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Human plasma derived exosomes: Impact of active and passive drug loading approaches on drug delivery

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ABSTRACT

The aim of the current study was to explore the potential of human plasma-derived exosomes as versatile carriers for drug delivery by employing various active and passive loading methods. Exosomes were isolated from human plasma using differential centrifugation and ultrafiltration method. Drug loading was achieved by employing sonication and freeze thaw methods, facilitating effective drug encapsulation within exosomes for delivery. Each approach was examined for its effectiveness, loading efficiency and ability to preserve membrane stability. Methotrexate (MTX), a weak acid model drug was loaded at a concentration of 2.2 μM to exosomes underwent characterization using various techniques such as particle size analysis, transmission electron microscopy and drug loading capacity. Human plasma derived exosomes showed a mean size of 162.15 ± 28.21 nm and zeta potential of −30.6 ± 0.71 mV. These exosomes were successfully loaded with MTX demonstrated a better drug encapsulation of 64.538 ± 1.54 % by freeze thaw method in comparison 55.515 ± 1.907 % by sonication. *In-vitro* drug release displayed 60 % loaded drug released within 72 h by freeze thaw method that was significantly different from that by sonication method i.e., 99 % within 72 h (p value 0.0045). Moreover, cell viability of exosomes loaded by freeze thaw method was significantly higher than that by sonication method (p value 0.0091) suggested that there was membrane disruption by sonication method. In conclusion, this study offers valuable insights into the potential of human plasma-derived exosomes loaded by freeze thaw method suggest as a promising carrier for improved drug loading and maintenance of exosomal membrane integrity.

1. Introduction

Exosomes are small extracellular vesicles derived from cells typically ranging from 30 to 150 nm, have gained considerable attention as a potential carrier for drug delivery (He et al., 2022, Liu et al., 2023). These drug delivery systems are biocompatible and possess low immunogenicity (Qin and Xu, 2014, Ha et al., 2016). They provide enhanced stability and protection for drugs, improve drug solubility and prolong circulation time (Clemmens and Lambert, 2018, Du et al., 2023). Exosomes can be sourced from various types of stem cells on the one hand and different body fluids including plasma on the other hand (Lopez-Verrilli et al., 2016). Plasma or serum-derived exosomes have been implicated in numerous physiological and pathological conditions, making them attractive targets for biomarker discovery and disease diagnosis (Muller et al., 2014, Semreen et al., 2018).

Plasma-derived exosomes have attracted considerable attention as promising candidates for targeted drug delivery due to their distinct attributes such as stability, biocompatibility, and ability to overcome

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biological barriers (Goetzl et al., 2016, Shams et al., 2023). Maximizing their therapeutic potential, necessitates efficient loading of drugs into exosomes. Xi et al., 2021 have been investigated various loading methods including direct incubation, electroporation, sonication, extrusion and genetic engineering of exosome-producing cells. Understanding and optimizing these methods are essential for enhancing drug loading efficiency and ensuring the therapeutic efficacy of the loaded exosomes (Xi et al., 2021).

Active drug loading approaches i.e., electroporation, sonication, and extrusion can be employed to disrupt the exosome membrane and facilitate drug entry (Mehryab et al., 2020). These methods offer higher drug loading efficiency, however, careful optimization and membrane recovery steps are required to ensure the integrity and functionality of both the exosomes and the loaded drugs (Donoso-Quezada et al., 2020, Mehryab et al., 2023). Passive drug loading approaches i.e., co-incubation, freeze thaw method, pH gradient loading includes the inherent biophysical characteristics of exosomes, allowing for the encapsulation of diverse therapeutic agents without the need for complex modifications. These methods are simple, reduced probability of affecting exosome stability, and minimal alteration of the vesicle's biological properties The freeze–thaw method is a technique utilized for loading drugs into exosomes by taking advantage of the freeze–thaw-induced destabilization of the exosome membrane. This process allows for the incorporation of drugs into the interior of exosomes. When subjected to freezing, the formation of ice crystals exerts mechanical stress on the exosome membrane, causing temporary disruptions (Fu et al., 2020). Subsequent thawing leads to the resealing of the membrane, effectively trapping the drugs within the exosomes. It involves minimal additional steps and does not rely on complex equipment or reagents. This characteristic makes the technique easily accessible and cost-effective, as it can be performed in a standard laboratory setting (Xu et al., 2020). Moreover, studies have demonstrated that the freeze–thaw method has minimal impact on exosome integrity and functionality (Zeng et al., 2023). The choice of drug loading method depends on various factors, including the physicochemical properties of the drug, the desired loading efficiency, and the intended therapeutic application. Optimization of drug loading methods is crucial to maximize loading efficiency, minimize drug leakage, and preserve exosome integrity (Xu et al., 2020). MTX is a weak acid drug with pka 4.6 to 5.5, log P of −1.85 and 454 D molecular weight and is chosen as a representative model drug for evaluating drug loading strategies for weak acid drug into exosomes. (Gaurav et al., 2021, Sullivan et al., 2021). In this research work, MTX is solubilize in phosphate buffer saline pH 7.4. The evaluation of MTX loading strategies in exosomes provides valuable insights into optimizing drug delivery for high molecular weight hydrophilic drugs.

This research article comprehensively evaluates potential of human plasma derived exosomes as a drug delivery carrier. Meanwhile, this study also investigates the impact of various drug loading methods on human plasma derived exosomes functionality. For this purpose, freeze thaw and sonication method was utilized to load MTX in human plasma derived exosomes. The outcome was evaluated by various characterization techniques including particle size analysis, morphology, drug release and cytotoxicity studies.

2. Materials & Methods

2.1. Materials

Methotrexate (MTX) was a gift sample from Werrick Pharmaceuticals, Islamabad, Pakistan. Human plasma was provided by a volunteer with informed consent from KRL hospital, Islamabad, Pakistan. Monobasic potassium phosphate was purchased from Merck, Germany. Bovine serum albumin, coomassie brilliant blue, Tween 20, Sodium hydroxide and Calcium chloride were purchased from Sigma Aldrich, USA. Pierce fast western blot kit ECL substrate (cat#35055), Bradford reagent, MTT assay kit and DMEM media were supplied by Merck Traders.

2.2. Methods

2.2.1. Isolation of exosomes from human plasma

Exosomes from the human plasma was isolated by ultracentrifugation method followed by dialysis and ultrafiltration method with slight modification (Kang et al., 2019). In brief, 5 mL of human plasma sample was subjected to differential centrifugation under control conditions at 4 ℃. Differential centrifugation (Hermle GmbH Z-326 K, Germany) was performed with different centrifugal speed and time i.e., 300 rpm for 10 min to remove cellular debris. Supernatant was centrifuged again at 10,000 rpm for 10 min to remove microvesicles, dead cells and large particles. The resultant supernatant was centrifuged at 100,000 rpm for 70 min to isolate exosomes. Exosomes were further purified by using 10kD dialysis membrane for 42 to 72 h using distilled water as medium. Exosomes sample was placed inside dialysis bag and tied closely with thread and dip in 300 mL of distilled water. After 24 h distilled water will be changed and final sample was collected after 72 h. Sample of exosomes upon purification changed color from yellow to white. Isolated exosomes were filtered by using 0.22 μm syringe filters and stored at −20 ℃ for further use.

2.2.2. Formulation of MTX mediated exosomes

2.2.2.1. Sonication method. MTX loaded exosomes were also formulated by active loading approach i.e., sonication method (Kim et al., 2016, Mehryab et al., 2023). For this a probe sonicator (Biobase Lab, UCD-150) was used to allow drug loading as sound waves open the pores of exosomal membrane. For sonication, 2.2 μM of MTX stock solution and 100 μL of exosomes were mixed and final volume makeup to 5 mL with phosphate buffer saline. This drug containing exosomal solution subjected to 6 cycles of sonication with 20 % amplitude with 30 sec on/off for 3 min. After every cycle 2 min cooling time was allowed. After sonication, MTX loaded exosomes were placed in incubator (Memmert IN30) at 37℃ for 1 h to restore membrane integrity. Drug loaded exosomes were subjected to centrifugation at 10,000 rpm for 15 min with three wash cycles. Collected pellet was stored at −20℃ for further use. Sonication frequency amplitude was selected as a critical variable to evaluate its effect on selected response variables i.e., particle size, PDI and zeta potential.

2.2.2.2. Freeze thaw method. MTX mediated exosomes were formulated by using passive loading method of freeze and thaw cycles along with calcium chloride ($CaCl_2$) transfection with slight modifications (Zhang et al., 2017). MTX stock solution with concentration of 1 mg/mL was prepared. Aliquot of 2.2 μM of MTX stock solution and 100 μL of exosomes were mixed. Therefore, 200 μM $CaCl_2$ was added in the prepared mixture and final volume was made to 5 mL with phosphate buffer saline 7.4. This mixture was subjected to multiple freeze and thaw cycles to incorporate drug into the exosomes. Mixture was placed at −20℃ for 30 min followed by heat cycle at 25℃ for 30 min. These steps were repeated three times. In order to remove free drug, drug loaded exosomes were centrifuged (Hermle GmbH Z-326 K, Germany) at 10,000 rpm for 15 min with three wash cycles. Collected pellet was stored at −20℃ for further use. For the optimization, exosomes concentration, drug concentration and $CaCl_2$ concentration were used as a critical variable that can affect particle size, PDI and zeta potential selected as response variables. Details of critical variables were added in results section.

2.2.3. Characterization of human plasma derived exosomes

2.2.3.1. Particle size and surface charge analysis. Size of isolated exosomes were determined by dynamic light scattering (DLS) using Zeta

sizer (Malvern Panalytical, ZS90 Worcestershire, UK) provides results of size range and surface charge of exosomes. PDI was used to evaluate the size distribution of exosomes. For the analysis by zeta sizer, sample of exosomes were diluted in a defined ration of 1:10 with ultrapure distilled water. Sample was placed in the cuvette of zeta sizer and readings were recorded in the form of graphs (Nazir et al., 2018). Particle size, PDI and zeta potential of MTX loaded exosomes of all the formulations by sonication and freeze thaw method was also evaluated by zeta sizer in a similar manner with dilution ratio of 1:10 with ultrapure distilled water.

2.2.3.2. Morphological analysis. Morphology of blank and MTX loaded exosomes were visualized by transmission electron microscopy (TEM) (He et al., 2023). For TEM analysis, sample was lyophilized and mounted on the sample stub. Samples were placed over copper grid after staining with phosphotugistic acid. Prepared sample were observed under different resolution by transmission electron microscope (JEM-2100; Jeol Ltd., Japan).

2.2.3.3. Protein quantification of exosomes by bradford assay. Quantitative analysis of protein content of exosomes was performed by bradford assay with slight modification (Dash et al., 2021). In this method, a calibration curve of bovine serum albumin with five known concentrations were constructed. Proteins samples were allowed to react with Bradford reagent for 10 min at room temperature and readings of absorbance was recorded at 595 nm using UV–visible spectrophotometer (Shimadzu UV1700).

2.2.3.4. Nanodrop analysis. Protein and nucleic acid content of exosomes was quantified by using nanodrop analysis (Ezdrop 1000, blue ray biotech) (Patel et al., 2019). Nanodrop quantify protein content at 280 nm and nucleic acid content at 260 nm. By comparing the absorbance of sample with known concentrations of standard, protein and nucleic acid content of isolated exosomes were determined.

2.2.3.5. Western blot analysis. Sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis was utilized for protein quantification of human plasma derived exosomes. The SDS-PAGE gel was prepared with 10 % resolving and 5 % stacking gel percentages, for exosome analysis. The gel was casted and allowed to polymerize. Sample were denatured by using non-reducing buffer. Exosomes sample and molecular markers were loaded into the wells of SDS-PAGE gel. SDS-PAGE electrophoresis was performed at a constant voltage until the dye front reaches the bottom of the gel. This process separates the exosomal proteins based on their molecular weight. Following electrophoresis, the gel was removed from the cassette, and the separated proteins were visualized using Coomassie Brilliant Blue or silver staining. An image of the stained gel was captured and the banding pattern of exosomal proteins and molecular weight marker bands was analyzed.

Molecular characterization of exosomes was conducted using western blotting, focusing on their transmembrane protein marker CD63 (Wang et al., 2022, Bakadia et al., 2023, Fujimura et al., 2023). Following electrophoresis, the proteins were transferred to a PVDF membrane in the Bio-Rad blot module, utilizing transfer buffer. To probe the antigens, a primary antibody against CD63 was applied in a 1:1000 dilution followed by a rabbit anti-mouse secondary antibody in a 1:2000 dilution conjugated with horseradish peroxidase (HRD). This process occurred in phosphate-buffered saline with added tween 20 (PBST) blocking buffer containing 5 % non-fat milk. For visualization, equal volumes of peroxide solution and luminol enhancer solution were added, and the ChemiDoc XRS + gel documentation system was used.

2.2.4. Drug loading studies of MTX mediated exosomes

Drug loading was determined by direct method that involved measuring drug concentration in exosomes pellet with UV–Visible spectrophotometer (Shimadzo UV1700) (Aqil et al., 2017, Gul et al., 2018, Qi et al., 2020). For this purpose, MTX loaded exosomes were centrifuged at 10,000 rpm for 15 min to remove free drug from the sample. Absorbance of all known solutions were determined by using UV–Visible spectrophotometer (Shimadzu UV1700) at 303 nm. Drug concentration was determined in supernatant and exosomal pellet and percentage encapsulated and drug loading was calculated by following equations.

$$Encapsulation\ Efficiency = \frac{Total\ MTX\ concentration - Concentration\ of\ MTX\ in\ supernatant}{Total\ MTX\ concentration} \times 100$$

$$Loading\ capacity = \frac{Total\ MTX\ concentration\ in\ exosomes\ pellet}{Total\ protein\ content\ in\ exosomes\ pellet} \times 100$$

Total protein content in exosomes pellet was measured by Bradford assay.

2.2.5. In-vitro drug release studies at pH 7.4

Drug release studies were performed by using dialysis membrane diffusion method (Gul et al., 2018, Ghadi et al., 2019, He et al., 2023). Lyophilized formulation of MTX loaded exosomes were added in a concentration of 5 mg in dialysis bags immersed in phosphate buffer pH 7.4. Dialysis bag assembly was kept at 37 °C and at 50 rpm. Samples were collected at predetermined time intervals 0, 0.5, 1, 2, 4, 6, 12, 24 and 48 h. Samples were evaluated by UV–Visible spectrophotometer at λ_{max} 303 nm. Results of drug released were further evaluated by application of drug release kinetics models by DDsolver.

2.2.6. Biocompatibility assay

Biocompatibility assay was utilized to check the biocompatibility of human plasma derived exosomes and MTX loaded exosomes on HEK cell lines by MTT assay (Ebrahimnejad et al., 2010, Rayamajhi et al., 2019, Nazir et al., 2020). To establish HEK cell cultures for the MTT assay, cells were cultivated at a density of 2.5×10^4 cells/well in essential medium DMEM to sustain their viability and overall health. Cells were placed in CO_2 incubator (Mermmert GmbH) to facilitate growth. For the MTT assay, these cells were added into 96-well plates with density of 25,000 cells, encouraging their adherence and formation of a monolayer. Cell viability of different concentrations of MTX and the prepared exosomes-based formulations were evaluated by adding 200 μL of each sample in 96-well plate incubated with cells. Sample loaded 96-well plate was incubated for 48 h in CO_2 incubator. DMEM media was used as control. After 48 h, 100 μL MTT dye was added. The reagent undergoes metabolic conversion within viable cells, resulting in the development of insoluble formazan crystals that display distinct color alteration. After 3 h of incubation, absorbance was measured at 570 nm using microplate reader. IC50 of MTX and its formulations were determined by plotting the results in the form of graph.

2.2.7. Statistical analysis

GraphPad Prism v8.0 was used to perform statistical analysis. All readings were assessed in term of mean and standard deviation of three

experiments (n = 3). Comparative analysis was analyzed by one-way ANOVA test with significant results at p value < 0.5.

3. Results

3.1. Characterization of exosomes

3.1.1. Particle size, size distribution and surface charge analysis

Exosomes were isolated on the basis of density of cellular constituents present in the plasma by ultracentrifugation. Differential centrifugal forces separate cellular debris, apoptotic bodies and microvesicles from the sample to isolate exosomes. The results of particle size analysis of human plasma derived exosomes indicate an average size of 142.2 ± 16.71 nm, PDI 0.741 ± 0.152 and zeta potential −26.8 ± 0.88 mV. Results of optimized MTX loaded by sonication also depict nano-size carrier with average size of 166.2 ± 7.49 nm, PDI 0.384 ± 0.045 and negative zeta potential –23.5 ± 0.42 mV. Size analysis of MTX loaded exosomes by freeze thaw method showed particle size in the range of 162.15 ± 28.21 nm, PDI 0.472 ± 0.024 and zeta potential −30.6 ± 0.71 mV. Size distribution of exosomes is summarized in Table 1.

For sonication method, sonication frequency was used as a variable from 10 % to 50 % amplitude with constant sonication time. Results showed that with increase in sonication frequency average particle size was reduced, however PDI was increased from 0.384 to 0.663 and zeta potential was reduced to from –23 to −12 mV. So, minimum optimized amplitude of 20 % seems more appropriate for drug loading as illustrated in Fig. 1.

For passive loading, drug, exosomes and $CaCl_2$ concentration were investigated as a critical variable that can affect particle size, PDI, and zeta potential of exosomes during drug loading. When concentration of exosomes varied from 50 μL to 200 μL, average particle size was reduced from 400 nm to 162 nm. Further increase in exosomes concentration from 300 to 400 μL resulted in increased average particle size 275 nm to 363 nm respectively. These results suggest that too low or too high concentration of exosomes results in increased particle size along with variation in size distribution and reduced zeta potential shown in graphs of Fig. 2a, 2b and 2c.

As the concentration of $CaCl_2$ increases from 100 to 500 μL, particle size increases from 234 nm to 875 nm along with PDI increased to 1 that indicates heterogenous size of particles. Zeta potential also reduced by increasing calcium chloride concentration to −6.45 mV. This reduce zeta potential may be due to positive charge of increase calcium ions that results in more particle aggregation. Results were presented in graph of Fig. 2d, 2e, and 2f.

Drug concentration also affects the average particle size, PDI and zeta potential. As the concentration of MTX increases from 1.1 μM to 22 μM, the average size also increased from 215 nm to 482 nm, PDI from 0.611 to 1 and zeta potential reduced from −20 to −9.76 mV as shown in Fig. 2g, 2 h and 2i.

Table 1
Particle size, size distribution and surface charge analysis.

Exosomes (μL)			Size (nm) ± SD	PDI ± SD	Zeta Potential ± SD (+/-mV)
Particle size, size distribution and surface charge analysis of human plasma derived exosomes					
100			142.2 ± 16.71	0.741 ± 0.152	−26.8 ± 0.88
Drug (μM)	**Exosomes (μL)**	**Sonication**	**Size (nm) ± SD**	**PDI ± SD**	**Zeta Potential ± SD (+/-mV)**
Particle size, size distribution and surface charge analysis by sonication					
2.2	100	0.2	166.2 ± 7.49	0.384 ± 0.045	–23.5 ± 0.42
Particle size, size distribution and surface charge analysis by freeze thaw method					
Drug (μM)	**Exosomes (μL)**	**$CaCl_2$ (μL)**	**Size (nm) ± SD**	**PDI**	**Zeta Potential ± SD (+/-mV)**
2.2	100	200	162.15 ± 28.21	0.472 ± 0.024	−30.6 ± 0.71

3.1.2. Morphological analysis

The morphology of exosomes in the samples were investigated by using TEM. The TEM images revealed oval structures with a cup-shaped morphology, which is a distinctive characteristic of exosomes. The individual exosomes observed had diameters ranging from 60 to 150 nm as shown in Fig. 3. The surface of the exosomes appeared smooth and uniform, indicating the preservation of their lipid bilayer structure. The TEM analysis provided high-resolution images that allowed for the visualization of exosome morphology and size distribution. Images of TEM for blank exosomes, MTX loaded exosomes by freeze thaw and sonication are shown in Fig. 3. Morphology images indicate exosomes loaded by sonication are small in size but depicts some aggregation that may be due to loss of membrane integrity during sonication stress.

3.1.3. Protein quantification of exosomes by bradford assay

The results of Bradford assay revealed protein concentration in the exosome sample was 2.75 ± 1.12 mg/ml. This finding indicates a notable protein content within the exosomes.

3.1.4. Nanodrop analysis

For an exosome sample, the nanodrop analysis revealed information about the concentration of nucleic acids (e.g., RNA or DNA), protein and the presence of contaminants. The results indicate the dsDNA, RNA and protein concentration in sample was 597.11 ng/μL, 628.29 ng/ μL and 3.11 mg/mL respectively (Table 2). Fig. 4 illustrates results of nanodrop analysis.

3.1.5. Western blot analysis

Exosome samples containing approximately 2.75 to 3.74 mg of protein in 1 mL of suspension were subjected to 10 % PAGE (Polyacrylamide Gel Electrophoresis) analysis. The sample contains protein from a wide range of peptides, spanning from 15 kDa to 130 kDa with characteristics bands in 25 to 30 KDa region.

The Western blot analysis of plasma derived exosomes further unveiled bands that corresponded distinctly with the exosomal markers, CD9 and CD63. The SDS-PAGE gel quantified molecular weight of bands at approximately 50 kDa and 25 kDa for CD63 and CD9, respectively, aligning accurately with their known molecular weights illustrated in Fig. 5. The presence of CD63 bands in the exosome-enriched samples suggests successful isolation of exosomes from human plasma sample by differential ultracentrifugation followed by dialysis method.

3.2. Drug loading studies of MTX mediated exosomes

The encapsulation efficiency achieved by active MTX loading was 55.515 ± 1.907 %. This indicates sonication method is successful for loading hydrophilic drug into exosomes. The encapsulation efficiency of MTX in exosomes using the passive method was determined to be 64.538 ± 1.54 %. This indicates that approximately 64 % of the hydrophilic drug was successfully encapsulated within the exosomes (Table 3).

3.3. In-vitro drug release studies

The *in-vitro* drug release investigation pertaining to exosomes demonstrated a gradual and sustained release pattern observed over a span of 72 h. Within this timeframe, approximately 60 % of the encapsulated MTX was gradually released from exosomes loaded by the freeze–thaw method. However, in stark contrast, exosomes loaded via the sonication method

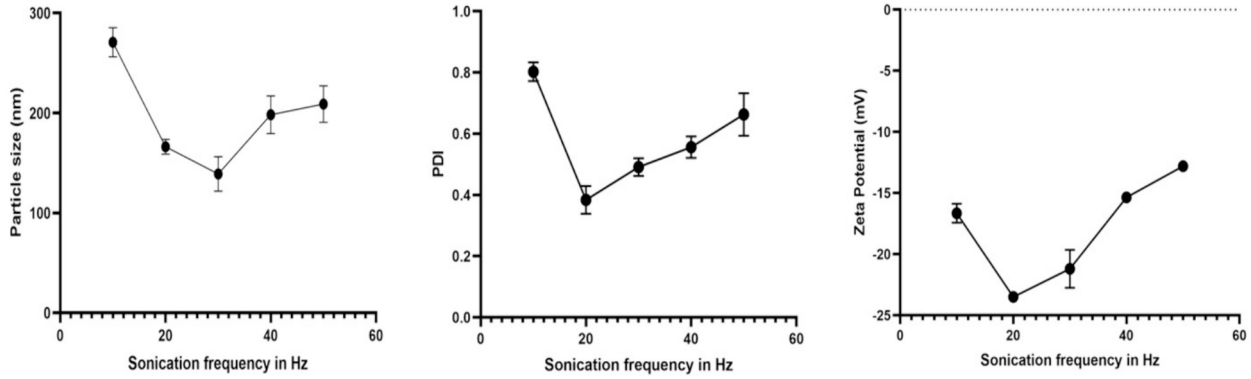

Fig. 1. Effect of sonication frequency in hertz (Hz) on average particle size, PDI and zeta potential.

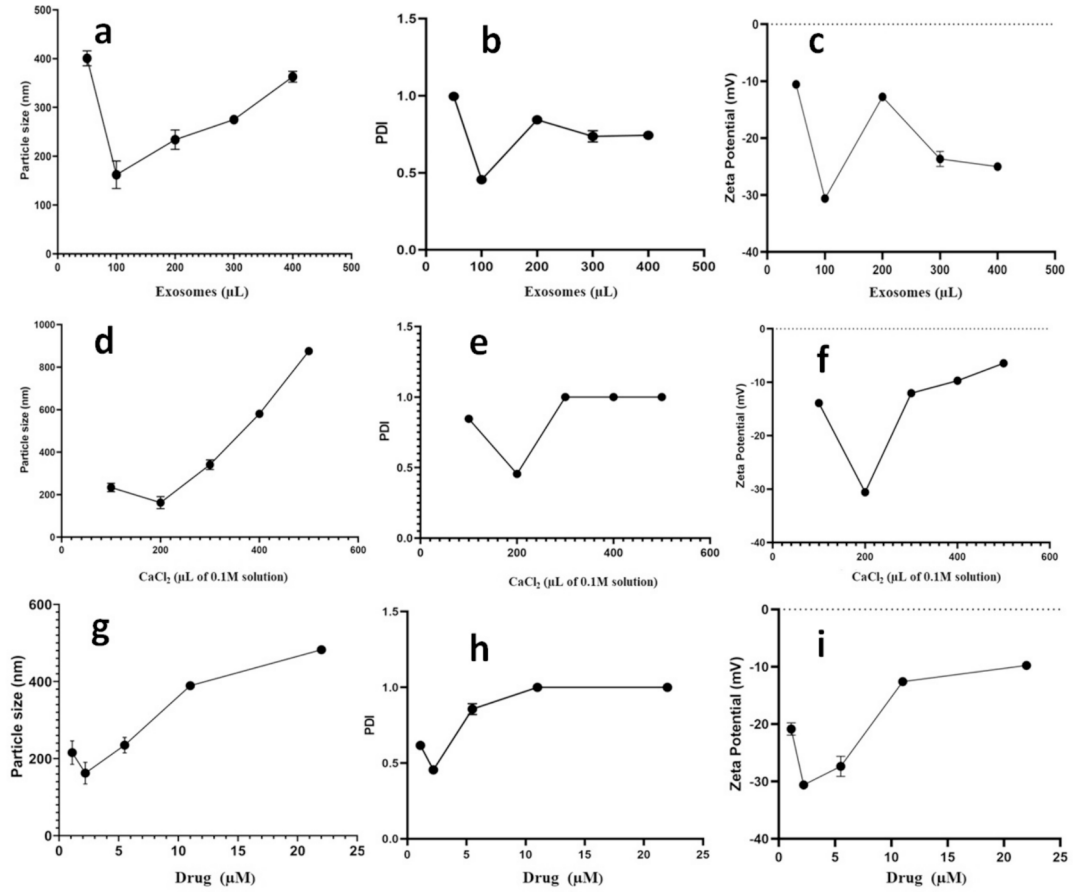

Fig. 2. Optimization of particle size, PDI and zeta potential of MTX loaded exosomes by freeze thaw method a) effect of exosomes concentration on particle size b) effect of exosomes concentration on PDI c) effect of exosomes concentration on zeta potential d) effect of $CaCl_2$ concentration on particle size e) effect of $CaCl_2$ concentration on PDI f) effect of $CaCl_2$ concentration on zeta potential g) effect of drug concentration on particle size h) effect of drug concentration on PDI i) effect of drug concentration on zeta potential.

exhibited an accelerated drug release profile, with nearly 99 % of the entrapped MTX released within the same 72-hour duration, as illustrated in Fig. 6. Evidently, the drug release kinetics from exosomes employing the sonication method exceeded that of the freeze–thaw approach, and statistical analysis yielded a significant difference between the two (p-value 0.0045 significant results as $p < 0.05$), underscoring distinct release mechanisms. Mechanism of drug release from exosomes was evaluated by applying kinetic models using DD solver. MTX loaded by sonication method released from exosomes according to Hixon-crowell model and MTX loaded by freeze thaw method release drug according to Higuchi model as shown in Table 4 and Fig. 7.

3.4. Biocompatibility assay

A biocompatibility assay was performed on HEK (Human Embryonic Kidney) cell lines to assess the potential cytotoxic effects of blank and drug loaded exosomes. The results of the biocompatibility assay revealed that the presence of blank exosomes did not lead to significant cytotoxicity or adverse effects on the viability of the HEK cells. Even when exposed to the highest tested concentration of blank exosomes, the cells exhibited a high level of viability and maintained their normal cellular morphology. These findings indicate that the blank exosomes demonstrated biocompatibility with HEK cell lines, suggesting a

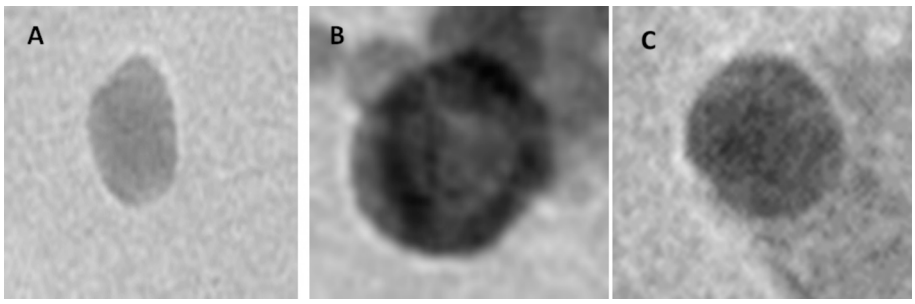

Fig. 3. Morphology of Human Plasma Derived Exosomes by TEM Images. (A) Human plasma derived exosomes (B) MTX loaded exosomes by sonication (C) MTX loaded exosomes by freeze thaw

Table 2
Nanodrop analysis for protein and nucleic acid quantification.

Sample	dsDNA (ng/µL)	RNA (ng/µL)	Protein (mg/mL)
Human plasma derived exosomes	597.11 ± 10.31	628.29 ± 13.56	3.11 ± 1.88

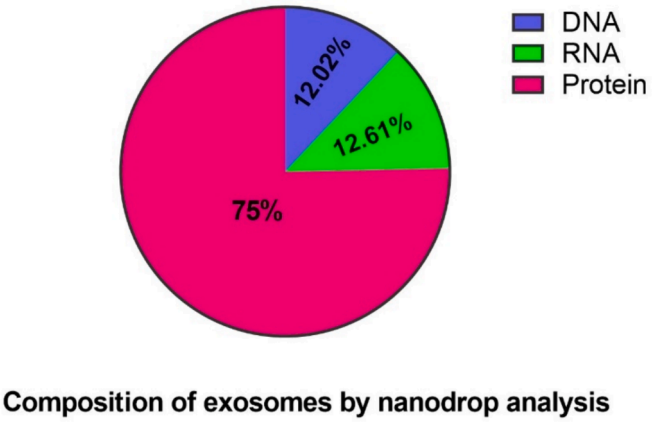

Fig. 4. Nanodrop analysis of human plasma derived exosomes.

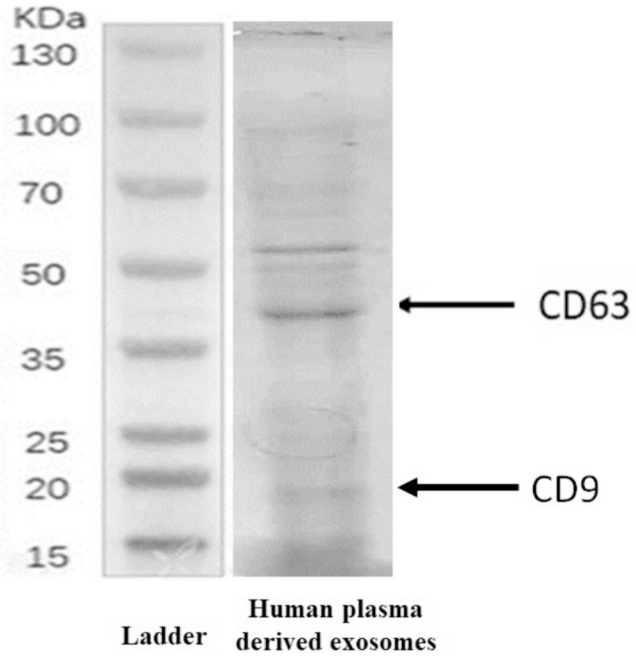

Fig. 5. Western blot of human plasma derived exosomes. The CD9 and CD63 marker of human plasma derived exosomes was identified in exosomes isolated from ultracentrifugation method followed by dialysis.

favorable safety profile. MTT assay of MTX loaded exosomes by freeze thaw and sonication method showed significant improvement in cell viability as compared to pure MTX as shown in graph of Fig. 8.

4. Discussion

The purpose of current research study was to isolate exosomes from human plasma and investigate active and passive drug loading strategies to encapsulate hydrophilic drug MTX. The first objective was achieved by using ultracentrifugation and dialysis technique to isolate exosomes from human plasma. The ultracentrifugation method followed by dialysis has established itself as a fundamental technique for isolating exosomes from human plasma. In a study, it was emphasized its significance in extracting exosomes from cell culture supernatants and biological fluids, citing its popularity due to its capacity to generate reasonably pure exosome fractions (Théry et al., 2006). However, Lobb et al. (2015) acknowledged the challenges intrinsic to ultracentrifugation, notably the balance between purification and yield. Although ultracentrifugation can yield relatively pure exosomes, it suffers from time-intensive processes and limited scalability, particularly concerning larger sample volumes (Lobb et al., 2015). Despite its limitations, the ultracentrifugation method retains its significance for exosome isolation from human plasma, especially when prioritizing high purity, as highlighted in the context of drug delivery research (Kalluri and LeBleu, 2016).

MTX loading was achieved by two approaches active and passive. Sonication was employed as active loading approach. This method utilizes the ultrasonic waves to disrupt biological membranes and create pores in the lipid bilayer of exosomes for the entrapment of hydrophilic drugs and genetic material that have limitations of simple diffusion (Kim et al., 2016). This process created transient disruptions in the exosomal lipid bilayer, facilitating the entrapment of hydrophilic drugs within the vesicles. Freeze-thaw method was utilized for efficient drug loading as passive approach, particularly for small molecules with water-soluble properties and genetic materials (Zhang et al., 2017). The method's simplicity and compatibility with a wide range of drugs make it an appealing option for drug encapsulation. The freeze–thaw method minimizes the risk of altering exosome integrity and cargo. Additionally, the absence of specialized equipment or reagents simplifies its implementation.

Particle size, PDI and zeta potential of plasma derived exosomes during drug loading may varied due to different approaches applied to facilitate drug inward movement. Particle size of isolated exosomes was smaller than MTX loaded exosomes as shown in Table 1. The size increase after drug loading into exosomes was also reported in other studies (Kumar et al., 2022). The increase in particle size is associated with drug loading owing to surface adsorption or encapsulation of drug

Table 3
Encapsulation Efficiency (EE) and Loading capacity (LC).

Absorbance	Concentration (µg/ml)	Encapsulation efficiency	EE ± SD	Loading capacity	LC ± SD
Encapsulation Efficiency (EE) and Loading capacity (LC) of exosomes loaded by Sonication					
0.265	4.663	53.368	55.515 ± 1.907	17.13	16.33 ± 0.709
0.248	4.298	57.018		15.77	
0.252	4.384	56.159		16.10	
Encapsulation Efficiency (EE) and Loading capacity (LC) of exosomes loaded by Freeze Thaw method					
0.207	3.417	65.826	64.538 ± 1.54	19.88	19.27 ± 1.73
0.221	3.718	62.821		17.31	
0.211	3.503	64.967		20.62	

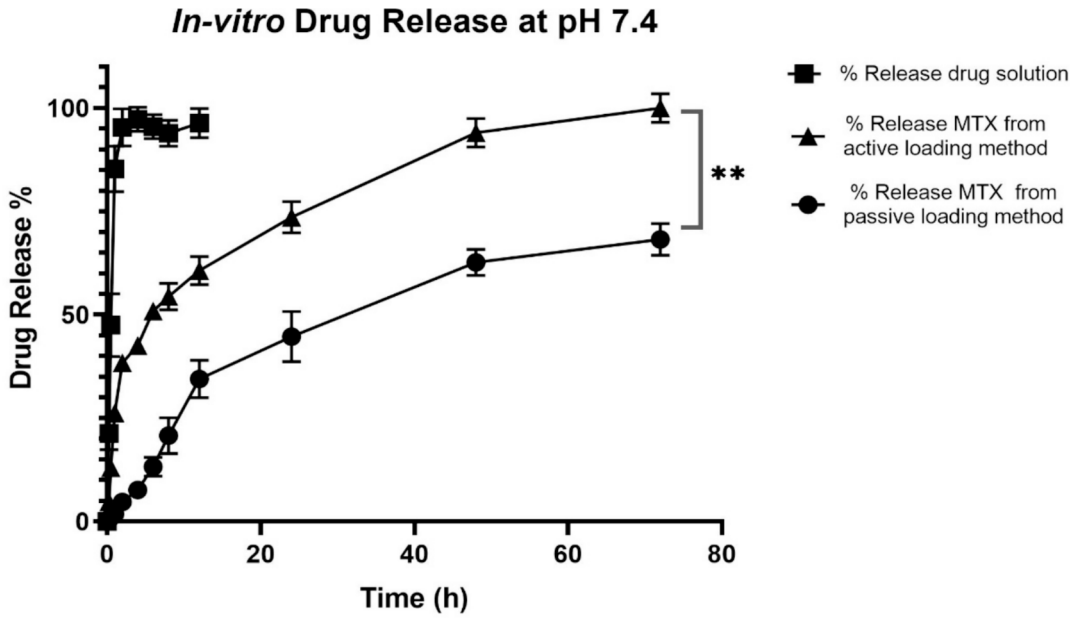

Fig. 6. *In-vitro* drug release of MTX from MTX solution, MTX loaded exosomes by freeze thaw method and MTX loaded exosomes by sonication methods at pH 7.4 **p ≤ 0.005.

Table 4
Kinetic model of drug release.

	Zero order		First order		Higuchi		Korsmeyer-peppas		Hixon crowell	
	R^2	k_1	R^2	k_2	R^2	k_4	R^2	k_5	R^2	k_3
MTX solution	0.543	13.43	0.9914	0.975	0.5461	7.713	0.7439	−1.88	0.2305	0.028
MTX Release from exosomes by sonication	0.7313	1.242	0.871	0.033	0.9221	11.95	0.6926	−0.195	0.9576	0.0703
MTX Release from exosomes by freeze thaw	0.888	1.02	0.9596	0.007	0.9694	9.201	0.7097	−0.025	0.9365	0.0221

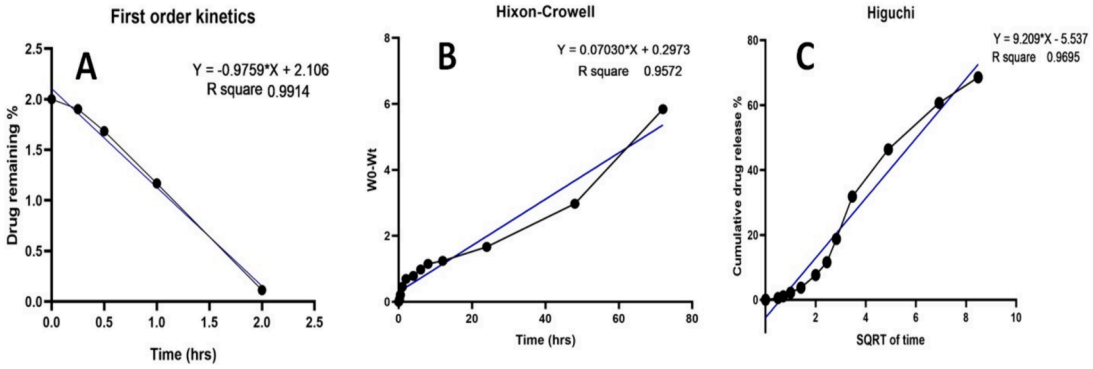

Fig. 7. Graphical representation of kinetic model of MTX release from various formulations A) Kinetic release model for MTX solution B) Kinetic release model for MTX loaded exosomes by sonication method and C) Kinetic release model for MTX loaded exosomes by freeze thaw method.

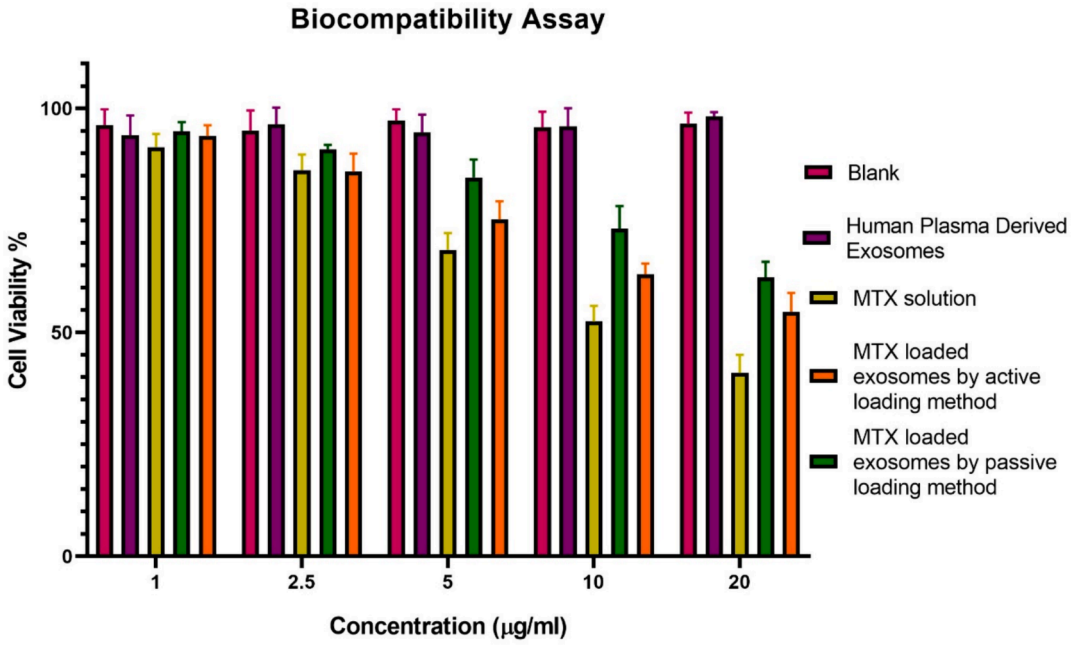

Fig. 8. Biocompatibility studies by MTT assay on HEK cell lines.

molecules into exosomes. Drug loading techniques also caused slight changes in exosomes to facilitate drug diffusion within exosomes. Meanwhile, particle size by active method was slightly larger than that by passive method. This size increase is also associated with exosomal membrane disruption due to sonication (Kumar et al., 2023). Polydispersity index PDI, suggested size distribution within narrow range for blank and drug loaded plasma derived exosomes as shown in Table 1. The size distribution displayed a Gaussian-like curve centered around average size indicating a homogeneous particle size distribution. The negative zeta potential signifies a net negative charge on the particles in the sample. This negative charge indicates good stability and repulsion between particles, which can contribute to effective dispersion and prevent agglomeration (He et al., 2023). The particle size analysis showed predominantly nanoscale particles with a narrow size distribution. Furthermore, the negative zeta potential suggests favorable stability of the particles. These findings are crucial for understanding the physical characteristics and behavior of the particles, with potential implications in for drug delivery purposes. Results of size distribution revealed that exosomes loaded by both methods were in nano range with negative zeta potential and uniform size distribution as PDI above 0.5 indicates heterogeneity in particle size may be due to agglomeration (Zeeshan et al., 2021).

Particle size, PDI and zeta potential of drug loaded exosomes was further optimized by hit and trial method. Optimization of drug loaded exosomes further help in understanding the reason of changes in particle size with various loading approaches (Kimiz-Gebologlu and Oncel, 2022). For sonication method, sonication frequency was used as a variable from 10 % to 50 % amplitude with constant sonication time. Results showed that with increase in sonication frequency average particle size was reduced, however PDI was increased from 0.384 to 0.663 and zeta potential was reduced to from –23 to −12 mV. Probe sonicator is an effective tool to reduce particle size and facilitate drug loading but sonication waves damage the biological membranes. So, minimum optimized amplitude of 20 % seems more appropriate for drug loading as illustrated in Fig. 1. As higher amplitude may result in more exosomal membrane damage. These findings were supported by a study that reported comparison of two methods for drug loading into exosomes derived from glioblastoma cells. His findings suggested that there was significant increase in particle size of exosomes loaded with paclitaxel by sonication method as compared to that of incubation method. In this study, agglomeration of exosomes were observed during TEM analysis (Salarpour et al., 2019). In another study, paclitaxel was incorporated into exosomes derived from macrophages cells and two drug loading approaches were compared. Results of this study also proposed that paclitaxel molecules exhibited enhanced diffusion across the tightly and intricately structured lipid bilayer during exosome membrane reorganization induced by sonication. However, due to exosomal membrane disorganization exosomes resulted in agglomeration (Kim et al., 2016). Another study reported that sonication might boost drug loading and exosome accumulation in target cells. However, this reorganization and disruption of exosome membrane uniformity could potentially impact on their recognition by the immune system (Haney et al., 2015). Thus, to achieve the full potential of sonication method there is need to develop appropriate techniques to recover exosomal membrane integrity after sonication process.

For passive loading, drug, exosomes and $CaCl_2$ concentration were investigated as a critical variable that can affect particle size, PDI, and zeta potential of exosomes during drug loading. Exosome's concentration is a key attribute and optimization of required concentration of exosomes is very important. Higher exosome concentrations may lead to increased drug loading capacity due to the greater number of available surface molecules for drug attachment. However, excessively high exosome concentrations might also lead to potential aggregation or reduced stability of the exosomes. When concentration of exosomes varied from 50 µL to 200 µL, average particle size was reduced from 400 nm to 162 nm. Further increase in exosomes concentration from 300 to 400 µL resulted in increased average particle size 275 nm to 363 nm respectively. These results suggest that too low or too high concentration of exosomes results in increased particle size along with variation in size distribution and reduced zeta potential shown in graphs of Fig. 2a, 2b and 2c. Similar findings related to drug and exosomes concentrations was also reported in another study that concluded that sirolimus loaded exosomes size greatly depends upon appropriate ratio of drug and exosomes (Mehryab et al., 2023). $CaCl_2$ is commonly used to induce the fusion of drug-loaded molecules with exosomes, facilitating the encapsulation of drugs. The concentration of calcium chloride influences the fusion efficiency and overall drug loading capacity of exosomes. As the concentration of $CaCl_2$ increases from 100 to 500 µL, particle size

increases from 234 nm to 875 nm along with PDI increased to 1 that indicates heterogenous size of particles. Zeta potential also reduced by increasing calcium chloride concentration to −6.45 mV. This reduce zeta potential may be due to positive charge of increase calcium ions that results in more particle aggregation. Results were presented in graph of Fig. 2d, 2e, and 2f. Drug concentration also affects the average particle size, PDI and zeta potential. As the concentration of MTX increases from 1.1 μM to 22 μM, the average size also increased from 215 nm to 482 nm, PDI from 0.611 to 1 and zeta potential reduced from −20 to −9.76 mV as shown in Fig. 2g, 2 h and 2i. These findings suggest that there is an appropriate drug to exosomes and $CaCl_2$ ratio is required to achieve optimized size, PDI and zeta potential. When drug and exosomes and pore inducing agents are in optimized ratio, drug loading is facilitated that resulted in uniform size exosomes with more negative charge indicates stability of formulation. However, when one variable is changed while keeping other constant then in too low or too high concentration of one factor leads to non-uniform sized exosomes that resulted in increased average size, higher PDI and reduced zeta potential indicates aggregation of exosomes when ratio of all variables was not appropriate.

Quantifying the protein content of exosomes is vital for understanding their composition and potential biological functions. It provides valuable insights into the cargo carried by exosomes, including proteins involved in cell signaling, RNA-binding proteins, and enzymes. Additionally, protein quantification enables comparisons between exosome samples, evaluation of purification methods, and assessment of exosome isolation protocols. Protein quantification of exosomes was performed by Bradford's assay, nanodrop analysis, SDS-PAGE and western blot. Western blot confirmed the specific surface protein markers present on exosomes isolated from human plasma. The outcome of protein analysis effectively validates the existence of CD63 and CD9 within the isolated exosome sample. The proficient identification of these markers stands as robust proof of successful exosome isolation, underscoring the crucial role of these vesicles in facilitating intercellular communication and their potential utility as biomarkers (Cumba Garcia et al., 2019).

The drug encapsulation efficiency is also a critical parameter that reflects the effectiveness of the loading methods. In this case, achieving a drug encapsulation efficiency of 64 % suggests a relatively efficient encapsulation of the drug within the exosomes. Optimizing drug loading efficiency is essential to maximize the therapeutic potential of exosomes as drug delivery vehicles. A higher drug loading efficiency indicates that a larger proportion of the drug payload is effectively encapsulated, increasing the likelihood of achieving the desired therapeutic effect. Active and passive approaches did not show any noticeable difference in drug loading capacity indicates both approaches can be utilized for loading hydrophilic drug within exosomes. Similar results was reported in another study, that revealed drug loading by active and passive method was slightly different (Kumar et al., 2023). However, drug loading by freeze thaw method was slightly higher as compared to that by sonication method. This might be explained by the fact that sonication method caused membrane disruption that may lead to relatively lower drug loading.

To evaluate the mechanism of drug release from exosomes *in-vitro* drug release study was performed and release of MTX loaded exosomes was compared with MTX solution. Drug release from MTX solution was immediate and 99 % drug released within 1 h. However, Drug released from exosomes in a sustain manner. Drug release of MTX loaded exosomes by active and passive methods was also compared to evaluate effect of drug loading approaches on *in-vitro* drug release. Results indicated that drug release from exosomes loaded by sonication method was 2 times faster as compared to that by freeze thaw method. These observations substantiate the likelihood of changes induced in the exosomal membrane architecture as a consequence of the sonication method. Applying drug release kinetic models through the utilization of DD solver, the MTX-loaded exosomes prepared via the freeze–thaw method adhered to the Higuchi release model. This suggests that the MTX release mechanism from human plasma-derived exosomes follows Fickian diffusion, whereby drug molecules diffuse as per the concentration gradient within the carrier. The rate of drug release in this scenario is directly proportional to the square root of time, implying a gradual escalation in drug liberation with the progression of time. Conversely, drug release from exosomes subjected to the sonication method was according to the Hixon-Crowell release model. In this context, drug release occurs through a combination of diffusion and erosion mechanisms, suggesting a potential compromise in exosomal membrane integrity due to the effects of sonication-induced disruption.

Influence of loading approaches on biocompatibility of drug loaded exosomes was assessed by biocompatibility studies on HEK cell lines. HEK cell lines represents normal cell lines and these cell lines were used to evaluated cytotoxicity of prepared formulations on various normal cells within human body. MTT assay was utilized to assess biocompatibility. Results of MTT assay indicates minimum cytotoxicity with blank exosomes as compared to pure drug solution. These results support the potential suitability of blank exosomes as carriers or vehicles for therapeutic cargo. MTT assay of MTX loaded exosomes by freeze thaw and sonication method showed significant improvement in cell viability as compared to pure MTX. These results are also supported by Yan et al., 2020, as cytotoxicity of dexamethasone reduced by incorporation into exosomes (Yan et al., 2020). Viability of MTX was higher by exosomes loaded by freeze thaw method as compare to sonication. These findings also suggested that in sonication method, stress results in loss of membrane integrity that causes to release drug earlier and produce more cytotoxicity. However, in freeze thaw method exosomes maintain their membrane integrity and release drug in a sustain manner over 48 h.

Based on results on current research work, it is concluded that exosomes isolated from human plasma can be utilized as an emerging carrier for drug delivery. Drug loading method should be selected according to the therapeutic needs. There was no significant difference in loading capacity by active and passive methods. However, drug release studies and cytotoxicity studies suggested that MTX loaded exosomes by active approach may result in exosomal membrane disruption. Further investigations are required to address the challenges of active drug loading approaches.

5. Conclusion

Within this study, exosomes derived from human plasma was isolated to utilized as a drug delivery carrier by active and passive loading approaches. Characterization results revealed nanosized human plasma derived exosomes with oval shape did not induce cytotoxicity in HEK cell lines. The findings of this study provide valuable insights into the safety and biocompatibility of human plasma derived exosomes, making them a promising option as safe carriers for various drug delivery applications. Our study concludes passive drug loading approaches are more promising owing to their improved attributes in terms of both drug loading capacity and the preservation of exosomal membrane integrity for human plasma-derived exosomes. These significant findings not only advance innovative strategies for leveraging human plasma-derived exosomes in drug delivery but also open up new avenues for therapeutic interventions across various medical conditions.

CRediT authorship contribution statement

Rabia Gul: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Hamid Bashir:** Formal analysis, Validation. **Muhammad Sarfraz:** Formal analysis, Visualization, Writing – review & editing. **Ahson Jabbar Shaikh:** Formal analysis, Investigation, Writing – review & editing. **Yousef A. Bin Jardan:** Formal analysis, Funding acquisition, Writing – review & editing. **Zahid Hussain:** Formal analysis, Visualization, Writing – review & editing. **Muhammad Hassham Hassan Bin Asad:** Data

curation, Investigation, Validation. **Faisal Gulzar:** Formal analysis, Writing – review & editing. **Bo Guan:** Data curation, Validation. **Imran Nazir:** Conceptualization, Resources, Supervision, Validation, Writing – review & editing. **Muhammad Imran Amirzada:** Conceptualization, Formal analysis, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

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