliable downstream functional characterization and therapeutic investigations.

## Materials and Methods

### 1. Plant materials and growth condition

Fresh *O. japonicus* samples used in this experiment were all collected from a standardized cultivation base located in Santai County, Sichuan Province, China. According to the differences in cultivation management practices, the samples were divided into two groups, including control group (CK) and plant regulators treated group (Treatment). All seedlings were transplanted in April 2023 and harvested in April 2024, corresponding to a 1-year cultivation cycle. Throughout the growth period, standard management protocols of the cultivation base were strictly implemented, including regular soil tillage (e.g., deep plowing and harrowing to improve soil structure), scientific water-fertilizer management (irrigation and fertilization adjusted based on the growth stage of *O. japonicus*), and integrated pest control. The treated group was the application of paclobutrazol during the specific growth stage, with a 15% paclobutrazol wettable powder (WP) solution sprayed on the foliage. The application rate was 0.6 kg active ingredient (a.i.) per hectare, and the spray was evenly distributed on both the adaxial and abaxial surfaces of leaves to ensure absorption.

### 2. Isolation of plant EVs by differential centrifugation

EVs were isolated from fresh roots of *O. japonicus* using a differential centrifugation protocol. Briefly, roots were weighed, thoroughly washed three times with deionized water, and sectioned into 0.5 cm pieces using sterile scissors. The tissue sections were then homogenized in ice-cold 1× phosphate-buffered saline (PBS, pH 7.4) using a mechanical homogenizer. The resulting homogenate was sequentially filtered through sterile gauze to remove large debris. The clarified filtrate was subjected to a series of centrifugation steps at 4°C: first at 1,000 × g for 10 min to remove large cellular fragments, followed by 5,000 × g for 20 min to pellet smaller debris and organelles, and then 10,000 × g for 40 min to remove larger vesicles and apoptotic bodies. The supernatant obtained after the 10,000 × g centrifugation was filtered through a sterile 0.22 μm membrane and then carefully transferred to ultracentrifuge tubes (Beckman, Cat# 355642). Ultracentrifugation was performed at 150,000 × g for 90 min at 4°C using a Beckman Optima XE ultracentrifuge. The resulting pellet, containing crude EVs (designated as the initial pellet), was resuspended in 1.5 mL of ice-cold PBS to obtain the P1 EV fraction.

To further purify the EVs and remove potential soluble contaminants or aggregates, the P1 EV fraction was purified by a second ultracentrifugation step under identical conditions (150,000 × g, 90 min, 4°C) and resuspended to yield the P2 EV fraction, which was used for subsequent analyses.

### 3. Density gradient fractionation separates plant EVs

EV subpopulations were further purified from the P1 fraction (obtained via differential centrifugation) using discontinuous sucrose density gradient ultracentrifugation. Sucrose working solutions (8%, 30%, 45%, and 60% w/v) were prepared in sterile 1× phosphate-buffered saline (PBS, pH 7.4) and filtered (0.22 µm). A discontinuous gradient was meticulously constructed in ultracentrifuge tubes (Beckman, Cat# 355642) by sequentially layering 2 mL of each sucrose solution, beginning with 60% at the bottom, followed by 45%, 30%, and finally 8% at the top, ensuring minimal disruption of the interfaces. The P1 EV fraction (1.5 mL) was carefully loaded onto the pre-formed gradient.

Gradients were subjected to ultracentrifugation at  $150,000 \times g$  for 120 min at 4°C using a Beckman Optima XE ultracentrifuge with a fixed-angle rotor (e.g., Type 70 Ti). Following centrifugation, distinct bands were visible. The fraction corresponding to the buoyant density interface between the 30% and 45% sucrose layers was collected by careful pipetting. This fraction was diluted > 10-fold with ice-cold PBS and pelleted by ultracentrifugation at  $150,000 \times g$  for 90 min at 4°C. The resulting pellet was resuspended in 1.5 mL of ice-cold PBS to yield the sucrose density gradient-purified EV fraction (designated P3).

To generate an ultra-purified fraction (P4), the P1 material underwent additional pre-clearing steps prior to density gradient fractionation. Specifically, multiple sequential centrifugation steps at  $10,000 \times g$  for 40 min (4°C) were performed to exhaustively remove residual large debris. The pre-cleared supernatant was then processed identically to the P1 fraction through sucrose density gradient fractionation (as described above for P3 generation). The final pellet from the post-gradient ultracentrifugation was similarly resuspended in 1.5 mL of ice-cold PBS to obtain the P4 EV fraction.

### 4. Enzymatic isolation of plant extracellular vesicles

EVs, designated here as *O. japonicus* Root-Derived Extracellular Nanovesicles (ORDENs), were isolated from fresh *O. japonicus* roots using an enzymatic digestion protocol. Briefly, roots were washed thoroughly with deionized water to remove soil contaminants, blotted dry, and weighed to record fresh biomass.

The cleaned roots were homogenized in ice-cold 1×PBS (pH 7.4) using a mechanical homogenizer at a tissue-to-buffer ratio of 1:2 (w/v). The resultant homogenate was filtered through sterile muslin cloth to remove large particulate matter. To digest the plant cell wall matrix and facilitate EV release, the filtrate was supplemented with the following enzymes (final concentrations): 30 mg/mL Cellulase and 2 mg/mL Pectinase. The enzymatic digestion proceeded for 12 h at room temperature (approximately 25°C) under gentle agitation. Following incubation, the digestate was centrifuged at  $3,000 \times g$  for 20 min at 4°C to pellet cellular debris, including residual cell wall fragments and protoplasts. The supernatant, enriched with released ORDENs, was then subjected to ultracentrifugation for EV isolation. Specifically, it was transferred to ultracentrifuge tubes (Beckman, Cat# 355642) and centrifuged at  $150,000 \times g$  for 90 min

at 4°C (Optima XE; Beckman) to pellet the ORDENs. The resulting pellet was subsequently processed according to the downstream purification or analysis requirements (e.g., resuspension in PBS, density gradient fractionation as described previously).

## **5. Transmission electron microscope analysis of plant EVs**

To confirm plant EVs morphology, 10 µL of sample suspension was dropped on the copper mesh with a liquid gun, timed for 10min, and the excess liquid was sucked away with a small piece of filter paper. Each sample was stained with 3% uranyl acetate for 1–3 min and used a filter paper to wick away excess staining liquid before being imaged at 100 KV using a Transmission Electron Microscope (FEI TECNAI G2 12).

## **6. Nanoparticle Concentration and Size Distribution by Nano-Flow Cytometry**

The particle size distribution and concentration of the isolated plant EVs were determined using nanoparticle flow cytometry (Flow NanoAnalyzer U30E, NanoFCM, China). Briefly, the EV sample was appropriately diluted with 1× PBS to a concentration within the instrument's optimal detection range (typically  $5 \times 10^8$  to  $5 \times 10^9$  particles/mL) to minimize coincidence events. A minimum of 10,000 events were recorded for each sample at a flow rate of 100 µL/min. The volumetric absolute count method was applied for direct concentration measurement without the need for external standards. The derived particle size was calculated based on the scattered light intensity.

## **7. Membrane staining efficiency assay**

To confirm the lipid membrane integrity of the isolated vesicles and distinguish them from non-lipid particles, the samples were stained with a lipophilic fluorescent dye. A 10 µM working solution of POMV Membrane Green Stains was freshly prepared by a 10-fold dilution of the stock solution with the provided Dilution C. Then, 10 µL of EVs (approximately  $2-5 \times 10^{10}$  particles/mL, pre-diluted with Diluent C if necessary) was incubated with 10 µL of the working solution at 37°C for 10 minutes in the dark. Following incubation, the stained sample was diluted 100-fold with 1× PBS and analyzed immediately on the Flow NanoAnalyzer. An unstained EV sample, processed identically but without the dye, was used as a negative control to set the fluorescence threshold. The percentage of membrane-positive particles was defined as the proportion of events exhibiting fluorescence intensity above the 99.99th percentile of the unstained control.

## **8. Small RNA library construction and sequencing**

The EVs were selected from each sample, and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for RNA extraction. Total RNA was extracted by using Trizol reagent (TIANGEN, Beijing, China). The RNA amount and purity of each sample was quantified using NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). The RNA integrity was assessed by Bioanalyzer 2100 (Agilent, CA, USA) with RIN number  $> 7.0$ , and confirmed by electrophoresis with denaturing agarose gel. The small RNA (sRNA) libraries were constructed following the manufacturer's instructions of TruSeq small RNA sample preparation kits (Illumina, San Diego, United States). All libraries were sequencing by Hiseq 2500 Sequencing & Pipeline filter in Lianchuan biotech Co., Ltd. (Hangzhou, China).

## 9. Bioinformatics analysis of small RNAs and identification of miRNA

Bioinformatics analysis of sRNA and miRNA identification were performed using the ACGT101-miR (v4.2) platform (LC Sciences, USA). Raw reads were processed by first removing adapter sequences, contaminants, and low-quality reads to obtain clean data, after which small RNAs within the plant-specific length of 18–25 nucleotides were retained. These filtered sequences were then aligned against mRNA, Rfam, and Repbase databases to exclude non-miRNA sequences such as rRNAs, tRNAs, snRNAs, snoRNAs, and repetitive elements. The remaining sequences were mapped to known miRNA precursors and the reference genome to identify known miRNAs and novel miRNAs derived from either the 3' or 5' arm of hairpin structures. The expression levels of miRNAs were normalized, and differential expression between comparison groups was evaluated using statistical methods such as Student's t-test or ANOVA, with significance thresholds set at  $p < 0.05$  or  $0.01$ . Finally, putative target genes of differentially expressed miRNAs were predicted and subjected to functional enrichment analysis to interpret their biological roles. To systematically analyze and identify potential biological pathways, the omics platform was used for GO and KEGG pathway enrichment analysis with the target genes of different miRNAs. GO enrichment analysis include three categories: biological process (BP), molecular functions (MF), and cell components. The GO enriched terms and KEGG pathway terms were identified with  $p$  values  $< 0.05$ . The Top 25 of KEGG enrichment were selected for further analysis.

## Results

## 1. Double ultrahigh-speed centrifugations enhance EVs purity at the expense of yield

Differential ultracentrifugation, a standard method for EV isolation, was systematically evaluated by comparing single (P1) versus double (P2) ultracentrifugation wash steps for yield, purity, and integrity of EVs derived from *O. japonicus* roots. Fresh roots were processed via washing, dicing, PBS homogenization, juice extraction, and filtration (Fig. 1A). Sequential centrifugation steps ( $1,000 \times g/10$  min  $\rightarrow 5,000 \times g/20$  min  $\rightarrow 10,000 \times g/40$  min) removed cellular debris, organelles, and protein complexes. The supernatant was then subjected to ultracentrifugation ( $150,000 \times g/2$  h) to pellet the initial EV fraction (P1). For P2, the P1 pellet was resuspended in PBS and subjected to a second identical

ultracentrifugation. Visual inspection revealed significantly reduced turbidity in the P2 fraction compared to P1 (Fig. 1B). Transmission electron microscopy (TEM) confirmed higher contamination in P1, with abundant protein aggregates and fibrils, while P2 exhibited cleaner vesicle morphology (Fig. 1C). Nanoparticle tracking analysis (NTA) quantified this improvement: P2 EVs displayed a smaller mean diameter (67.5 nm vs. P1: 61.5 nm) and reduced concentration ( $1.27 \times 10^{12}$  vs. P1:  $5.05 \times 10^{12}$  particles/mL), reflecting the removal of non-vesicular material (Fig. 1D). Fluorescent membrane labeling further validated enhanced purity, with 49.0% of P2 particles retaining intact membranes versus 46.4% in P1 (Fig. 1E). This demonstrates that the additional wash step effectively depletes co-precipitated contaminants (e.g., apoptotic bodies, microvesicles), albeit at the cost of reduction in particle yield.

## 2. Enhanced EV purity through density gradient centrifugation and apoplastic

Fluid Pre-clearing to achieve higher purity EVs suitable for downstream applications, sucrose DG-UC was optimized. Sucrose gradients (60%/45%/30%/8%) were layered, followed by loading of EVs pre-isolated via ultracentrifugation. Ultracentrifugation migrated EVs to their characteristic buoyant density (1.10–1.18 g/mL), visible as a discrete band at the 30–45% sucrose interface (Fig. 2B). This band was aspirated, diluted in PBS, and repelleted to yield purified fractions (P3, P4). Crucially, we refined apoplastic washing fluid (AWF) preparation to minimize input debris. Iterative centrifugation of crude AWF (AWF1) at  $10,000 \times g/40$  min – repeated until no visible pellet formed – produced markedly clarified AWF2 (Fig. 2B). EVs derived from both AWF sources banded identically during DG-UC. TEM imaging revealed superior vesicle integrity and reduced fibrillar/protein contamination in the P4 fraction compared to P3 (Fig. 2C). NTA confirmed P4 EVs were smaller (62.4 nm vs. P3: 73.4 nm) and more concentrated ( $1.16 \times 10^{12}$  vs. P3:  $2.8 \times 10^{11}$  particles/mL) (Fig. 2D), consistent with effective contaminant removal. Fluorescent labeling demonstrated significantly higher membrane integrity in P4 (75.3% labeled vesicles) versus P3 (65.1%) (Fig. 2E), quantitatively validating the enhanced purity achieved by combining pre-cleared AWF2 with DG-UC.

## 3. Enzymatic Digestion Enhances EV Release and Purity

To address the challenge of efficiently liberating EVs embedded within the robust plant cell wall matrix, we evaluated an enzymatic pre-digestion strategy. This approach utilizes hydrolytic enzymes, including cellulase and pectinase, to degrade the primary structural components of the cell wall, thereby facilitating the release of entrapped vesicles into solution while simultaneously reducing co-isolation of intracellular contaminants [32]. Building on its successful application in species like *Catharanthus roseus* [33], we adapted this method for *O. japonicus* roots, yielding the P5 EV fraction. TEM confirmed that P5 EVs exhibited classic cup-shaped exosome morphology with minimal non-vesicular debris (Fig. 3A). NTA revealed a homogenous size profile, with the majority of particles ranging from 45–150 nm and a peak diameter of  $77 \pm 27.3$  nm (Fig. 3B). Crucially, membrane integrity assays using fluorescent dyes

demonstrated that 83% of particles in the P5 fraction were intact, labeled vesicles (Fig. 3C), indicating superior vesicle integrity and purity compared to fractions isolated by ultracentrifugation alone (P1, P2) or density gradient centrifugation (P3, P4). This confirms enzymatic digestion as a highly effective pre-processing step for isolating high-quality EVs from complex plant tissues.

## 4. High-quality miRNA sequencing data validates the effectiveness of our isolation method

We successfully isolated EVs from the roots of *O. japonicus* using our optimized extraction protocol. To rigorously evaluate the reliability and reproducibility of our method, high-quality miRNA sequencing was performed on EVs derived from two biologically independent replicates for both control and paclobutrazol-treated groups. Overall, 9,074,206 to 29,418,203 raw reads were obtained from these four sRNA libraries. The GC content of each sample was between 55.19%–56.17%, and the Q30 value exceeded 95% (Supplementary Table 1). After eliminating 3' adapters and low - quality sequences, 27.00 M clean reads were obtained. Compared the clean reads sequences with the RFam and Repbase databases, and removed the non-miRNA sequences from the clean data.

After filtering out the junk reads, repeats, and adapters sequences, 3,944,772 to 13,379,771 clean reads were obtained (Supplement Table 2). Then, the remaining sequences were aligned to mRNA, Rfam and Repbase database to discard ncRNAs (rRNA, tRNA, snoRNA, and snRNA,), other Rfam RNA, and repeat sequences, and the filtered sequences were used for miRNAs identification.

A total of 228 expressed miRNAs were identified in all samples (Supplementary Table 3). All these miRNAs were divided into five groups, then 201 pre-miRNAs (1, 13, 79, 33 and 75 in gp1, gp2a, gp2b, gp3 and gp4, respectively) and 174 unique miRNAs (2, 14, 59, 35 and 64 in gp1, gp2a, gp2b, gp3 and gp4, respectively) were obtained (Supplementary Table 4). Interestingly, the number of unique miRNAs in gp4 (novel miRNA group) was the largest. All mapped sRNAs within the length range of 18–24 nt were counted for the total and unique reads, most of samples showed the highest abundance at 21-nt (Supplementary Fig. 1A and Table 5). A total of 142 known and 86 novel mature miRNAs were consistently identified across all replicates, confirming that our EV extraction method effectively captures a diverse miRNA population. The known miRNAs were classified into 32 families, with miR2592 (9 members), miR168 (8 members), and miR156 (7 members) being the most abundant (Supplementary Table 6). The length distribution of miRNAs showed that those sequences of 21 nt were the most abundant size class of the unique miRNAs followed by 18, 22, 19 and 20 nt (Supplementary Fig. 1A). Both known and novel miRNAs showed a typical length distribution, further supporting the successful enrichment of EV-derived miRNAs. Further, to reveal the conservation of identified miRNAs with other species in this study, we compared pre-miRNAs with other species in miRbase, and found that 139 miRNAs were highly conservative with their homologue in soybean (*Glycine Max*) (Supplementary Fig. 1B). To enhance the reliability and stability of miRNAs identified here, the miRNAs expressed only in a

library of two biological repeats were filled out. The Venn analysis showed that 79 known miRNAs were identified both in the control and treatment groups, while 47 and 8 miRNAs were expressed only in treatment group and control group, respectively (Fig. 4A). Expression analysis showed that 78 known miRNAs were differentially expressed in the control and treatment groups. 13 miRNAs were differentially expressed in the treatment group, with 6 up - regulated and 7 down - regulated ( $p < 0.05$ ; Supplementary Table 7 and Fig. 4B). Among them, 9 were known Differentially expressed miRNAs (DEMs) from 7 miRNA families. miR156 and miR159 families had 2 members each, and the other 5 families possessed 1 member each (Fig. 4C & D).

## **5. Functional analysis and validation of known and unknown miRNA in extracellular vesicles.**

To explore the specific functions of candidate miRNAs mentioned above, their target genes were screened and obtained from degradome data. The GSTAr (v1.0) was used to predict the target genes of the known and novel miRNAs. A total of 232 target genes were predicted. Then, functional classification of target genes was performed by GO analysis under biological processes (BP), cell components (CC) and molecular functions (MF) categories (Table 1). GO enrichment analysis of the target genes of differentially expressed miRNAs revealed that in the biological process category, multiple processes related to leaf development (GO:0048366), root development (GO:0048364), and the cell cycle were significantly enriched. In the cellular component category, terms like extracellular region (GO:0005576) and exosome (GO:0000178) were enriched, indicating that some target gene products function in extracellular vesicles (exosomes). In the molecular function category, functional groups such as sesquiterpene synthase activity (GO:0010334) and P-type calcium transporter activity (GO:0005388) were significantly enriched (Fig. 5A). Interestingly, it was showed that most target genes of identified miRNAs were enriched in sesquiterpene synthase activity and sesquiterpene biosynthetic process

Table 1  
GO enrichment

| GO ID                                                    | GO Term                                                                               | GO Category | Rich Factor | <i>P</i> value | Sig. Sign. <i>P</i> |
|----------------------------------------------------------|---------------------------------------------------------------------------------------|-------------|-------------|----------------|---------------------|
| GO:0051762                                               | sesquiterpene biosynthetic process                                                    | BP          | 0.375       | 7E-18          | ****                |
| GO:0010334                                               | sesquiterpene synthase activity                                                       | MF          | 0.3214      | 4E-17          | ****                |
| GO:0005388                                               | P-type calcium transporter activity                                                   | MF          | 0.1923      | 2E-16          | ****                |
| GO:0008408                                               | 3'-5' exonuclease activity                                                            | MF          | 0.1525      | 6E-14          | ****                |
| GO:0004527                                               | exonuclease activity                                                                  | MF          | 0.1047      | 2E-12          | ****                |
| GO:0010507                                               | negative regulation of autophagy                                                      | BP          | 0.375       | 3E-12          | ****                |
| GO:0033897                                               | ribonuclease T2 activity                                                              | MF          | 0.3333      | 6E-12          | ****                |
| GO:0010168                                               | ER body                                                                               | CC          | 0.2727      | 2E-11          | ****                |
| GO:0042325                                               | regulation of phosphorylation                                                         | BP          | 0.3846      | 2E-10          | ****                |
| GO:0009048                                               | dosage compensation by inactivation of X chromosome                                   | BP          | 0.8         | 2E-10          | ****                |
| GO:1904872                                               | regulation of telomerase RNA localization to Cajal body                               | BP          | 0.8         | 2E-10          | ****                |
| GO:0071044                                               | histone mRNA catabolic process                                                        | BP          | 0.6667      | 7E-10          | ****                |
| GO:0090503                                               | obsolete RNA phosphodiester bond hydrolysis, exonucleolytic                           | BP          | 0.6667      | 7E-10          | ****                |
| GO:0000178                                               | exosome (RNase complex)                                                               | CC          | 0.5714      | 2E-09          | ****                |
| GO:0045339                                               | farnesyl diphosphate catabolic process                                                | BP          | 0.5         | 3E-09          | ****                |
| GO:0004532                                               | RNA exonuclease activity                                                              | MF          | 0.5         | 3E-09          | ****                |
| GO:0071048                                               | nuclear mRNA surveillance                                                             | BP          | 0.5         | 3E-09          | ****                |
| GO:0016075                                               | rRNA catabolic process                                                                | BP          | 0.1071      | 1E-08          | ****                |
| GO:0031597                                               | cytosolic proteasome complex                                                          | CC          | 0.1053      | 1E-08          | ****                |
| GO:0036402                                               | proteasome-activating activity                                                        | MF          | 0.1053      | 1E-08          | ****                |
| GO:0045899                                               | positive regulation of RNA polymerase II transcription preinitiation complex assembly | BP          | 0.1053      | 1E-08          | ****                |
| GO:0006401                                               | RNA catabolic process                                                                 | BP          | 0.1053      | 1E-08          | ****                |
| ‘**’ indicates $p < 0.005$ , ‘***’ indicates $p < 0.001$ |                                                                                       |             |             |                |                     |

| GO ID                                                    | GO Term                                                    | GO Category | Rich Factor | <i>P</i> value | Sig. Sign. <i>P</i> |
|----------------------------------------------------------|------------------------------------------------------------|-------------|-------------|----------------|---------------------|
| GO:0045338                                               | farnesyl diphosphate metabolic process                     | BP          | 0.1786      | 1E-08          | ****                |
| GO:0016838                                               | carbon-oxygen lyase activity, acting on phosphates         | MF          | 0.3636      | 2E-08          | ****                |
| GO:0007155                                               | cell adhesion                                              | BP          | 0.1667      | 2E-08          | ****                |
| GO:0031595                                               | nuclear proteasome complex                                 | CC          | 0.0968      | 2E-08          | ****                |
| GO:0005887                                               | plasma membrane                                            | CC          | 0.0159      | 5E-08          | ****                |
| GO:0006364                                               | rRNA processing                                            | BP          | 0.0317      | 9E-08          | ****                |
| GO:0005576                                               | extracellular region                                       | CC          | 0.0093      | 1E-07          | ****                |
| GO:0004521                                               | RNA endonuclease activity                                  | MF          | 0.0682      | 1E-07          | ****                |
| GO:0000325                                               | plant-type vacuole                                         | CC          | 0.0349      | 2E-07          | ****                |
| GO:0008540                                               | proteasome regulatory particle, base subcomplex            | CC          | 0.0638      | 2E-07          | ****                |
| GO:0007568                                               | obsolete aging                                             | BP          | 0.0632      | 2E-07          | ****                |
| GO:0017025                                               | TBP-class protein binding                                  | MF          | 0.0571      | 4E-07          | ****                |
| GO:0032211                                               | negative regulation of telomere maintenance via telomerase | BP          | 0.1667      | 5E-07          | ****                |
| GO:0005622                                               | intracellular anatomical structure                         | CC          | 0.0132      | 5E-07          | ****                |
| GO:0000956                                               | nuclear-transcribed mRNA catabolic process                 | BP          | 0.16        | 6E-07          | ****                |
| GO:0071034                                               | CUT catabolic process                                      | BP          | 0.1538      | 7E-07          | ****                |
| GO:0004321                                               | fatty-acyl-CoA synthase activity                           | MF          | 0.1143      | 2E-06          | ****                |
| GO:0019888                                               | protein phosphatase regulator activity                     | MF          | 0.0625      | 3E-06          | ****                |
| GO:0035327                                               | euchromatin                                                | CC          | 0.1111      | 3E-06          | ****                |
| GO:0016207                                               | 4-coumarate-CoA ligase activity                            | MF          | 0.1081      | 3E-06          | ****                |
| GO:0005773                                               | vacuole                                                    | CC          | 0.0113      | 3E-06          | ****                |
| GO:0009851                                               | auxin biosynthetic process                                 | BP          | 0.1026      | 4E-06          | ****                |
| GO:0008285                                               | negative regulation of cell population proliferation       | BP          | 0.0568      | 4E-06          | ****                |
| ‘**’ indicates $p < 0.005$ , ‘***’ indicates $p < 0.001$ |                                                            |             |             |                |                     |

| GO ID                                                | GO Term                                                                | GO Category | Rich Factor | <i>P</i> value | Sig. Sign. <i>P</i> |
|------------------------------------------------------|------------------------------------------------------------------------|-------------|-------------|----------------|---------------------|
| GO:0030433                                           | ubiquitin-dependent ERAD pathway                                       | BP          | 0.0377      | 5E-06          | ****                |
| GO:0071035                                           | nuclear polyadenylation-dependent rRNA catabolic process               | BP          | 0.0889      | 7E-06          | ****                |
| GO:0071028                                           | nuclear mRNA surveillance                                              | BP          | 0.0816      | 9E-06          | ****                |
| GO:0061088                                           | obsolete regulation of sequestering of zinc ion                        | BP          | 0.08        | 1E-05          | ****                |
| GO:0043231                                           | intracellular membrane-bounded organelle                               | CC          | 0.0123      | 1E-05          | ****                |
| GO:0004816                                           | asparagine-tRNA ligase activity                                        | MF          | 0.0667      | 2E-05          | ****                |
| GO:0006421                                           | asparaginyl-tRNA aminoacylation                                        | BP          | 0.0667      | 2E-05          | ****                |
| GO:0000175                                           | 3'-5'-RNA exonuclease activity                                         | MF          | 0.0656      | 2E-05          | ****                |
| GO:0000460                                           | maturation of 5.8S rRNA                                                | BP          | 0.0548      | 5E-05          | ****                |
| GO:0005777                                           | peroxisome                                                             | CC          | 0.0145      | 5E-05          | ****                |
| GO:0031408                                           | oxylipin biosynthetic process                                          | BP          | 0.0513      | 6E-05          | ****                |
| GO:0004252                                           | serine-type endopeptidase activity                                     | MF          | 0.0191      | 6E-05          | ****                |
| GO:0008559                                           | ABC-type xenobiotic transporter activity                               | MF          | 0.0488      | 7E-05          | ****                |
| GO:0009850                                           | auxin metabolic process                                                | BP          | 0.0488      | 7E-05          | ****                |
| GO:0009695                                           | jasmonic acid biosynthetic process                                     | BP          | 0.0482      | 8E-05          | ****                |
| GO:0010321                                           | regulation of vegetative phase change                                  | BP          | 0.3333      | 0.0001         | ***                 |
| GO:0000176                                           | nuclear exosome (RNase complex)                                        | CC          | 0.0435      | 0.0001         | ***                 |
| GO:0005385                                           | zinc ion transmembrane transporter activity                            | MF          | 0.0421      | 0.0001         | ***                 |
| GO:0048366                                           | leaf development                                                       | BP          | 0.0199      | 0.0002         | ***                 |
| GO:0003916                                           | DNA topoisomerase activity                                             | MF          | 0.2         | 0.0003         | ***                 |
| GO:0010043                                           | response to zinc ion                                                   | BP          | 0.0328      | 0.0003         | ***                 |
| GO:0046686                                           | response to cadmium ion                                                | BP          | 0.0091      | 0.0004         | ***                 |
| GO:0003917                                           | DNA topoisomerase type I (single strand cut, ATP-independent) activity | MF          | 0.1176      | 0.0009         | ***                 |
| ** indicates $p < 0.005$ , *** indicates $p < 0.001$ |                                                                        |             |             |                |                     |

| GO ID                                                    | GO Term                                                  | GO Category | Rich Factor | <i>P</i> value | Sig. Sign. <i>P</i> |
|----------------------------------------------------------|----------------------------------------------------------|-------------|-------------|----------------|---------------------|
| GO:0016485                                               | protein processing                                       | BP          | 0.0242      | 0.001          | **                  |
| GO:0048658                                               | anther wall tapetum development                          | BP          | 0.1111      | 0.001          | **                  |
| GO:0030527                                               | structural constituent of chromatin                      | MF          | 0.1053      | 0.0012         | **                  |
| GO:0048364                                               | root development                                         | BP          | 0.0126      | 0.0018         | **                  |
| GO:0043682                                               | P-type divalent copper transporter activity              | MF          | 0.0833      | 0.0019         | **                  |
| GO:0004477                                               | methenyltetrahydrofolate cyclohydrolase activity         | MF          | 0.0769      | 0.0022         | **                  |
| GO:0004488                                               | methylenetetrahydrofolate dehydrogenase (NADP+) activity | MF          | 0.0769      | 0.0022         | **                  |
| GO:0010076                                               | maintenance of floral meristem identity                  | BP          | 0.0741      | 0.0024         | **                  |
| GO:0009554                                               | megasporogenesis                                         | BP          | 0.0606      | 0.0035         | **                  |
| GO:0030163                                               | protein catabolic process                                | BP          | 0.0162      | 0.0045         | **                  |
| GO:0050105                                               | L-gulonolactone oxidase activity                         | MF          | 0.0526      | 0.0046         | **                  |
| GO:0052694                                               | jasmonoyl-isoleucine-12-hydroxylase activity             | MF          | 0.5         | 0.0053         | **                  |
| GO:0016874                                               | ligase activity                                          | MF          | 0.0154      | 0.0053         | **                  |
| GO:0035066                                               | positive regulation of histone acetylation               | BP          | 0.0476      | 0.0057         | **                  |
| GO:0003885                                               | D-arabinono-1,4-lactone oxidase activity                 | MF          | 0.0476      | 0.0057         | **                  |
| GO:2000012                                               | regulation of auxin polar transport                      | BP          | 0.0465      | 0.0059         | **                  |
| GO:0016020                                               | membrane                                                 | CC          | 0.0052      | 0.0073         | **                  |
| ‘**’ indicates $p < 0.005$ , ‘***’ indicates $p < 0.001$ |                                                          |             |             |                |                     |

Subsequently, KEGG analysis was used to explore the metabolic pathways regulated by target genes, and showed that 18 metabolic pathways were significantly enriched (Supplementary Table 8). Interesting, most pathways were enriched in Metabolism, especially in metabolism of terpenoids and polyketides and biosynthesis of other secondary metabolites (Fig. 5C&D), the Sesquiterpenoid and triterpenoid biosynthesis (map00909) accounts the most (Table 2). Combine with the GO analysis, it was found that target genes were enriched in biosynthesis and activity of Sesquiterpenoids and triterpenoids, which are two crucial classes of secondary metabolites in plants (Ref). Sesquiterpenoids are usually involved in plant defense, insect attraction, and antifeedant responses, whereas triterpenoids, including

saponins (such as ginsenosides and soyasaponins), sterols, play key roles in defense, signaling, and interactions with microorganisms (Ref). This indicates that miRNAs in exosomes can target the biosynthetic genes of these pathways, and suggests that *O. japonicus* plants can remotely and precisely "turn off" or "downregulate" these metabolic processes in recipient cells.

Table 2  
KEGG enrichment analysis of DE miRNA target genes.

| Pathway                                                  | KEGG Level 2                                | Pathway Name                                  | Rich Factor | P value     | Sig.Sign.P |
|----------------------------------------------------------|---------------------------------------------|-----------------------------------------------|-------------|-------------|------------|
| map00909                                                 | Metabolism of terpenoids and polyketides    | Sesquiterpenoid and triterpenoid biosynthesis | 0.0523      | 7.54311E-10 | ****       |
| map00525                                                 | Biosynthesis of other secondary metabolites | Acarbose and validamycin biosynthesis         | 0.1613      | 1.71603E-08 | ****       |
| map00523                                                 | Metabolism of terpenoids and polyketides    | Polyketide sugar unit biosynthesis            | 0.1389      | 3.76866E-08 | ****       |
| map00521                                                 | Biosynthesis of other secondary metabolites | Streptomycin biosynthesis                     | 0.1111      | 1.19904E-07 | ****       |
| map03050                                                 | Folding, sorting and degradation            | Proteasome                                    | 0.0209      | 7.38953E-06 | ****       |
| map00515                                                 | Glycan biosynthesis and metabolism          | Mannose type O-glycan biosynthesis            | 0.0833      | 0.00011423  | ***        |
| map04392                                                 | Signal transduction                         | Hippo signaling pathway - multiple species    | 0.2857      | 0.000139345 | ***        |
| map04111                                                 | Cell growth and death                       | Cell cycle - yeast                            | 0.25        | 0.000185479 | ***        |
| map04113                                                 | Cell growth and death                       | Meiosis - yeast                               | 0.1538      | 0.00051233  | ***        |
| map00910                                                 | Energy metabolism                           | Nitrogen metabolism                           | 0.0265      | 0.000666004 | ***        |
| map00220                                                 | Amino acid metabolism                       | Arginine biosynthesis                         | 0.0229      | 0.001152312 | **         |
| map00592                                                 | Lipid metabolism                            | alpha-Linolenic acid metabolism               | 0.0159      | 0.00424437  | **         |
| ‘**’ indicates $p < 0.005$ , ‘***’ indicates $p < 0.001$ |                                             |                                               |             |             |            |

## Discussion

Exosomes are nanoscale vesicles secreted by cells, encapsulating bioactive molecules such as proteins, lipids, and nucleic acids (including miRNAs). Their lipid bilayer membrane structure protects the internal miRNAs from degradation in the extracellular environment and enables uptake by recipient cells through endocytosis, thereby facilitating intercellular communication. In plants, the functional study of EVs, similar to exosomes, is emerging as a hot research field. Robust isolation of high-purity EVs from plant tissues remains a significant methodological challenge, hindering the exploration of their diverse roles in intercellular communication, development, and stress responses [26, 32, 34]. This study addresses this gap by presenting a systematic optimization and comparative evaluation of EVs isolation protocols specifically tailored for the fibrous roots of *O. japonicus*, a homology plant of food and medicine. Our integrated approach, refining both tissue pre-processing and downstream purification strategies, demonstrably enhances EV yield while critically minimizing co-isolated contaminants, as rigorously validated through multi-modal characterization (NTA, TEM, small RNA-Seq) and adherence to MISEV guidelines [33, 35, 36].

The initial hurdle in plant EV research is obtaining uncontaminated AWF [26]. While sap-rich tissues often allow direct juice expression, low-sap tissues like *O. japonicus* roots necessitate alternative approaches. We critically compared juice extraction/cell disruption and enzymatic maceration (cellulase-pectinase). Juice extraction offered procedural simplicity and high throughput, yielding larger quantities of crude EVs (Fig. 1A). However, this method inevitably co-isolated substantial amounts of plant-derived contaminants, primarily cellulose and pectin fragments, as evidenced by TEM and impurity analysis (Fig. 1C&2C). In contrast, enzymatic digestion specifically targeted the major cell wall components, facilitating the efficient release of EVs entrapped within the wall matrix while significantly reducing contamination from intracellular macromolecules and wall debris [28]. This resulted in EVs of markedly higher purity (Fig. 3A), albeit at the expense of a reduced final yield (Fig. 3B). This clear trade-off between yield and purity underscores the importance of selecting the pre-processing method based on the specific downstream application requirements (e.g., bulk characterization vs. detailed molecular profiling).

DC is widely employed for plant EV isolation [15, 17, 37–39], but standard protocols require optimization for specific tissues. Our optimized DC protocol for *O. japonicu* roots involved sequential steps ( $1,000 \times g/10$  min,  $5,000 \times g/20$  min,  $10,000 \times g/40$  min,  $150,000 \times g/2$  h). Crucially, we implemented a post-ultracentrifugation wash step: resuspending the initial EV pellet in PBS followed by a second ultracentrifugation. This simple yet effective modification significantly reduced soluble contaminants and aggregated material co-pelleted during the first spin, leading to a substantial improvement in EV purity (Fig. 1D&E), despite an expected reduction in absolute particle number. This refinement is particularly valuable for applications demanding high sample purity.

To achieve the highest purity essential for confident molecular characterization and functional studies, we employed and optimized sucrose DG-UC, building upon existing methods [37, 40]. Our key innovation

was the introduction of an additional high-speed centrifugation ( $10,000 \times g$ ) step prior to loading the crude EV sample onto the sucrose gradient (60%/45%/30%/8%). This pre-clearing step maximized the removal of residual plant debris and large aggregates from the AWF, resulting in a cleaner input for the density gradient (Fig. 2B). DG-UC leverages the characteristic buoyant density of plant EVs (1.10–1.18 g/mL, corresponding to the 30%–45% sucrose interface) to effectively separate them from co-sedimenting contaminants with overlapping sedimentation coefficients, such as protein complexes, lipoprotein aggregates, and residual small debris [31, 41, 42]. EVs isolated from the optimal density fraction (P4) exhibited superior purity and were therefore selected for in-depth small RNA sequencing analysis.

Small RNA sequencing of P4 EVs served a dual purpose: providing insights into the EV miRNA cargo and establishing a novel, molecular-based quality assessment metric. We identified 228 mature miRNAs (142 known + 86 novel), with the miR2592, miR168, and miR156 families being predominant (Table S3&S6). The remarkable abundance of miR2592, rarely a major player in model plants, highlights potential unique regulatory networks in medicinal species like *O. japonicus* and serves as a distinctive molecular signature of its root EVs. The prevalence of miR168, a known regulator of *AGO1* and thus global RNA silencing efficiency [43, 44], suggests a potential mechanism for selective miRNA sorting into EVs or a role in modulating recipient cell silencing machinery. Among 13 DEMs identified in response to treatment ( $|\log_2FC| > 1$ , FDR < 0.05; Fig. 4), two belonged to the miR156 family. This is mechanistically significant as miR156 targets *SPL* transcription factors to repress developmental transitions [45–48]. Its altered abundance in EVs provides a compelling molecular link to the observed paclobutrazol-induced root thickening phenotype in *O. japonicus*, further corroborated by GO term enrichment for "root development" (GO:0048364). Strikingly, target genes of these DEMs were significantly enriched in terms directly associated with EV biology ("exosome" GO:0000178, "extracellular region" GO:0005576; Fig. 5). This strongly suggests that these DEMs, shuttled within EVs, may orchestrate the intercellular transport and regulation of key enzymes, such as monoterpene synthases, involved in specialized metabolism. Furthermore, KEGG pathway analysis revealed that DEMs target genes were significantly enriched in biosynthesis pathways for pharmacologically active compounds, particularly sesquiterpenoids/triterpenoids (map00909) (Fig. 5D). This positions EVs and their miRNA cargo as key regulators of the valuable secondary metabolites characteristic of *O. japonicus*.

In summary, this study delivers an optimized, integrated pipeline for isolating high-purity EVs from the challenging matrix of *O. japonicus* roots, establishing a viable source for plant-derived EVs with potential biotechnological and therapeutic applications. However, the field of plant EV research continues to face substantial methodological hurdles: (1) The inherent complexity of plant extracts, rich in polysaccharides, phenolics, and other secondary metabolites, presents persistent challenges for achieving absolute purity and significantly increases processing complexity and cost. (2) A critical limitation is the current lack of conserved, plant-specific EV protein markers analogous to CD63/TSG101 in animal systems. Identification still heavily relies on physical characteristics (NTA size distribution, TEM morphology) and cargo analysis, necessitating complementary approaches like the miRNA profiling demonstrated here. Our findings that *O. japonicus* root EVs carry functionally relevant miRNAs, including

development-regulating miR156 and those targeting medicinal compound pathways, provide compelling evidence that paclobutrazol may modulate root architecture, in part, by altering the EV-mediated intercellular trafficking of regulatory RNAs. This underscores EVs as active signaling entities in plants. Future research must focus on elucidating the precise loading mechanisms of specific miRNAs into EVs, their uptake and functional impact in recipient cells, and their definitive roles in mediating developmental and metabolic reprogramming within plant tissues.

## Conclusions

We conducted a comparative analysis of three methods for isolating EVs from *O. japonicus* roots. Our results demonstrate that the isolated EVs exhibit sufficient yield, purity, and structural integrity for downstream analyses. Utilizing this optimized approach, we performed small RNA sequencing on *O. japonicus* roots-derived EVs. This detailed characterization of EV miRNA profiles will advance our understanding of vesicle-mediated signaling and the mechanistic actions of agrochemicals.

## Abbreviations

AWF: Apoplastic washing fluid; BP: Biological process; CC: Cell components; CK: Control group; DC: Differential centrifugation; DEMs: Differentially expressed miRNAs; DG-UC: Density gradient ultracentrifugation; EV: Extracellular vesicle; miRNA: microRNA; MF: molecular functions ; nt: Nucleotide; NTA: Nanoparticle tracking analysis; ORDEN: *Ophiopogon japonicus* Root-Derived Extracellular Nanovesicle; PBS: Phosphate-buffered saline; PDEN: Plant-derived exosome-like nanovesicle; PEV: Plant extracellular vesicle; sRNA: Small RNA; TEM: Transmission electron microscope; WP: Wettable powder.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

Raw Illumina sequence data were deposited in the Short Read Archive of the NCBI database (Biosample accession numbers are SAMN50730936, SAMN50730937, SAMN50730938, SAMN50730939). MiRNA data are available via NCBI with accession (PRJNA1309075).

### Competing interests

The authors declare no conflict of interest.

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## Authors' contributions

Y.X. carried out the design of this research work and writing this manuscript; Y.X., X.Z and S.Y.C. performed the experiments; Z.H.L., Q.Z. and L.Q.F. curated the data; F.Q.L. and Y.X. performed the formal analysis and validated the results; B.J.X. administered the project; Y.T.M., T.Z and B.J.X. supervised the study; Y.X. B.J.X. wrote the main manuscript text; T.Z. B.J.X. reviewed and edited the manuscript. All authors read and approved the final manuscript.

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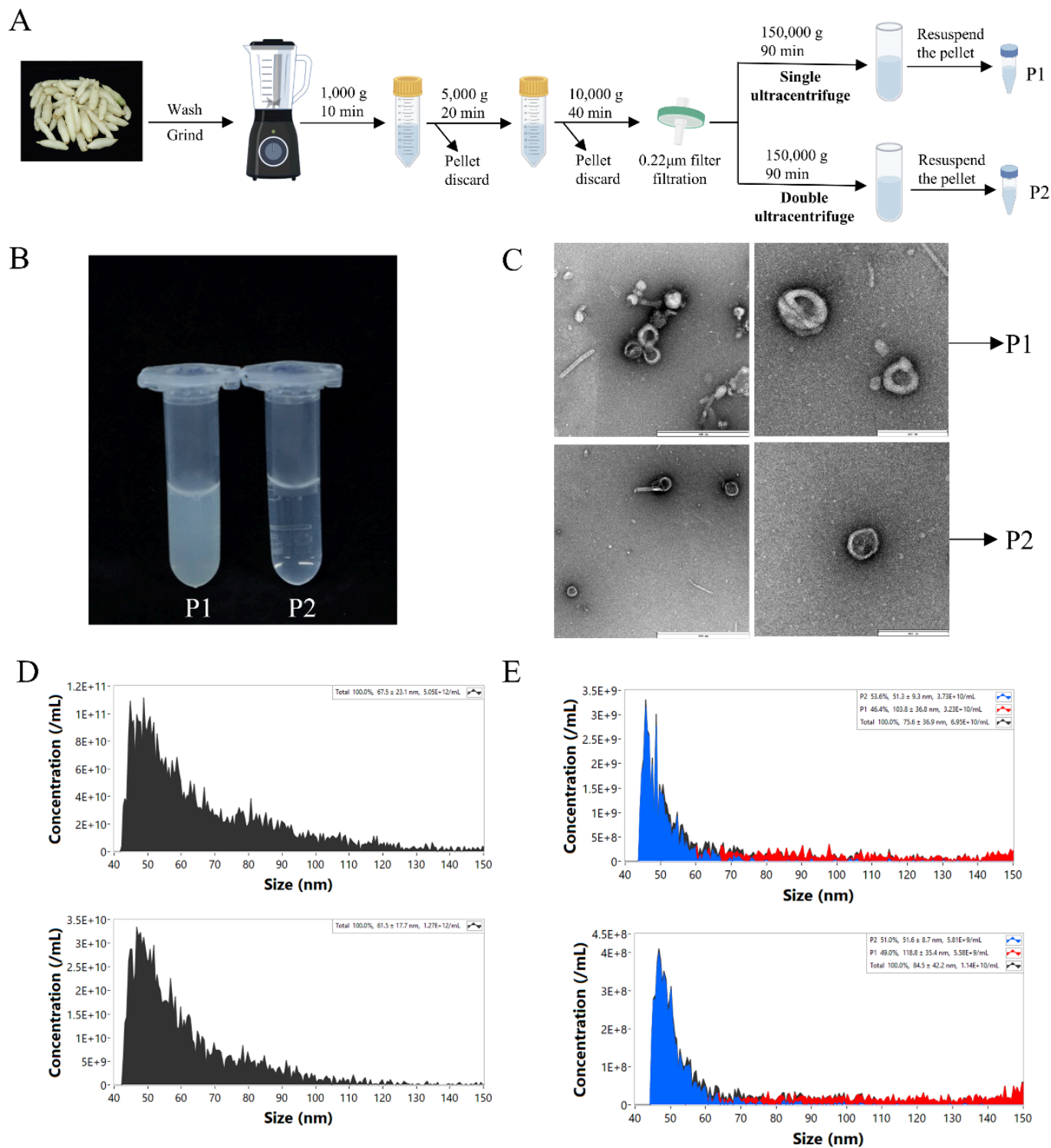
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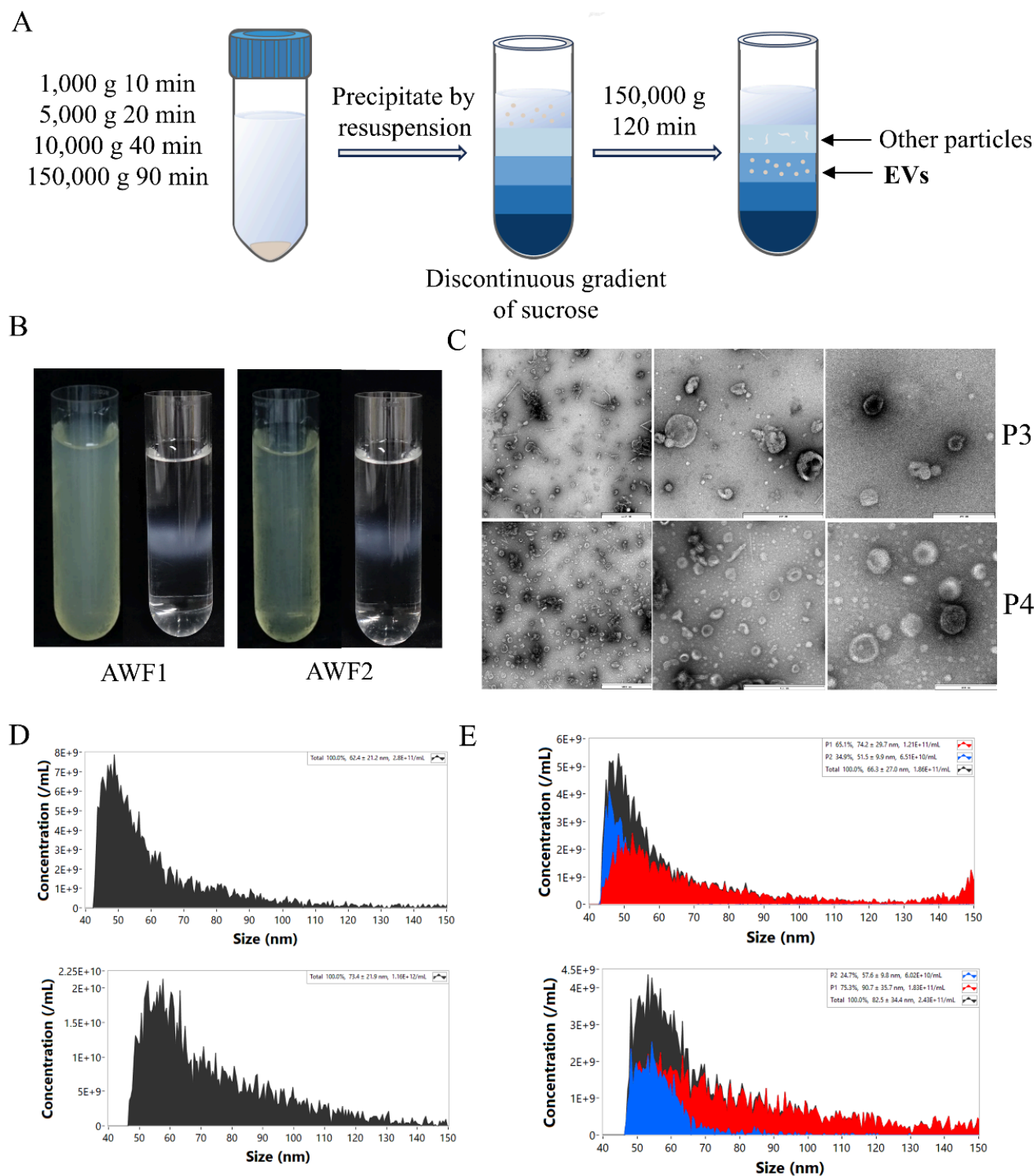
Figures



## Figure 1

### Double ultrahigh-speed centrifugations increase EVs purity but decrease yield

(A) Images show the various steps involved in the extraction of extracellular vesicles using ultracentrifugation. (B) Comparison of the color of extracellular vesicles extracted by two different ultracentrifugation methods. Both methods used the same weight of roots (50g). (C) Representative TEM images of extracellular vesicles separated by two different ultracentrifugation methods. Scale bars, 600 and 200 nm. (D) Particle size distribution graphs of extracellular vesicles extracted by two different ultracentrifugation methods. (E) Particle size distribution graphs of extracellular vesicles extracted by two different ultracentrifugation methods after being labeled with fluorescent dyes (Red: fluorescently labeled membrane-bound particles, i.e., extracellular vesicles; Blue: particles not labeled with fluorescent dyes).



**Figure 2**

### Purification of EVs by sucrose density gradient centrifugation

(A) The image shows the various steps involved in the extraction of extracellular vesicles using sucrose density gradient centrifugation. (B) Left: Comparison of the color of plant tissue extracts obtained by two different pretreatment methods. Right: After resuspension of plant tissue extracts obtained by two

different pretreatment methods and subsequent ultracentrifugation, sucrose density gradient purification results in bands converging at the same location (30 – 45%). Both methods used the same weight of roots (50 g). (C) TEM images of extracellular vesicles separated by two different ultracentrifugation methods. Scale bars, 1000, 600, and 200 nm. (D) Particle size distribution graphs of extracellular vesicles extracted by two different sucrose density gradient centrifugation methods. (E) Particle size distribution graphs of extracellular vesicles extracted by two different sucrose density gradient centrifugation methods after being labeled with fluorescent dyes (Red: fluorescently labeled membrane-bound particles, i.e., extracellular vesicles; Blue: particles not labeled with fluorescent dyes).

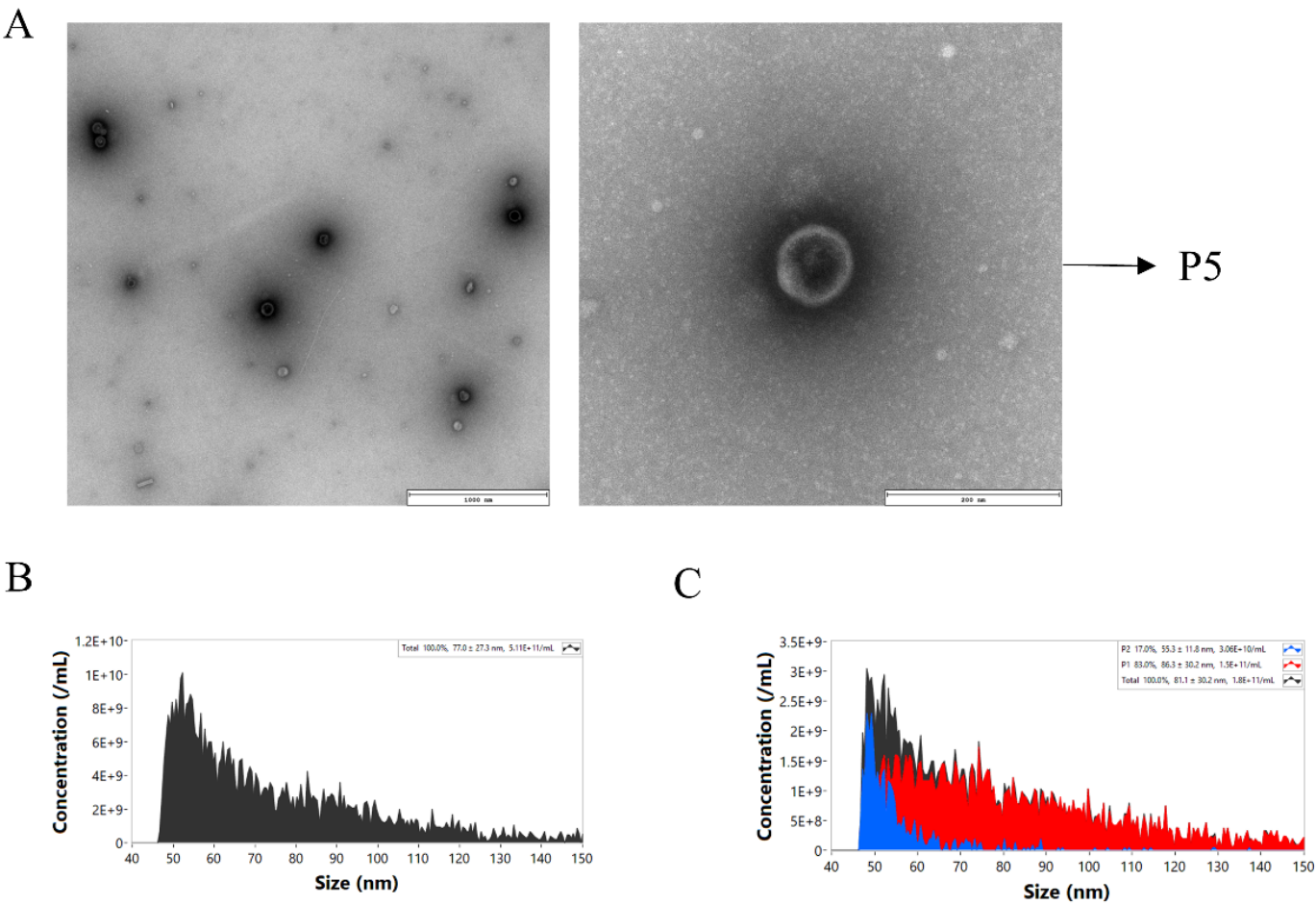
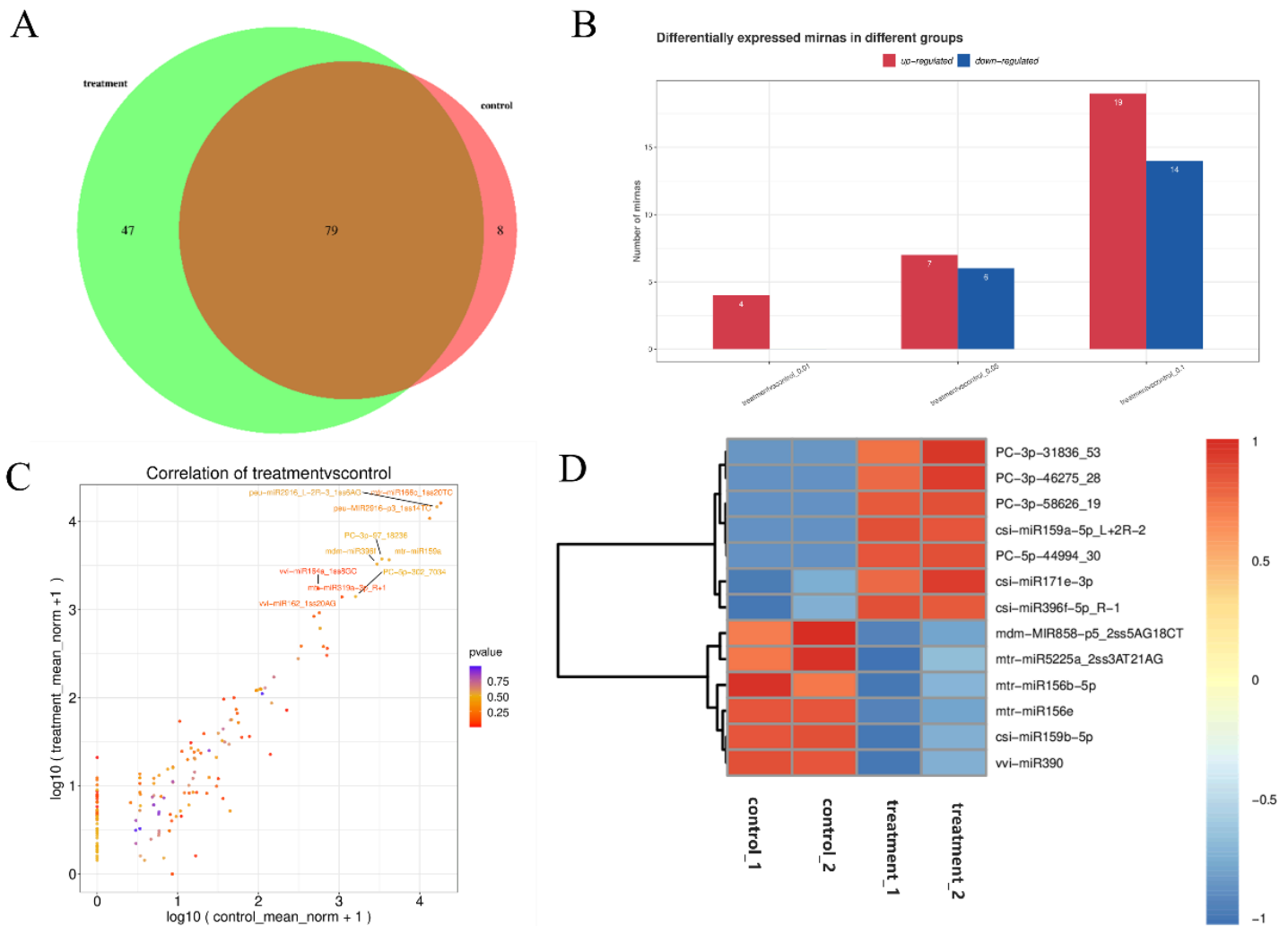


Figure 3

### Extraction of EVs by enzymatic method

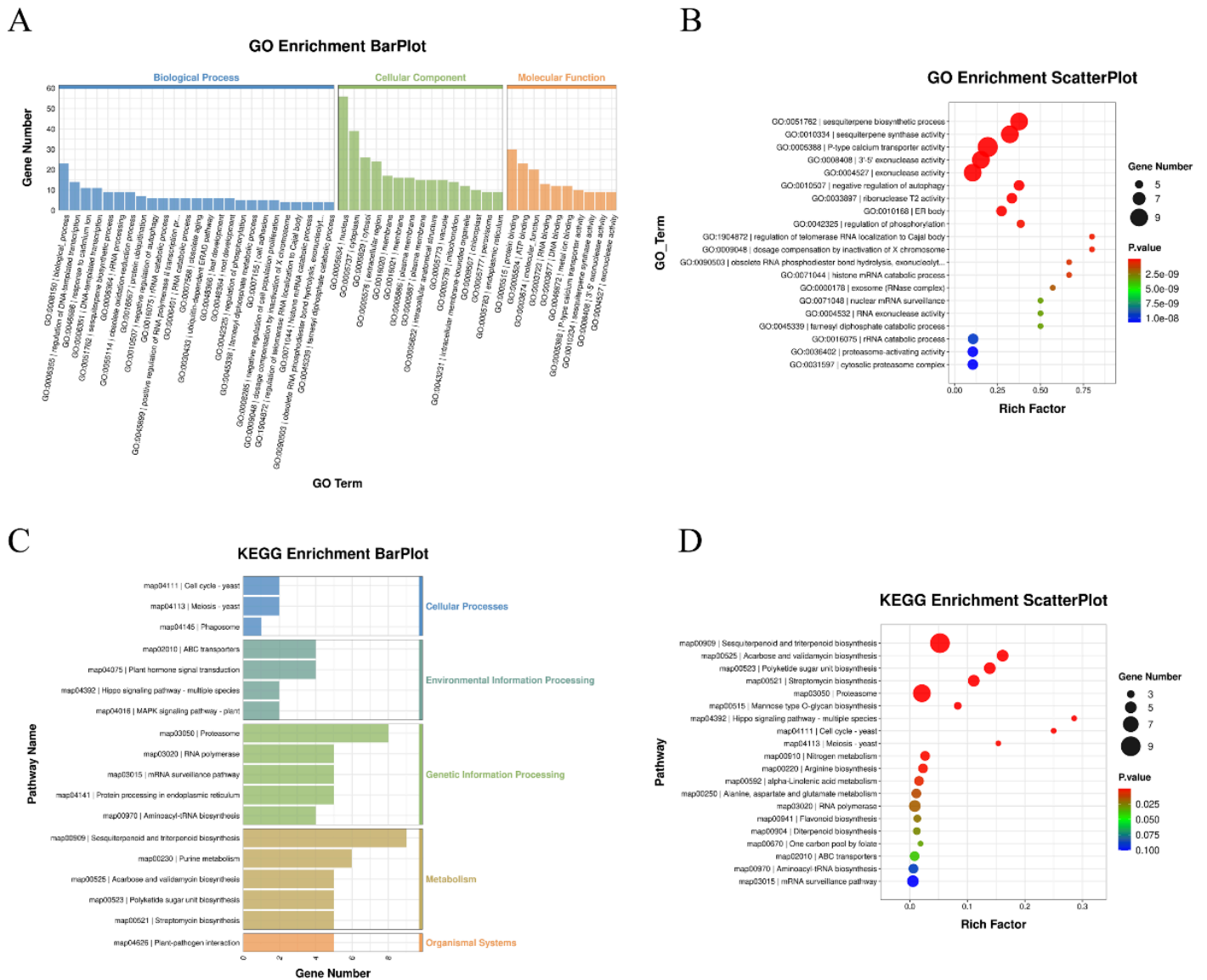
(A) Representative transmission electron microscopy (TEM) images of extracellular vesicles isolated by enzymatic method. Scale bars, 1000 and 200 nm. (B) Particle size distribution graph of extracellular vesicles extracted by enzymatic method. (C) Particle size distribution graph of extracellular vesicles extracted by enzymatic method after being labeled with fluorescent dyes (Red: fluorescently labeled membrane-bound particles, i.e., extracellular vesicles; Blue: particles not labeled with fluorescent dyes).



**Figure 4**

**Analysis of known miRNA expression and differential expression between control and treatment groups.**

(A) Venn diagram of miRNAs identified in control and treatment groups. (B) DEMs between groups. (C) Correlation of miRNA expression between treatment and control groups. (D) Significance and expression of key DEMs.



**Figure 5**

**GO and KEGG enrichment analysis of DE miRNA target genes.**

(A) GO enrichment bar plot. (B) GO enrichment scatter plot. (C) KEGG enrichment bar plot. (D) KEGG enrichment scatter plot.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFiles.zip](#)