

Optimisation of Preprocessing Protocol to Enhance Yield and Purity of Exosomes in Buffalo Milk: An In-vitro Study

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ABSTRACT

Introduction: Milk-Derived Exosomes (MDEs) are nano-sized Extracellular Vesicles (EV) with significant potential as natural drug delivery systems due to their biocompatibility, stability, and ability to cross biological barriers. However, efficient isolation of high-purity exosomes from buffalo milk is challenging because of its high fat and casein content. Optimising preprocessing strategies for casein removal is therefore critical.

Aim: To compare acetic acid precipitation and Ethylenediaminetetraacetic acid (EDTA) precipitation as pre treatment strategies for casein removal and to evaluate their effectiveness in isolating high-purity buffalo MDEs.

Materials and Methods: This in-vitro study was conducted at the Department of Biochemistry, Dr. Prabhakar Kore Basic Science Research Centre (Dr PK BSRC), KAHER, Belagavi, Karnataka, India, from December 2024 to March 2025. Here, fresh buffalo milk was defatted using differential centrifugation, followed by casein removal through either acetic acid precipitation or EDTA precipitation. Exosomes were subsequently isolated from the whey fraction using the ExoEnrich™ (Serum/Plasma) exosome isolation kit. Particle size, size distribution, and zeta potential were analysed using Dynamic Light Scattering (DLS). Morphological characterisation was performed by Transmission

Electron Microscopy (TEM), and the exosomal surface marker CD81 was confirmed using flow cytometry.

Results: Both isolation methods yielded nanosized vesicles within the expected exosomal range. Acetic acid-treated samples showed a mean particle size of 146 nm and a zeta potential of -20.55 mV, whereas EDTA-treated samples exhibited a slightly larger size of 154 nm with a zeta potential of -17.07 mV. TEM analysis revealed characteristic spherical, cup-shaped vesicles in both groups. Flow cytometry confirmed CD81-positive exosomal populations. Overall, acetic acid precipitation resulted in higher exosome purity compared to EDTA precipitation.

Conclusion: The present study demonstrates that both acetic acid- and EDTA-mediated precipitation enable successful isolation of buffalo milk-derived exosomes. Acetic acid precipitation showed comparatively improved purity and favourable physicochemical properties in the current experimental setting. However, rather than establishing definitive methodological superiority, these findings emphasise the role of casein removal chemistry in influencing vesicle characteristics and enrichment efficiency. Further studies are required to confirm these observations across broader conditions.

Keywords: Acetic acid, Casein, Dynamic light scattering, Flow cytometry, Milk-derived exosome, Transmission electron microscope

INTRODUCTION

Exosomes are nano-sized EVs (30-180 nm) involved in intercellular communication through the transfer of biologically active molecules such as proteins, lipids, Deoxyribonucleic Acid (DNA), messenger Ribonucleic Acid (mRNA), and microRNAs [1]. Their biocompatibility, stability, and ability to cross biological barriers, including the blood-brain barrier and placental barrier, have attracted considerable attention for applications in drug delivery, diagnostics, and immunotherapy [2,3]. Engineered exosomes have demonstrated efficient targeted delivery; for example, dendritic cell-derived exosomes expressing Lysosome-associated membrane protein 2 (Lamp2b) fused with Rabies Virus Glycoprotein (RVG) peptide enabled neuron-specific Small Interfering RNA (siRNA) transport across the blood-brain barrier, resulting in effective gene silencing in mouse brain tissue [4]. Similarly, aptamer-functionalised, doxorubicin-loaded exosomes have shown enhanced cytotoxicity against colorectal cancer cells [5].

Milk has emerged as a natural and scalable source of exosomes suitable for therapeutic applications. MDEs exhibit high stability under gastrointestinal conditions, making them promising candidates for oral drug delivery systems [6]. Aqil F et al., reported improved loading efficiency, stability, bioavailability, and tissue distribution of drugs such as curcumin when delivered using MDEs [7]. Moreover,

bovine milk provides a safe and cost-effective source for large-scale production; however, traditional ultracentrifugation-based isolation methods remain time-consuming and challenging to standardise for large-scale applications [8].

Isolation efficiency and purity of MDEs are strongly influenced by sample preprocessing and removal of interfering components such as fat globules and casein micelles. Standardisation of isolation protocols in accordance with the minimal experimental requirements proposed by the International Society for EVs remains essential [9]. Previous studies indicate that pre-analytical factors, including sample handling and processing conditions, significantly affect exosome recovery and purity [8,10]. Removal of cream prior to storage improves yield, and differential ultracentrifugation combined with filtration has shown advantages over precipitation-based methods in bovine milk [10]. In addition, acetic acid-mediated casein removal combined with size-exclusion chromatography has been reported to produce highly pure cow milk-derived EVs, emphasising the importance of efficient casein removal [11,12].

Milk composition varies considerably between species and can influence exosome isolation outcomes. Buffalo milk contains higher levels of fat, protein, and total solids compared with cow milk, which may further complicate efficient exosome purification [13]. Despite increasing interest, standardised and scalable protocols for isolating buffalo MDEs remain limited. Additionally, Acetic acid precipitation and

EDTA precipitation methods are previously used for exosome isolation from cow milk [11,12]. EDTA-based precipitation has been reported to facilitate exosome isolation by chelating divalent cations, thereby disrupting casein micelle structure and reducing protein contamination. In contrast, acetic acid precipitation is a widely used, cost-effective approach that induces casein aggregation through pH adjustment, but may differentially affect exosome integrity and yield [14].

The study hypothesised that efficient removal of casein and associated milk components during preprocessing significantly improves the purity and recovery of buffalo MDEs. Therefore, the present study aimed to systematically compare acetic acid precipitation and EDTA precipitation as preprocessing strategies to optimise the isolation of high-purity exosomes from buffalo milk.

MATERIALS AND METHODS

This in-vitro study was conducted in Dr. P K Basic Science Research Centre, KLE Academy of Higher Education and Research, Belagavi, Karnataka, India from December 2024 to March 2025. Ethical approval was not required for this study as it does not involve any in-vivo study or animal handling.

Study Procedure

Sample collection: Fresh raw buffalo milk (250 mL) was procured from a licensed local dairy farm in Belagavi, India, and collected as a commercially available food product and processed immediately after collection without any storage; therefore, no animal handling or experimental procedures were involved, and ethical approval was not required. For each experiment, 50 mL of milk was used for processing. Milk samples were obtained from three independent batches collected on different days, and all experiments were performed in biological triplicates unless otherwise specified.

Buffalo milk samples were obtained from three independent milk batches. Each batch was processed independently to account for biological variability. For each isolation method (acetic acid precipitation and EDTA precipitation), experiments were performed in triplicate technical replicates per batch, resulting in a total of $n = 9$ independent measurements per group. No samples were excluded from analysis.

Protocol conditions, including centrifugation speed, temperature, filtration steps, and isolation procedures, were kept identical between groups to enable direct comparison of preprocessing strategies. The two groups were compared based on Size, Polydispersity Index (PDI), Zeta Potential, TEM, and Flow cytometry to allow assessment of vesicle enrichment and morphology.

Defatting of Milk: Defatting of milk was carried out as previously described [15]. Briefly, raw milk was centrifuged twice at $1,200 \times g$ for 10 minutes at 4°C to remove fat globules, somatic cells, and large debris, and the resulting defatted milk was collected for further processing.

Removal of Casein from Defatted Milk:

- **Acetic acid precipitation:** Defatted milk was treated with 17.5 N acetic acid at a 1:100 (v/v) ratio and centrifuged at $4,500 \times g$ for 30 minutes at 4°C to precipitate casein and residual lipid components. The obtained whey fraction was sequentially filtered through $0.45 \mu\text{m}$ and $0.22 \mu\text{m}$ membrane filters to remove remaining debris and large particles [11].
- **EDTA precipitation:** Defatted milk was treated with 250 mM EDTA-3Na and incubated for 15 minutes at room temperature to disrupt casein micelles through calcium chelation. Samples were subsequently centrifuged at $4,500 \times g$ for 30 minutes at 4°C . The resulting whey was sequentially filtered using $0.45 \mu\text{m}$ and $0.22 \mu\text{m}$ membrane filters [11].

Isolation of Milk-Derived Extracellular Vesicles (MEVs): The MEVs were isolated from clarified whey using the ExoEnrich™ (Serum/

Plasma) exosome isolation kit based on colloidal destabilisation-mediated precipitation. Whey samples were processed according to the manufacturer's protocol, in which addition of the proprietary reagent induces destabilisation of colloidal particles, enabling selective precipitation of EVs. The resulting pellet was resuspended in sterile Phosphate-Buffered Saline (PBS) for downstream characterisation.

- **Determination of particle size and zeta potential:** The particle size, PDI, and zeta potential of the exosomes were determined using a Zetasizer Nano ZS (Malvern Instruments, UK). Exosome suspensions were suitably diluted in PBS, and all measurements were performed at 25°C .
- **Transmission Electron Microscope (TEM):** The morphology of the isolated exosomes was examined using a transmission electron microscope (TEM, HT7700, Hitachi, Japan). Exosomal samples were negatively stained for two minutes on Formvar-carbon copper grids, followed by air drying before TEM imaging.
- **Surface marker analysis by flow cytometry:** The exosome marker CD81 was identified using flow cytometry (BC Dx Flex flow cytometer). For the detection of CD81, a well-established exosomal surface marker, the exosome suspension was incubated overnight at 4°C with Fluorescein Isothiocyanate (FITC)-conjugated anti-CD81 antibody (BD551108), allowing specific binding with minimal non-specific interactions. Prior to sample acquisition, the flow cytometer was calibrated using $100\text{--}200 \text{ nm}$ polystyrene beads to ensure optimal sensitivity and reliable detection of nanosized vesicles. The stained exosome suspension was then transferred into flow cytometry-compatible tubes and analysed using a 488 nm excitation laser with emission detection at $\sim 530 \text{ nm}$ for FITC fluorescence. Unstained exosome samples, and single-colour compensation controls were included to validate fluorescence specificity and minimise background noise. Data were analysed based on forward/side scatter properties and FITC fluorescence intensity to accurately identify CD81-positive exosomal populations and distinguish them from non specific events or debris.

STATISTICAL ANALYSIS

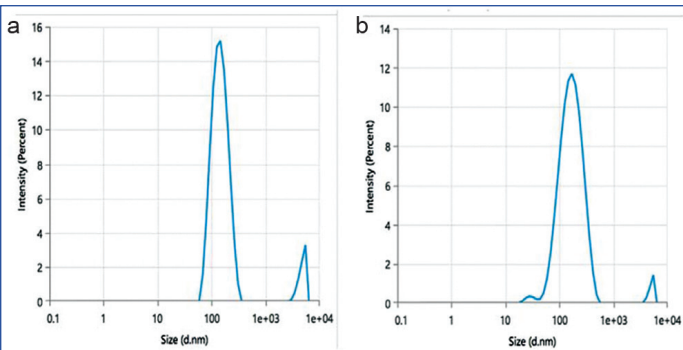
Statistical analysis was performed using GraphPad Prism 8.0.2. The data obtained from the two experimental groups were analysed using an unpaired (Independent samples) t-test to compare differences between the acetic acid precipitation and EDTA precipitation methods. A p-value <0.05 was considered statistically significant.

RESULTS

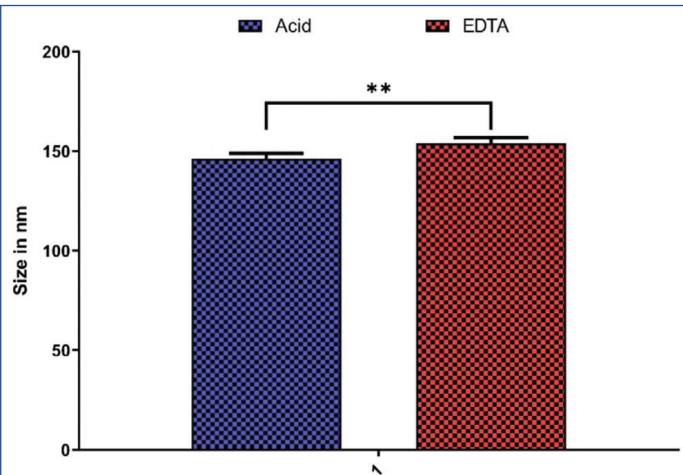
Two different preprocessing methods, acetic acid precipitation and EDTA precipitation, were employed for casein removal prior to MEV isolation. MEVs were subsequently isolated from the respective whey fractions. Exosome yield and enrichment were evaluated using protein concentration as one of the parameters along with physicochemical characterisation parameters rather than considering protein content alone as a direct indicator of EV purity. The measured protein concentrations of acetic acid precipitation and EDTA precipitation were $21.53 \pm 2.154 \text{ mg/mL}$ and $19.14 \pm 1.49 \text{ mg/mL}$, respectively and statistically non significant with p-value 0.378.

Exosome Size and Zeta Potential Analysis

The DLS analysis was performed to determine particle size, PDI and zeta potential of exosomes obtained following acid and EDTA precipitation. The average hydrodynamic diameter of exosomes isolated using acetic acid precipitation and EDTA precipitation was $146 \pm 4.5 \text{ nm}$ and $154 \pm 5.2 \text{ nm}$, respectively [Table/Fig-1a,b], which



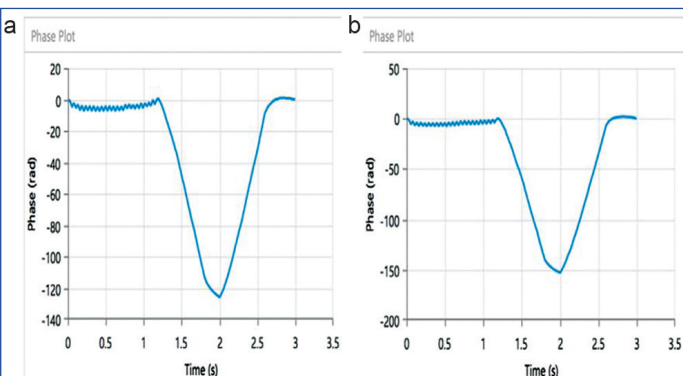
[Table/Fig-1]: Dynamic Light Scattering (DLS) analysis of exosomes isolated by two different precipitation methods: a) Size distribution profile of exosomes isolated by acid precipitation showing an average particle size of 146 nm; b) Size distribution profile of exosomes isolated by EDTA precipitation with an average particle size of 154 nm.



[Table/Fig-2]: Comparison of exosome particle size obtained using acetic acid and EDTA precipitation methods. Bar graph represents the mean vesicle diameter (nm) measured for acid-processed and EDTA-processed samples, expressed as mean \pm SD (146 \pm 4.5 nm and 154 \pm 5.2 nm) respectively. EDTA-isolated vesicles showed a larger average size compared to acid-isolated vesicles. ** indicates a statistically significant difference between the two groups.

falls within the expected nanoscale range (30-180 nm) reported for EVs [1]. The t-test showed statistically significant difference ($p < 0.01$) among the two groups as presented in [Table/Fig-2]. The PDI values were 0.2783 \pm 0.033 for acetic acid precipitation and 0.3247 \pm 0.022 for EDTA precipitation, indicating a moderate particle size distribution in both preparations, with the difference being statistically non significant ($p = 0.1207$).

Zeta potential measurements showed mean surface charges of -20.55 ± 1.8 mV for acetic acid-treated samples and -17.07 ± 1.3 mV for EDTA-treated samples [Table/Fig-3a,b]. Both preparations exhibited negative surface charge values consistent with colloidal stability of EV suspensions. No marked aggregation was observed under the tested conditions.



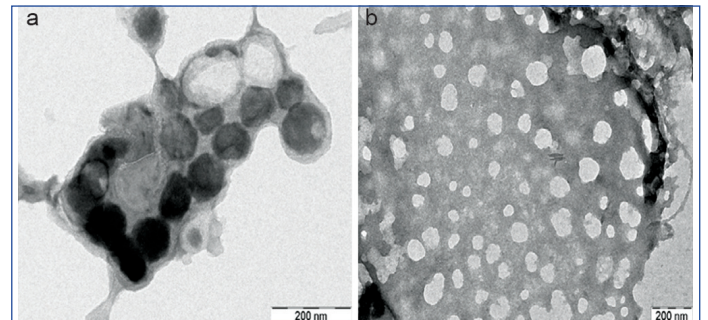
[Table/Fig-3]: Zeta potential analysis of exosomes isolated by different precipitation methods: a) Zeta potential distribution of exosomes isolated by acid precipitation showing a surface charge of -20.55 mV; b) Zeta potential distribution of exosomes isolated by EDTA precipitation with a surface charge of -17.07 mV.

Exosome Morphology by TEM

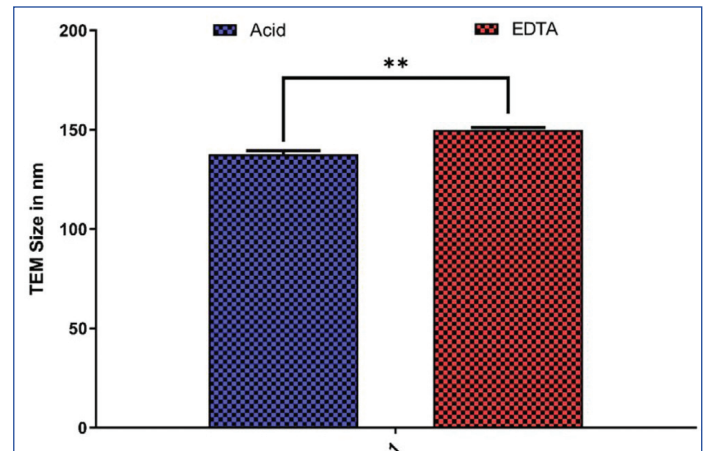
The TEM revealed vesicles with typical spherical to cup-shaped morphology, characteristic of exosomes. The observed particle diameters were approximately 137 \pm 4.2 nm for acetic acid-processed samples and 150 \pm 3.7 nm for EDTA-processed samples [Table/Fig-4a,b], supporting the size measurements obtained by DLS analysis. A statistically significant difference in TEM-determined particle diameter between the two groups was observed, as shown in [Table/Fig-5], based on statistical analysis ($p < 0.01$).

Surface Marker Characterisation

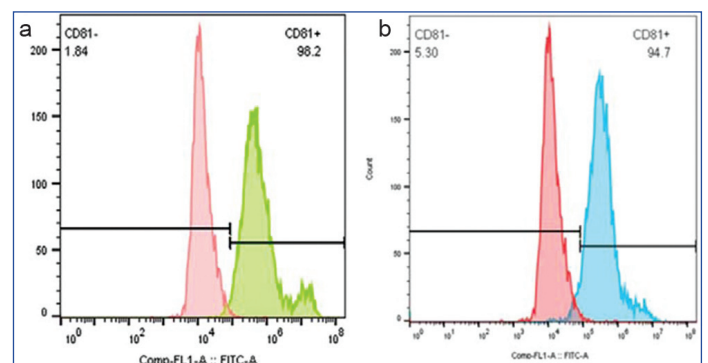
Flow cytometry analysis confirmed the presence of the exosomal surface marker CD81. A clear fluorescence shifts relative to the control indicated positive CD81 expression, confirming successful enrichment of EVs in both preparations [Table/Fig-6a,b]. A statistically significant difference between the two groups, as determined by flow cytometry, is presented in [Table/Fig-7], ($p = 0.0102$).



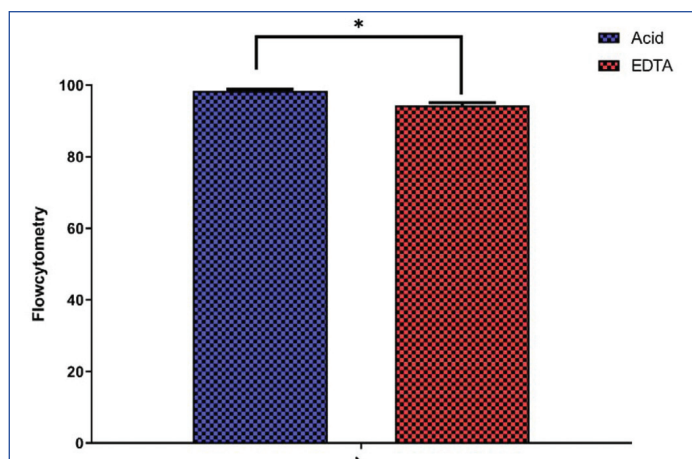
[Table/Fig-4]: TEM images of exosomes isolated by different precipitation methods: a) Acid-precipitated exosomes; and b) EDTA-precipitated exosomes showing a typical spherical, cup-shaped morphology characteristic of exosomal vesicles.



[Table/Fig-5]: TEM-based size comparison of exosomes isolated using acetic acid (137 \pm 4.2 nm) and EDTA precipitation (150 \pm 3.7 nm) methods. The bar graph represents the mean vesicle diameter (nm) measured from TEM analysis, expressed as mean \pm SD. Exosomes obtained through EDTA precipitation exhibited a slightly larger average size compared to those isolated using acid precipitation. ** indicates a statistically significant difference between the two groups.



[Table/Fig-6]: Flow cytometric characterisation of exosomes showing CD81 surface marker expression. Representative flow cytometry histograms/dot plots demonstrate positive CD81 expression on isolated exosomes, evidenced by a clear shift in fluorescence intensity compared to the unstained/control sample.



[Table/Fig-7]: Flow cytometry-based comparison of CD81 expression in exosomes isolated using acetic acid (98.2%) and EDTA precipitation (94.7%) methods. The bar graph represents the relative fluorescence intensity indicating CD81-positive Extracellular Vesicles (EV), expressed as mean±SD. Both isolation methods demonstrated successful enrichment of exosomes, with a statistically significant difference observed between the two groups ($p < 0.05$), indicated by*.

DISCUSSION

Efficient removal of casein and associated milk components remains a critical step for obtaining enriched Buffalo MEV preparations due to the complex biochemical composition of milk. In the present study, acetic acid- and EDTA-mediated precipitation was comparatively evaluated as preprocessing strategies prior to MEV isolation. Both approaches successfully enabled downstream vesicle recovery; however, differences in physicochemical characteristics suggest that pre treatment chemistry influences vesicle enrichment and suspension stability thus accepting the hypothesis. Previous study have highlighted that effective casein removal is critical for improving EVs recovery from cow milk matrices, with acid-mediated precipitation reported to enhance the removal of contaminating milk proteins compared to centrifugation-based approaches alone [12].

Particle size analysis confirmed that vesicles isolated using both preprocessing methods fell within the characteristic nanoscale range reported for exosomes. The observed mean particle diameters of 146 ± 4.5 nm for the acetic acid method and 154 ± 5.2 nm for the EDTA method are consistent with previously documented EV size distributions [16]. The PDI values indicate moderately heterogeneous vesicle populations in both preparations, with acetic acid precipitation producing a relatively more uniform size distribution compared to EDTA precipitation [17]. These findings indicate that both approaches were effective in preserving vesicle integrity during isolation. The small variation in particle size between the two groups may be attributed to differences in preprocessing conditions, but overall suggests comparable performance of the two strategies in obtaining vesicles within the expected exosomal size range and PDI.

The slightly larger particle size observed in EDTA-treated samples may be attributed to differences in isolation chemistry, which can influence vesicle aggregation and the apparent hydrodynamic diameter. Similar effects of precipitation-based isolation approaches on vesicle size distribution and aggregation behaviour have been reported previously [14,18]. Morphological analysis further revealed that EVs isolated using both acetic acid and EDTA precipitation predominantly exhibited spherical structures, consistent with the typical morphology of exosomes. However, vesicles obtained following EDTA precipitation occasionally displayed a rough surface appearance. This may be related to the calcium-chelating action of EDTA, which disrupts Ca^{2+} -dependent casein micelle structures and alters protein-protein interactions through changes in electrostatic bonding, thereby influencing vesicle surface characteristics [14]. Comparable observations have been reported by Agrawal AK et al., who demonstrated that precipitation-based isolation methods can affect vesicle size distribution and morphology due to co-precipitation or aggregation phenomena [16].

Western blotting is considered the gold-standard method for exosomal surface marker detection [10]; however, flow cytometry can also be used as a complementary technique for confirming the presence of exosomal markers. In this study, flow cytometric analysis demonstrated positive expression of the exosomal marker CD81 in both preparations; however, acetic acid precipitation showed higher expression (98.2%) compared to EDTA precipitation (94.7%), suggesting relatively better enrichment of EV populations with the acetic acid precipitation method. Detection of canonical markers remains an essential validation step in EV characterisation, consistent with previous reports by Burkova EE et al., supporting the reliability of the characterisation method used [19].

Overall, the present study demonstrates that both acetic acid- and EDTA-mediated precipitation enables successful isolation of buffalo milk-derived EVs. Rather than establishing methodological superiority, the findings highlight how differences in casein removal chemistry influence vesicle characteristics and enrichment efficiency, providing a foundation for future optimisation and large-scale standardisation of MDE isolation protocols.

Limitation(s)

However, certain limitations should be acknowledged. Although reproducibility was supported through processing independent milk batches under identical experimental conditions, inherent biological variability in milk composition, arising from differences in animal source, lactation stage, and storage conditions, may influence isolation outcomes and underscores the need for standardised pre-analytical handling. Furthermore, purity assessment was limited by the absence of advanced particle normalisation and comprehensive Nanoparticle Tracking Analysis (NTA)- based quantification. Vesicle characterisation was primarily based on physicochemical parameters and single-marker analysis; therefore, future studies incorporating multi-marker validation and more extensive analytical approaches will be essential to strengthen methodological robustness and translational applicability.

CONCLUSION(S)

The present study suggests that acetic acid-mediated casein precipitation as an effective pre-treatment strategy for the isolation of high-purity exosomes from buffalo milk. Compared to EDTA precipitation, acetic acid treatment yielded exosomes with improved purity, optimal nanoscale size distribution, stable negative surface charge, and well-preserved spherical morphology. The successful detection of the exosomal marker CD81 further confirmed the integrity and identity of the isolated vesicles. The findings emphasise the impact of preprocessing chemistry on vesicle characteristics in complex milk matrices; however, further quantitative and functional studies are required to establish statistical and biological significance.

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- iThenticate Software: Mar 23, 2026 (2%)

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