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Bovine Milk Derived Exosomal - Curcumin Exhibiting Enhanced Stability, Solubility, and Cellular Bioavailability

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Abstract

Curcumin is a promising anti-cancer drug with limited aqueous solubility and decreased bioavailability. Milk Extracellular Vesicles (MEVs) used as a drug-delivery vehicle, were precipitated with Poly-Ethylene-Glycol (PEG) MW3000 from milk-whey. The yield of MEVs was 200.0 ± 0.85 mg/liter of whey. MEVs were spherical, double-membrane structures with a size range of 92 ± 30 nm/Nanoparticle-Tracking-Analysis (NTA) and 87 ± 40 nm/Dynamic-Light Scattering (DLS). A 70% loading efficiency was achieved with a passive loading of curcumin. The mean size of mev-curcumin was 152 ± 49 nm/NTA and 113 ± 67 nm/DLS. A six-fold increase of cytotoxicity and a two fold increase in curcumin accumulation were observed by mev-curcumin over native curcumin in MDA-MB231, a breast cancer cell-line. Thus, we report the development of a curcumin formulation with improved solubility, stability, and cellular permeability for breast-cancer treatments.

Abbreviations

Cur: Curcumin; DLS: Dynamic Light Scattering; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; Mev: Milk extracellular vesicle or bovine EV; Mev-curcumin: Milk exosomal curcumin; NTA: Nanoparticle Tracking Analysis; TEM: Transmission Electron Microscope

Introduction

Breast Cancer (BC) is the most common cancer in women with increased morbidity and mortality globally. BC's standard treatment includes surgery, chemotherapy, hormonal therapy, and radiotherapy, and the choice of treatment is arrived based on its clinical and histopathological features [1,2]. However, treatment resistance quickly sets in as the disease progresses in almost 30% of BC patients. Novel therapeutic options, including curcumin, were investigated as a potential therapeutic for treating BC [2-5].

Curcumin is an active phenolic compound isolated from *Curcuma longa* [6]. Curcumin has diferuloylmethane, dimethoxy-curcumin, and bisdemethoxycurcumin in a ratio of 77:17:6 [7]. It exhibits anti-proliferative, antioxidant, anti-inflammatory, and anti-cancer properties [8-12]. Curcumin has been shown to inhibit migration, invasion, and apoptosis of various cancers, including BC [8]. Curcumin's clinical utility has been limited due to its low aqueous solubility and stability, leading to low bioavailability [13]. Improved curcumin stability is achieved with different approaches, including structure modification and reformulation with nanotechnology-based strategies [14,15]. However, no developed curcumin formulation exhibited the required drug stability, improved aqueous solubility, increased bioavailability, and proper tissue distribution. MDA-MB-231 is an aggressive triple-negative breast cancer cell line commonly used to develop and test novel therapeutic approaches in BC [1].

Extracellular Vesicles (EVs), including exosomes, microvesicles, and apoptotic bodies, are membrane vesicles found in all biological fluids, including milk [16]. EVs play a significant role in contributing to cellular homeostasis. Their endogenous cellular origin results in increased biocompatibility and limited immunogenicity, making them ideal candidates as a drug delivery vehicle. EVs have been used to deliver drugs, proteins, siRNAs, miRNAs, etc. EVs can be further bioengineered to enhance their penetrance and targetability [17]. However, the lack of optimized isolation protocols and lack of cheaper source material for EVs purification severely hampered the

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up scaling of EVs based drug delivery systems.

Milk is a natural source of nutrients and carries essential factors, including proteins, nucleic acids, vitamins, lipids, and other metabolites needed for growth and immune system development. Milk naturally contains secreted exosomes like any other body fluids [18]. It is known that milk-derived exosomes are intact after absorption from the gastrointestinal tract [19]. Milk is a highly scalable source for exosome isolation due to its economies of cost and production. Further, Mevs has a unique ability to withstand the acidic pH present in the stomach, which means Mevs effectively stand a chance to act as oral drug delivery vehicles to absorb drugs from the intestine [18]. Thus, bovine Mevs represent a viable alternative natural drug delivery vehicle because of their cost-effectiveness, biocompatibility, stability, tumor-targeting ability, and negligible toxicity. Mevs has been shown promising both for hydrophilic and lipophilic agents, including oncology drugs. Research studies advocated using Mevs for the delivery of oncology drugs, including paclitaxel, doxorubicin, and curcumin [20-22]. Further, the short and long-term stability status of exosome curcumin is unknown, and the detailed characterization of the formulation must be explored.

Herein, we endeavor to establish an ideal method for isolation, purification, characterization, and Mev-curcumin formulation development. Characterization of Mevs and its curcumin formulation was conducted to prove its short and long-term stability, efficacy, and bioavailability in the MDA-MB-231 cell line. This study demonstrates the potential utility of using exosomes from edible sources as a natural drug delivery vehicle for improvising oral drug delivery of curcumin in BC.

Materials and Method

Isolation of milk Exosomes - Fresh buffalo milk was differentially centrifuged at 5000 g for 10 min at 4°C to remove the upper-fat layer, lower cellular debris, including proteins. The middle fraction was collected in a beaker, and the pH was lowered to 4.5 by addition of 0.2N HCl (Fischer Scientific, MA, USA) for precipitating out the milk proteins. Clear milk whey was obtained by filtering it through cheesecloth. The filtered milk whey was then sequentially passed through a series of filters (1, 0.8, 0.4) µm, and finally through a 0.22 µm filter associated with a sterile borosilicate vacuum-assisted filtration unit. The final filtered whey was mixed with a 12% 3K PEG precipitation solution at a 1:1 ratio and incubated overnight at 4°C. After overnight incubation, the exosome pellet was obtained by centrifuging at 10000 g for 60 min at 4°C. All PEGs were obtained from M/s Sigma, MO, USA. Different molecular weights (MW 3K, 4K, 6K, 8K, 2K) PEG was initially used at various percentages (8%, 12%, and 16%) with 1M NaCl (Sisco Research Laboratories (SRL), Mumbai, India) to precipitate out Mevs.

Biochemical characterization

Protein estimation: Total protein concentration of Mevs was estimated using a micro-BCA Protein estimation kit (G Biosciences, MO, USA), according to the manufacturer's instructions.

Lipid estimation: Total lipid concentration of Mevs was determined by Phosphovanillin assay. Lipid standard, DHA (Docosahexaenoic Acid) (Sigma) was dissolved in chloroform (Rankem, India) and evaporated to dryness at 90°C in a dry bath. 250 µl of 96% H_2SO_4 (Rankem) was added to the sample in a 1:1.5 ratio and incubated for 20 min at 90°C. 220 µl of the above solution was added to 110 µl of vanillin reagent (200 µg vanillin in 17% H_3PO_4). Absorbance was read at 540 nm after a 10 min incubation.

Acetylcholine esterase activity (AChE): Acetylcholine esterase activity is used as a generic assay to estimate exosome formulation purity. AchE is concentrated in the exosomes membranes and is used as a measure of exosome purity. 20 µl of exosome fraction was added to 1.25 mM Acetyl thiocholine iodide and 0.1 mM of 5'-dithiol-bis (2-nitro benzoic acid (Sigma) adjusted to a final volume of 300 µl, and the change of absorbance at 412 nm was monitored for every 5 min in the kinetic assay.

Biophysical characterization

TEM: Transmission Electron Microscopy was performed to assess the size and shape of intact exosomes. Briefly, 10 µl of exosome solution was fixed with 1% glutaraldehyde for 5 min on 400 mesh copper grids (FCF400-Cu, Electron Microscopy Sciences, Hatfield, PA). 2% Uranyl acetate was used to stain the grid after washing it with water. After drying, the grids were examined under a transmission electron microscope (JEM-2100, JEOL Ltd. Tokyo, Japan), and images were captured for further analysis.

Nanoparticle tracking analysis (NTA): Size distribution analysis of isolated milk exosomes particles was determined by NTA, utilizes the light-scattering characteristics of macrovesicle preparations undergoing Brownian motion. Sample (diluted 1 µl in 999 µl 1X PBS) was injected under a continuous flow into the sample chamber of an LM10 size analyzer (Nanosight, Amesbury, UK). Light scattering was monitored at 488 nm laser light for their relative size and concentration measurement.

Dynamic light scattering (DLS): Light scattering properties of exosomes, i.e., size distribution, aggregate formation, and average diameter, were quantified through (Nicomp Z3000, Entegris, MA, USA). 50 µl of the Mevs sample was made to 3 ml, with PBS. At least three independent aliquots were quantified, and results were analyzed as a number weighted distribution curve for evaluating the size of exosomes.

Molecular characterization

QPCR analysis: Total RNA was extracted from isolated Mevs using TRIzol™ LS Reagent (Invitrogen, California, USA). Reverse transcription of RNA to cDNA was conducted with a high-capacity cDNA Reverse Transcription kit (Invitrogen), according to the manufacturer's instruction. cDNA was amplified using Sapphire Amp fast PCR master mix (Takara Bio Inc. Shiga Prefecture, Japan) in the RT PCR instrument (Applied Biosystem 7500 Real-Time System), and Cycle threshold (Ct) values were then plotted for each primer. Bovine gene primers used for amplification (Bio serve, India) and listed in the Table 1.

Exosomal curcumin formulation and its stability

Exosomal curcumin formulation was prepared in a ratio of 5:1 with the curcumin-exosome mixture and incubated at RT for 30 min. The unbound curcumin was removed by passing through the exosome spin column. The resulting supernatant was filter sterilized and stored at - 80°C for further preclinical studies. The short-term stability of Mev-curcumin was established by spectral analysis. In brief, free curcumin, exosomes, and Mev-curcumin were taken in 100 µl of PBS, and absorption was recorded in a plate reader (Synergy H1 Hybrid, Biotek, UK) at 430 nm [23]. OD value was obtained for every 30 min till 5 h and 24-, 48-, and 72- hours' time points. Respective graphs were plotted to estimate the stability of the Mev-curcumin

Table 1: Primer sequences.

GENE	FORWARD	REVERSE
Bovine Casein	CTCAAGAAGTCCTCAATGAA	TCCAGATAACCCAGGTAAC
Bovine β-Actin	CCTCACGGAACGTGGTTACA	TCCTTGATGTCACGCACAATTT
Bovine 18s rRNA	AGAAACGGCTACCACATCCA	CACCAGACTTGCCCTCCA
Bovine GAPDH	GGGTCATCATCTCTGCACCT	ATCCACAGTCTTCTGGGTGG

formulation loading efficiency.

HPLC analysis: Loading efficiency and long-term stability of Mev-curcumin were quantified using reverse-phase high-performance liquid chromatography (Prominence series, Shimadzu Corporation, Japan). The analysis was performed using Phenomenex, Kinetex 5 µm C18 (250 × 4.6 mm), analytical column using PDA (Photodiode Array Detector) detector. The mobile phase consists of 0.1% orthophosphoric acid and acetonitrile (50:50 ratio). The method was linear from 20 µg/ml to 1000 µg/ml, with a correlation coefficient above 0.99. Mev-curcumin was lysed in 10% Triton X100 in methanol and loaded onto the HPLC column. The analysis was conducted at a 1 ml/min flow rate at 425 nm wavelength.

Loading efficiency: Loading efficiency was calculated as the leftover unloaded curcumin from the standard curve and subtracting the value of the original amount loaded.

% Recovery of curcumin = [(Initial amount loaded- Remaining amount)/Remaining amount] × 100

Cell culture

MDA-MB-231 cells were cultured in RPMI 1640 (Hyclone GE Healthcare) medium containing 10% FBS and other essential nutrients. They were incubated in a CO_2 incubator (Thermo Scientific) at 37°C and 85% relative humidity. The cells were maintained by splitting for every three days in a split ratio of 1:5.

Intracellular uptake of Mev into cells

BODIPY TR Ceramide loaded Mev was prepared as per manufacturers' recommendations. Unbound curcumin is removed through the exosome spin column (Thermo scientific). 0.5 Million cells/well seeded in a 12 well plate and incubated overnight. The cells were incubated with 25 µg/ml of Mev and with PBS in control cells for 1, 2, and 4 hours. After incubation, the cells were washed twice with 1X PBS. The cells were then fixed with 4% formaldehyde, followed by permeabilization with 0.2% Triton X-100 (Sigma), and then stained with DAPI (G Biosciences). The cells were then captured under a fluorescence microscope (Nikon Eclipse Ti, Tokyo, Japan) in 40X, and images were analyzed later.

Quantification of intracellular Mev-curcumin uptake

The MDA-MB-231 cells were split when the culture flask was approximately 80% confluent. 0.5 Million cells/well were seeded in a 12 well plate and incubated overnight in a CO_2 incubator at 37°C and 85% relative humidity. After overnight incubation, the cells were treated with 25 µg/ml of Exosomal curcumin formulation, free curcumin, and only exosome and media controls, along with 2.5% DMSO as a vehicle, for 4 h. The cells were lysed with 100% methanol for an hour. After incubation, the lysed cells were spun in Eppendorf tubes at 5000 rpm for 10 min at 4°C. The supernatant containing intracellular curcumin in methanol was subjected to absorption at 430 nm, against a curcumin standard (50 µg/ml to 0.7 µg/ml). The curcumin concentration in the cells was then interpolated with the standard curve of curcumin, and intracellular curcumin was quantified in µg/ml/0.5 million cells.

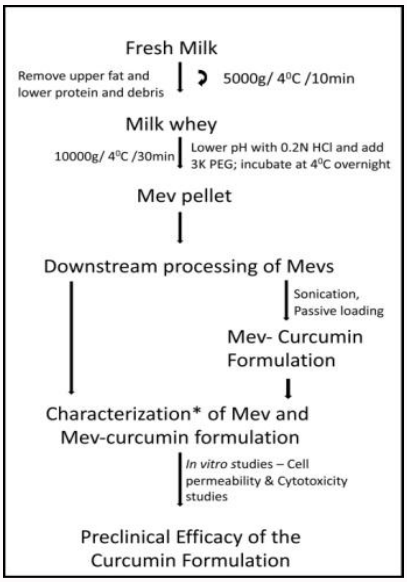


Figure 1: Workflow schematic of Milk exosome curcumin (Mev-curcumin) preparation, characterization, and preclinical efficacy - Flowchart depicting workflow for isolation and characterization* of Milk extracellular vesicles (Mev), followed by the curcumin formulation development and establishment of its efficacy *in vitro*. (*characterization - Biochemical, Biophysical, and molecular biology)

Cytotoxicity assay

Cytotoxicity assay was performed to compare the cytotoxic potential of exosomal curcumin against free curcumin in MDA-MB-231 cells. The cells were placed in a 96 well plate and incubated overnight in a CO_2 incubator. Cells were incubated with varying concentrations of free curcumin and Mev-curcumin in PBS along with media and Exosome controls. After incubation, for 48 h, cells were rinsed with 1X PBS and then with 90 µl of serum-free RPMI 1640 media without phenol red. 10 µl of MTT reagent was added to all the wells to a final concentration of 0.5 mg/ml and incubated for additional 4 h, followed by recording absorption at 570 nm. The percent inhibition was calculated for respective concentrations using the formula,

% inhibition = [(A control well- A treated well)/ A control well] × 100

where A is the absorbance at 570 nm

Statistics: Statistical significances of difference throughout this study were calculated using a student's t-test, two-way ANOVA, and one-way variance analysis. A p-value of <0.005 was considered statistically significant. Prism Software (version 8.4, San Diego, CA, USA) was used in data analysis.

Results

Workflow and isolation of bovine milk extracellular vesicle (Mevs)

Polyethylene Glycol (PEG) was traditionally used to concentrate viruses; the above protocol is adopted to precipitate Mevs from milk whey. PEGs of different MW (3K to 20K) were prepared in varying concentrations of 8%, 12%, and 16% in 0.5M NaCl, evaluated for their ability to precipitate Mevs from whey. Characterization of Mevs and Mev-curcumin formulation was conducted at biophysical, biochemical, and molecular biology level. Curcumin loading was performed with passive loading, sonication, and freeze-thaw methods to identify the ideal drug loading method to prepare the formulation. The Mev-curcumin formulation was characterized to establish its physical and functional efficacy in terms of solubility, stability, intracellular permeability, and cytotoxicity in MDA-MB-231 cells (Figure 1).

Biochemical characterization

A 12 % solution of 3K PEG yielded a clear and transparent EV pellet stable in aqueous solutions (Figure 2A), contrasting with other PEGs. The obtained pellet was unstable and pelleted in aqueous solutions. The total Mevs protein ranged around 2852 ± 103 µg/ml quantified by the BCA method. Total Lipid content is measured by phosphor vanillin assay (Figure 2B). The lipid level ranged from 1738 ± 67 µg/ml. P/L ratio ranged between 1:1 and 1:2, a ratio of 3K PEG 12% isolated Mevs being 1.6 ± 0.1 (Figure 2C). The P/L ratio provides an estimate of EV integrity. Lower P/L ratios represent better exosome preparations [24]. Precipitation with other PEGs yielded higher P/L ratios indicating other protein contamination. The total yield of Milk EVs was 1900 ± 72 µg/ml of milk (Figure 2D). Acetyl Choline Esterase is an enzyme reported to be concentrated in EVs. (AChE) activity confirmed the purity of exosomes in the Mevs pellet. AChE activity of 3K PEG 12% isolated Mevs was 1900 ± 66 mU/ml/mg protein (Figure 2D). Cumulatively, based on the pellet quality, AChE activity, and P/L ratio, we decided to go ahead with PEG 3K for Mev isolation for all downstream procedures.

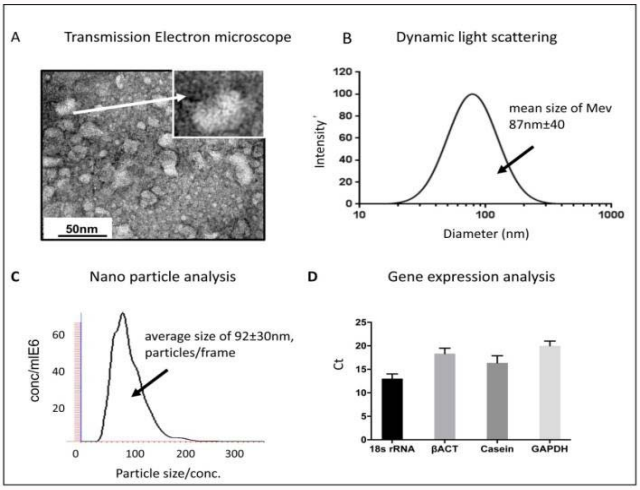
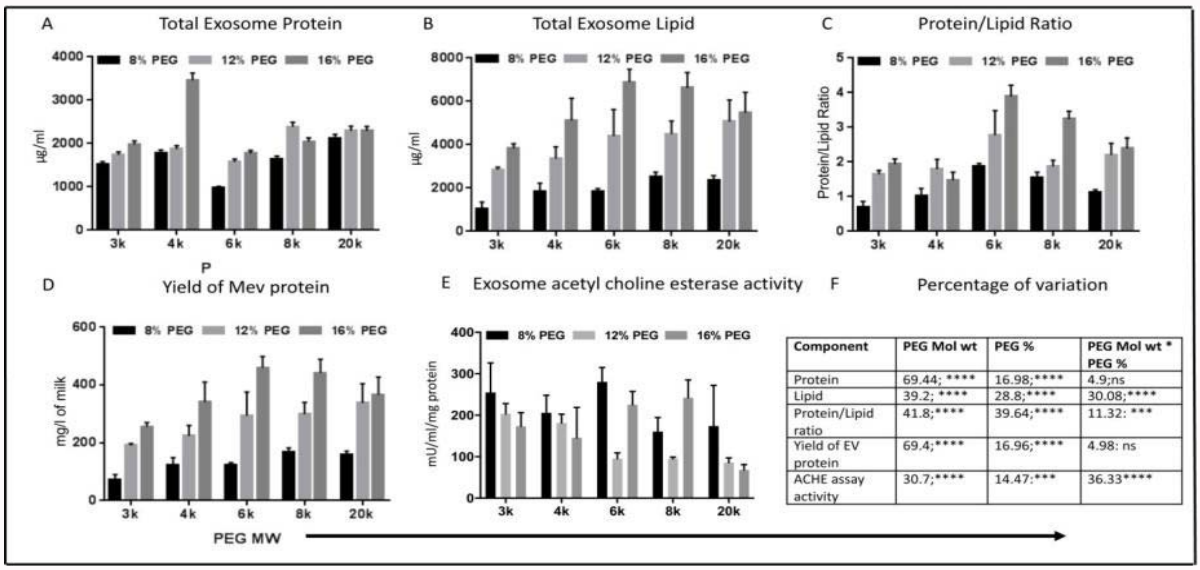


Figure 3: Biophysical characterization of precipitated Milk extracellular vesicle (Mev) - TEM images show intact round, double-membrane vesicles. All the vesicles were in size range of 30 nm to 150 nm; (scale =50 nm); (A). Dynamic light scattering showed a mean particle size of Mev around 87 nm ± 40 nm (B), and Nanoparticle tracking analysis established the average particle size of vesicles at 92 ± 30 nm; 214.24 particles/frame; the number of particles at 14.16 E8 particles/ml. (C). Molecular characterization showed the gene expression profile of bovine housekeeping genes from the RNA pool isolated from Mev (D). Graphs represent data of triplicate samples expressed as mean ± SEM (n=3).

Biophysical and molecular characterization

Transmission Electron Microscope (TEM) confirmed intact, spherical bilayered Mevs. Their size ranged from 30 nm to 150 nm (Figure 3A). However, the exosome surface has an irregular surface, which may be due to acidification with HCl. As quantified by Nanoparticle Tracking Analysis (NTA) and Dynamic Light Scattering



Component	PEG Mol wt	PEG %	PEG Mol wt * PEG %
Protein	69.44; ****	16.98;****	4.9;ns
Lipid	39.2; ****	28.8;****	30.08;****
Protein/Lipid ratio	41.8;****	39.64;****	11.32: ***
Yield of EV protein	69.4;****	16.96;****	4.98: ns
ACHE assay activity	30.7;****	14.47:***	36.33****

Figure 2: Biochemical characterizations of isolated Mevs - Mevs were precipitated with different molecular weight PEGs (MW3000, 4000, 6000 8000, and 20000) from whey. Mevs were precipitated with different concentrations of PEG (8%, 12%, and 16%) in 0.5M NaCl. The precipitated Mevs were quantified for their total protein contents (A), total lipid (B), Protein/Lipid ratio (C), the total yield of Mevs (D), ACHE Activity (E). Two-way ANOVA was conducted with PEG percentage and PEG molecular weight as the varying factors (F) with the above variables; significance (ns=0.1234, 0.0332*; 0.0021**, 0.0002***, <0.00001). 3K (12%) PEG yielded clear, transparent, and soluble vesicles with enhanced solubility in PBS and yielded lower P/L ratios with high acetyl-CoA esterase activity. Graphs represent data of triplicate samples expressed as mean ± SEM (n=3).

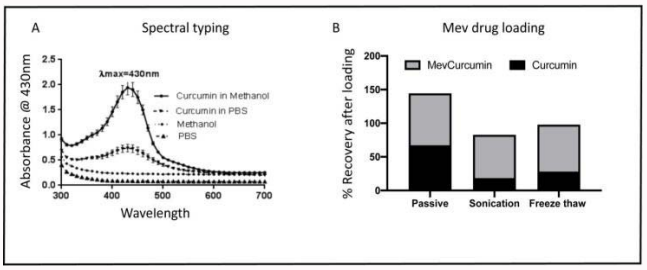
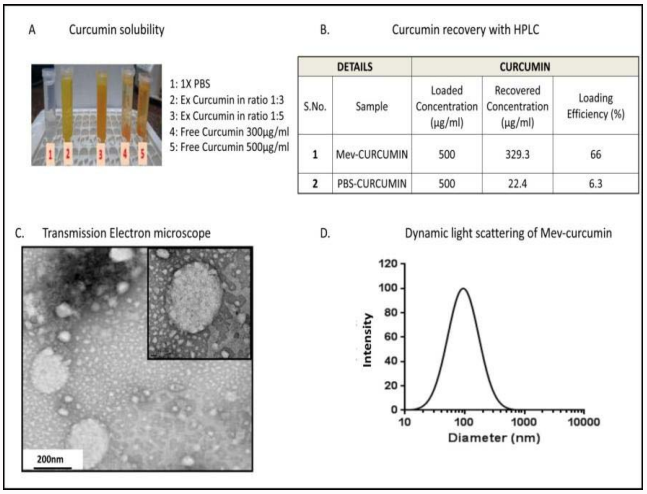


Figure 4: Enhanced Mev-curcumin stability over its parent curcumin molecule - (A) Spectral typing of free curcumin showed maximum absorption at 430 nm. The spectrogram was generated in the absorption range between 250 nm to 700 nm. PBS and methanol were used as controls. Differences between slopes were significant (P<0.0001) as per linear regression analysis (B). Curcumin was loaded into Mevs by different methods, including passive loading, sonication, and freeze-thaw methods. Stability was monitored by spectral analysis of curcumin for up to 300 min in all samples. A similar absorption trend was observed by all the methods with an insignificant trend in the passively loaded method, at 300 min(s). Passive loading protocol was used for the preparation of Mev-curcumin based on ease of preparation.



	DETAILS	CURCUMIN		
S.No.	Sample	Loaded Concentration (µg/ml)	Recovered Concentration (µg/ml)	Loading Efficiency (%)
1	Mev-CURCUMIN	500	329.3	66
2	PBS-CURCUMIN	500	22.4	6.3

Figure 5: Characterization of Milk encapsulated curcumin - (A) Representative photograph of curcumin and curcumin loaded Mevs at an exosome: curcumin ratio of 1:3 and 1:5. A homogenous orange-colored curcumin solution was observed with Mev-curcumin formulation, whereas only parent curcumin molecule precipitated out of the aqueous solution, (B). Loading efficiency of curcumin exosomes was calculated with HPLC; percent recovery was 66%, (C). TEM image of Mev-curcumin shows round, double-membrane vesicles; curcumin was found sticking to the vesicle surface, (Size =200 nm), (D) Dynamic Light Scattering yielded average size of Mev-curcumin; 113 ± 67 nm. All experiments were repeated at least thrice.

(DLS), the diameter and size of vesicles had an average diameter of 92 ± 30 nm and 87 ± 40 nm, respectively (Figure 3B). Data from NTA is in agreement with DLS data (Figure 3C). Gene expression data of the Mevs RNA pool showed that they have functional RNA. Gene expression of common housekeeping genes (βActin, 18S rRNA, and GAPDH) and casein were shown (Figure 3D).

Curcumin spectral typing and curcumin loading into Mevs

Spectral typing of curcumin yielded a spectrograph with a maximum absorbance of curcumin recorded at OD 430 nm (λmax). Representative spectrographs of curcumin and exosomes curcumin are shown (Figure 4A). Three different loading methods, i.e., sonication, FreezeThaw, and passive loading, were used for loading curcumin into Mevs (Figure 4B). Even though the above methods yielded comparable yields, we decided to move with passive loading due to ease of operation and the retention of the exosome membrane's integrity.

Encapsulation of curcumin into exosome

Mevs are exploited to serve as a natural drug delivery system for curcumin delivery, which is highly hydrophobic, having solubility/stability issues. Encapsulation of curcumin into Mev increased its solubility, which was evident by a homogenous orange-colored solution on longstanding at RT; in contrast, curcumin separated from the solution just after preparation (Figure 5A). Passive loading achieved a 70% loading efficiency. Both Spectroscopy and HPLC analyses were used to confirm curcumin's loading efficiency into exosomes (Figure 5B). The Mev-curcumin formulation was visible in TEM analysis in the range of 30 nm to 200 nm with no change in their morphology when compared to neat Mevs (Figure 5C). Curcumin was seen attached to the outer surface of Mevs. The mean size of curcumin loaded exosomes was 113 ± 67 nm according to DLS analysis (Figure 5D).

Exosome curcumin stability

Curcumin is a hydrophobic polyphenol compound that is insoluble in an aqueous solution. Though curcumin has reported anti-inflammatory properties, its unstable nature precludes it from being adopted into the clinical space. Short term stability studies were established with spectroscopy and long-term stability studies with HPLC. Curcumin and Mev-curcumin were incubated at 37°C for 150 min (Figure 6A). Long term stability studies were established for Mev-curcumin formulation stored at -70°C, -20°C, and 4°C and room temperature (Figure 6B), compared to free curcumin. The average percentage recovery after 14 days was (58, 54, 50, and 60) % at -70°C, -20°C, 4°C and RT. The above results confirm that exosome formulation has increased stability over its parent curcumin, and its stability increased with the exosomes encapsulation.

Intracellular uptake and fluorescence microscopic studies of Mev curcumin

The major hindrance to curcumin's adoption into clinical space

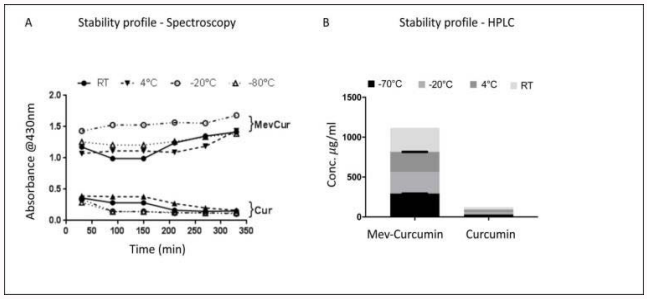


Figure 6: Improved stability of encapsulated Mev-curcumin - Short- and long-term stability of Mev curcumin over curcumin (A). The figure shows the increased stability of Mev-curcumin over its parent curcumin molecule by spectral analysis. The absorbance at 430 nm was monitored every 30 min up to 5 h to 6 h at different storage temperatures, i.e., Room Temperature (RT), 4°C, -20°C, -80°C. Increased stability was observed with Mev-curcumin at all the storage temperatures over curcumin. One-way ANOVA summary F=181.7; (P<0.0001): R2=0.9695 with post hoc test (Sidak multiple comparison test) among different groups between curcumin and Mev-curcumin were significant (P<0.0001); (B) Long term stability of Mev-curcumin formulation stored at different temperatures including, room temperature (RT), 4°C, -20°C, -70°C were quantified through HPLC analysis. There is an increased recovery of curcumin from Mev curcumin over parent curcumin stored at all temperatures. The percentage recovery of Mev-curcumin is 66%. Data were analyzed with two-way ANOVA using the Sidak multiple comparison test (ns=0.1234, 0.0332*; 0.0021**, 0.0002***, <0.00001). The graph represents data of triplicate samples expressed as mean ± SEM (n=3).

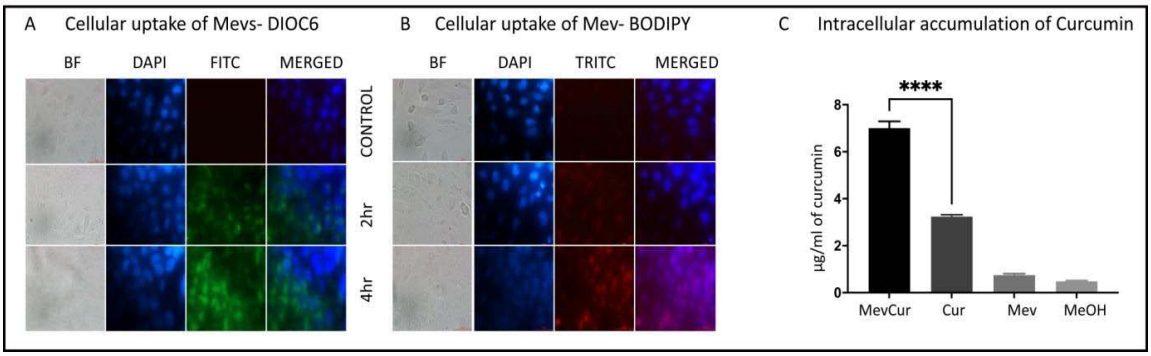


Figure 7: Enhanced cellular permeability of Mev-curcumin over curcumin - Fluorescence microscopy images of MDA-MB-231 cells captured at 2 h and 4 h in the FITC (DioC6) and TRITC (BODIPY) filter, after incubating with 100 µg/ml Mev. (A). Increased accumulation of DIOC6 (A) and BODIPY (B) stained Mev was observed in cells at 4 h (B); 40x magnification; scale =50 µm.
(C) Absorption spectra of intracellular curcumin accumulation of 0.5 × 10^6 cells MDA-MB-231 cells incubated with 25 µg/ml of Mev-curcumin and free curcumin for 4 h, collected, lysed, and curcumin was extracted by methanol lysis. Absorbance was measured at 430 nm, and intracellular curcumin was calculated from the curcumin standard curve. The figure represents triplicate samples' data expressed as mean ± SEM (n=3) unpaired t-test of Mev-curcumin *vs.* curcumin; p<0.0001.

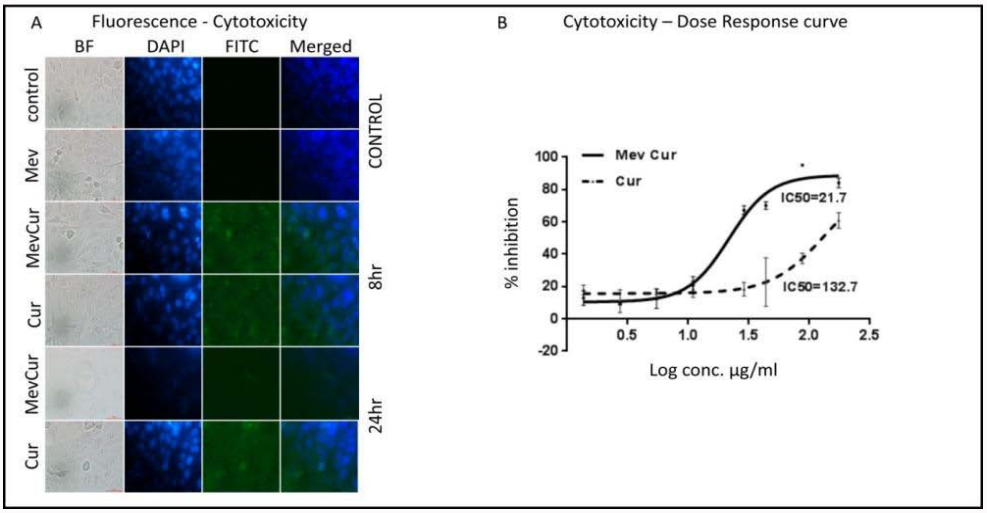


Figure 8: Increased cytotoxicity of Mev-curcumin over parent molecule - MDA-MB-231 breast cancer cells were treated with 20 µg/ml of Mev-curcumin and free curcumin for 24 h before fluorescent imaging. Fluorescence images were captured at 8 h and 24 h at 40x magnification (scale =50 µm); enhanced cytotoxicity is observed with Mev-curcumin than free curcumin as observed through (A). Dose-response curve of Mev-curcumin and free curcumin in MDA-MB-231 cells for 48 h treatment (B). The figure represents the mean ± SEM (n=3).

is its low systemic bioavailability due to the rapid first-pass effect and fast intestinal glucuronidation metabolism. DIOC6 and BODIPY TR Ceramide bound Mevs were used to establish their intracellular bioavailability, as evidenced by the accumulation of Mevs into cells. Kinetics of exosome accumulation into MDA-MB-231 cells was established through fluorescence microscopy, and accumulation maxima are achieved by 4 h (Figure 7A and 7B). Increased accumulation of Mev-curcumin is also shown through spectroscopy. Lysis of MDA-MB-231 cells with methanol showed increased curcumin accumulation in Mev-curcumin-treated cells (Figure 7C), compared to parent curcumin. The above studies establish that exosomes transverse the cell membrane and deliver their payload to cells better than its parent drug. The results indicate that the uptake increased in Mev-curcumin formulation when compared to free curcumin. There was a two-fold difference in the amount of curcumin uploaded when delivered through Mev.

Cytotoxicity of exosome curcumin

The cytotoxic effect of curcumin and Mev-curcumin was studied in a dose-response manner. The proliferation of MDA-MB231 cells was significantly inhibited by curcumin and exosome curcumin in a concentration-dependent manner. The fluorescence images of time-dependent cytotoxicity clearly show higher toxicity in Mev-curcumin in treated cells (Figure 8A). The IC50 value of free curcumin was 132.7 µg/ml, and exosome encapsulated curcumin was 21.7 µg/ml. There was a three-fold difference in IC50 obtained between free curcumin and exosome curcumin (Figure 8B). Cytotoxicity experiments show a lower IC50 value with Mev-curcumin with a six-fold difference *in vitro* efficacy with free curcumin in breast cancer cell line, MDA MB 231.

Discussion

The most preferred drug administration method is the oral drug route, but parenteral administration takes over due to stability and bioavailability issues associated with the drugs. Improved bioavailability can be achieved by developing particulate formulations, but most of them still have an impaired ability to pass the gastrointestinal mucosa. Evs can be used as effective vesicular vehicles and have the advantages of being endogenous, less immunogenic and natural but suffered from scalability dynamics [25].

In the present study, we optimized a separation protocol for the purification of Mev from milk whey (Figure 1). We identified 3K MW PEG as the ideal PEG for precipitating the Evs from milk whey (Figure 2). Detailed characterization of Mev at the biochemical, biophysical, and molecular levels was established (Figure 3). An ideal curcumin loading method was identified in terms of the ease and feasibility of loading curcumin (Figure 4). The curcumin loaded exosomes were also analyzed for their size, structure, and integrity (Figure 5). The short and long-term stability of Mev-curcumin was established by spectroscopy and HPLC (Figure 6). The cellular bioavailability and localization of Mev-curcumin were confirmed through exosome membrane dye BODIPY and DIOC6 incorporation (Figure 7). The cytotoxic potential of Mevcurcumin over its parent molecule was established in the MBA-MB-231 cell line by fluorescence imaging and MTT assay (Figure 8). Thus, we report establishing an easy workflow for the isolation of Mevs and the development of Mev-curcumin formulation.

An exosome yield of 200 mg/liter of milk was achieved with our optimized isolation protocol. Variability of exosome protein in different batches and other reported studies can be attributed to the animals' physical activity, nutrition, and metabolism status from which milk was collected [26,27]. The exosomal lipid concentration range is around 1 mg/ml to 2 mg/ml of whey. It is reported that the exosome membrane is enriched with specific lipids, including phosphatidylcholine, cholesterol, sphingomyelin, ceramides, etc [28]. The asymmetrical distribution of lipids on the exosome surface and interior significantly affects the curcumin binding and distribution and may account for minor differences from other studies [20,21,29-31]. The protein/lipid ratio represents the purity of the exosome preparations. Vesicular purity is further established by assessing acetyl-CoA esterase activity levels [32]. The functionality of isolated Mevs is established by detecting housekeeping genes, including 18S bactin, GAPDH, and casein. It is reported that 19,230 mRNAs and miRNAs are identified in milk exosomes. The biophysical parameters of Mevs are in line with published reports to appropriately classify our isolated EV fraction are in the exosome size range (50 nm to 150 nm) as confirmed by NTA and DLS and structure (TEM) [33].

EV morphology and integrity were retained in the formulation, and curcumin is found attached to the EV membrane surface. Consistent with similar studies, Mevs curcumin's size increased compared to Mevs but with no change in morphology [21]. The stability of Mevcurcumin is evident with the homogenous solution in contrast with the parent molecule (Figure 5A). Spectroscopy confirmed the short-term stability of the developed formulation (Figure 6). The concentration of curcumin in the exosome formulation was also monitored with HPLC analysis for up to 14 days. Storage at -70°C, -20°C, and 4°C did not yield any significant deterioration. There was no change in Mev-Curcumin concentration even upon 14 days of storage at different storage temperatures. Our formulation was comparatively stable even at 4°C, avoiding the need for low-temperature freezers for storage and distribution. BODIPY and DioC6 accumulated Mevs in the cells confirmed the cellular intake of Mev. A twofold increase in curcumin accumulation is observed in MDA-MB-231 cells shows increased delivery of payload through Evs. Six-fold cytotoxicity with Mev-curcumin shows an increased cytotoxic potential of the developed formulation and a direct correlation of improved bioavailability.

In the current study, we report the suitability of using Mevs for drug delivery with curcumin as a candidate drug [30]. The unique ability of Mevs to sustain gastric digestion and deliver the payload to the intestinal mucosa, coupled with the feasibility to be isolated in large quantities at a reasonable cost, makes them ideal candidates for oral drug delivery [29]. Optimizing a workflow for Mev isolation, purification, and characterization of efficient drug delivery systems will open indoors for reshelving many toxic oncology drugs shelved due to toxicity issues. Our data confirm that the delivery of curcumin by Mevs increases its solubility, stability, and bioavailability. By employing milk exosome as a delivery vehicle, therapeutic cargos can be shuttled orally as edible formulations. The limitation of the current study involves limiting the preclinical efficacy to *in-vitro* studies only. Further studies are needed to establish its clinical effectiveness and delivery potential in animal studies.

Conclusion

We demonstrate that bovine Milk could serve as a cost-effective source for harvesting milk exosomes. The exosomal formulation of curcumin enhanced its stability, solubility, bioavailability, and preclinical efficacy. We demonstrated that milk exosomes-based drug formulation would be a future solution to deliver drugs at reduced concentrations safely. Drugs notorious for their "brick dust status" (drugs with limited solubility) status could be converted to blockbusters if their solubility issues were resolved with vesicular delivery through Mevs.

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