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Original Article

Preparation and Evaluation of Exosomes Loaded with Combined Drugs against Colon Cancer

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ABSTRACT

Background: Nanoscale delivery systems such as milk exosomes, promising carriers for drug delivery, are used to deliver therapeutic drug molecules to the targeted organs. They possess characteristics that include low immunogenicity, specificity, and high bioavailability of cancer drugs. This research study unfolds the influence of the combination of phytoconstituents such as quercetin with an anticancer agent 5-fluoro uracil against the colon cancer cells HCT 116 and epithelial cells NCM 460 *in vitro* both individually and in combination when loaded into bovine exosomes. **Materials and Methods:** exosomes were isolated from a bovine source using the ultracentrifugation method and lyophilized. The selected drugs were loaded into exosomes by incubation, sonication, and freeze-thaw methods and morphologically characterized by SEM and zeta potential studies, FTIR studies, and *in vitro* diffusion studies. The study also highlights the *in vitro* cytotoxicity of the exosomal formulations. **Results:** The particle size of the pure exosomes, Exo 5FU and Exo QCT, was determined by Zeta sizer to be 125.7 nm, 157.8, and 142.4 nm, respectively. Further, the mean particle size of (5-FU+QCT) combination-loaded exosomes was 115.8 nm. The freeze-thaw method shows the highest possibility of drug loading. The flux of the release study is almost constant, with a permeability constant of 0.138. Further, the cytotoxicity study showed that Exo-Quercetin has an IC_{50} value, which is much less than pure Quercetin's IC_{50} value, indicating better activity. **Conclusion:** The present study indicated the feasibility of isolating exosomes from cow milk exploited in drug delivery systems as they remain unaffected by stomach acids. The exosomes were discrete, uniform, and stable in size. Selected drugs Quercetin and 5-FU and Combination were best loaded in exosomes by the freeze-thaw method. FTIR study showed that there is no or very little interaction as almost all the functional groups are the same in the case of pure drugs and the formulation. The cytotoxicity study showed that drugs loaded into exosomes had better IC_{50} values than the pure drugs indicating their role as potential applications in cancer therapy by reduced drug doses and minimum side effects.

Keywords: Exosomes, 5 Fluoro Uracil, Quercetin, Drug loading, MTT assay.

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INTRODUCTION

Exosomes are 80-200 nm diameter nanovesicles that are released by nearly every type of cell and facilitate humoral intercellular exchanges. Exosomes are considered a covenant delivery method for therapeutic nucleic acids and anticancer molecules to cell line cultures and laboratory animals due to their natural origin, high biocompatibility, and ability to modify their surface for targeted delivery.[1] Numerous physiologically significant substances, including lipids, DNA, proteins, mRNA, microRNA, and others, constitute exosomes.[2] Exosomes have the following advantages: (a) reduced immunogenicity;(b) simple entry into cells because of their biocompatible form and natural origin, which enable more effective cargo delivery; (c) a strong membrane with the ability to shield therapeutically important substances from deterioration; (d) the body's capacity for sustained circulation;(e) the potential could artificially alter their membrane in order to facilitate targeted distribution, allowing for tissue-specific or cell-specific distribution.[3] When it comes to milk exosomes, its benefit is that, as compared to culture fluid, milk is a less expensive source of exosomes and safer to use in preparation than blood or other biological fluids. Exosomes can also get through the placental and blood-brain barriers. Because of their capacity to pass the blood-brain barrier, delivery methods to brain tissue, such as those for Parkinson's and Alzheimer's disease, can be developed.[4] Nearly every bodily fluid has been shown to contain exosomes. Many studies use exosomes isolated from cell cultures despite the relatively low yield of exosomes from this source. Furthermore, vesicles from the serum used for cell culture may contaminate the system. The safety of using immortalized cell lines and exosomes produced from cancer in therapeutic applications is another

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issue.[5] Exosomes can be found in both semi-preparative and preparative amounts in milk, which is a special natural source of them. Studies conducted *in vitro* have exhibited the attraction of milk exosomes to epithelial cells, their ability to be absorbed by cells through the process of endocytosis, and, above all, their possibility for oral administration.

Exosomes Framework

Exosomes are carriers of membrane-bound, constituting metabolites, proteins, and nucleic acids that can be found in their cargo, which defines the pedigree of a cell and its corporeal condition. Exosomes are spheroidal when in solution, but during preparation when they are isolated by artificial drying, they seem bi-concave or cup-shaped.[6] The primary cytosolic and membrane-bound proteins included in exosomes are actin, Heat shock proteins (Hsp), integrins, Endosomal Sorting Complex needed for Transport (ESCRT) proteins (Alix, TSG101), members of the tetraspanin family (CD9, CD63, and CD81), and flotillins. However, heat shock proteins all exosomes share proteins including CD63, ESCRT, and cytoskeletal components.[7] Lipid components, including sphingomyelin, cholesterol, and ceramides, are also present in the stiff bilayer membrane of exosomes; these components impact cargo sorting, exosome release, shape, and signaling. Exosomes comprise a range of nucleic acids, including DNA, mRNA, and non-coding RNA species.[8] One of the most prevalent RNA species in exosomes is microRNA (miR). MiRs play a role in hematopoiesis, exocytosis, and angiogenesis, besides other biological activities. Cellular communication was mediated by exosomes. Additional exosomal RNA species that affect biological processes, specifically tumor development, are small nucleolar RNA, ribosomal RNA (rRNA), transfer RNA (tRNA), long non-coding RNA (lncRNA), small nuclear RNA (snRNA), and p-element-induced wimpy testis (piwi)-interacting RNA.[9] Thus, research has looked into their potential as a non-invasive tool for disease diagnosis and prognosis.

Biogenesis of Exosomes

Extracellular Vesicles (EVs) are micro vesicles formed from cells that are primarily made of lipids. They carry biologically active cargo, such as nucleic acids and proteins, to facilitate intercellular communication (Figure 1). EVs are typically divided into three types based on their origin, production, and size into exosomes (30-150 nm), plasma-membrane-budded microvesicles (50-1000 nm), and apoptotic bodies (1000-5000 nm).[10] Numerous cell types have been shown to secrete exosomes, which may be separated from cell culture supernatants and other bodily fluids such as blood, urine, and cerebrospinal fluid. Intraluminal Vesicles (ILVs) are formed within early endosomes through the plasma membrane's inward budding, which initiates the biogenesis of exosomes. The Endosomal Sorting Complex needed for Transport (ESCRT) machinery, lipids (such as ceramide), and tetraspanins8 are responsible for the deposition of proteins and macromolecules into small Intraluminal Vesicles (ILVs) in the endosomal system. After that, late endosomes develop into Multivesicular Bodies (MVBs).[11] MVBs ultimately have two fates: they can either be broken down by lysosomes or released as exosomes after fusion with ILVs and with the plasma membrane.

The machinery of the ESCRT is crucial to the process of biogenesis. Through RNA interference screening, 7 ESCRT proteins were discovered to influence exosome secretion in Hela cells. Exosome synthesis would be reduced by ESCRT-0 and ESCRT-I protein depletion. However, exosome secretion would be increased by ESCRTIII protein knockdown[12,13] Contrary to previous research that depended on exosome separation and measurement, it was reported that each breast cancer cell secretes 60-65 exosomes every hour, whereas non-cancer cells secrete exosomes at a rate that is 2.8 times higher.[14]

Through methodical research, our group has shown that cow's milk is a biocompatible and reasonably priced source for isolating exosomes in large quantities. We showed that milk exosomes could be used as a nanocarrier for tiny lipophilic or hydrophilic compounds. Furthermore, we demonstrated that milk exosomes may be loaded with a combination of chemotherapeutic agents and neutraceuticals to target tumor cells and increase treatment efficacy while lowering drug-related toxicity. To treat colon cancers *in vitro*, we discuss the usefulness of milk exosomes in delivering chemotherapeutic medicines like 5-Fluoro Uracil and phytoconstituents like Quercetin in this paper.

MATERIALS AND METHODS

Materials

Quercetin, 5-Fluoro Uracil were bought from Yarrow Chem Products, Mumbai. Methanol, Sodium Lauryl Sulphate (SLS), Phosphate buffer pH 7.4, Fetal Bovine Serum and dialysis membrane having molecular weight between 12000 and 14000 were obtained from Hi-Media labs. Double distilled water was used in the entire study. Colon cell lines HCT116 and NCM 460 were procured from National Centre for Cell Science, (NCCS) Pune and Incell, San Antonio, TX, USA. The cell lines were grown in Gibco's DMEM medium.

Isolation of Exosomes

In our investigation, Exosomes are separated using the Ultracentrifugation technique[15] from bovine milk using Avanti Jxn-26, a cooling centrifuge (Beckman Coulter) for the isolation.[1,16] The separation of exosomes is carried out in three steps, namely the Separation of fat, where the milk was centrifuged at 5000 RPM at 4°C for 15 min, followed by separation of High-molecular weight protein by centrifugation at 15000 rpm at 4°C for 60 min and finally pellet separation following centrifugation at 15000 rpm in 4°C for 90 min.[17-19] The supernatant was disposed of, and the final exosome pellet was collected. It was later purified

by washing in phosphate-buffered saline and stored at 2°C in a freezer until further use.

Lyophilization of Exosomes

A colloidal dispersion was created by dispersing the exosomes in an adequate amount of distilled water. Subsequently, a 0.2 µ membrane filter was used to filter the dispersion, and the filtrate was lyophilized. The resulting powder was measured for percentage yield and refrigerated at 0°C until needed.

Characterization

The freeze-dried exosomes are morphologically characterized for Scanning Electron Microscopy (SEM), Particle size analysis, and zeta potential to determine the particle size and stability of the formulation.[20]

Particle Size Analysis

The Horiba SZ-100 particle size analyzer (GITAM School of Science, GITAM Deemed to be University) was used to determine the size of exosomes.[21,22] The samples were put into a cuvette before being dispersed. For the purpose of detection, the analysis was run for roughly 60 sec at a 90° scattering angle. Particle size homogeneity was measured using the Polydispersity Index (PI).

Scanning Electron Microscopy

The exosomal formulations were imaged using a scanning electron microscope,[23] Tescan Mira. Briefly, each slide was covered with a concentrated aqueous suspension of samples, which was vacuum-dried. The sample was placed under a 20 nm thick coating of gold in a cathode evaporator. The apparatus ran at an acceleration voltage of 15 kV to record surface topography. An image processing application was used to take and process a microphotograph, from which individual the size of the different formulations was measured, and the surface characteristics were analyzed.[24]

Fourier Transform Infrared Spectroscopy (FTIR)

The FT-IR spectrophotometric approach was employed to look into any potential interactions between the drug and the exosome that had been manufactured.[24-26] FT-IR spectrophotometers employing Bruker Alpha II were used to measure the infrared spectra of pure drugs, pure exosomes, and drug-loaded exosomes. For sample analysis, the Attenuated Total Reflection (ATR) method was applied. The infrared spectrum was captured between 500cm^{-1} and 4000 cm^{-1}.

Analytical Method for the Estimation of 5-FU

A Reported UV spectrophotometric measuring absorbances at 269 nm and 265 nm in distilled water and phosphate-buffered saline (pH 7.4) for 5-Fluoro Uracil and Quercetin, respectively, was used to estimate the drugs. 10 mg of drugs were taken in a 10 mL volumetric flask and dissolved with Methanol and was made up to the volume, forming a concentration of 1000 µg/mL. Working standard concentrations were prepared, i.e., 2, 4, 6, 8, 10 µg/mL for standard curve construction.

Drug Loading in Exosomes

Selected drugs Quercetin and 5-FU were loaded in milk exosomes.[27-30] This is achieved by adopting physical methods for drug loading and their comparison- co-incubation, freeze-thawing, and ultrasonication. Co-incubation of drug loading is achieved by mixing the exosomes and drug solution, followed by incubation at 4°C. Three consecutive cycles of freeze and thaw technique at 0°C and room temperature is another technique employed for drug loading into exosomes. The loading efficiency is also compared using the ultrasonication technique,

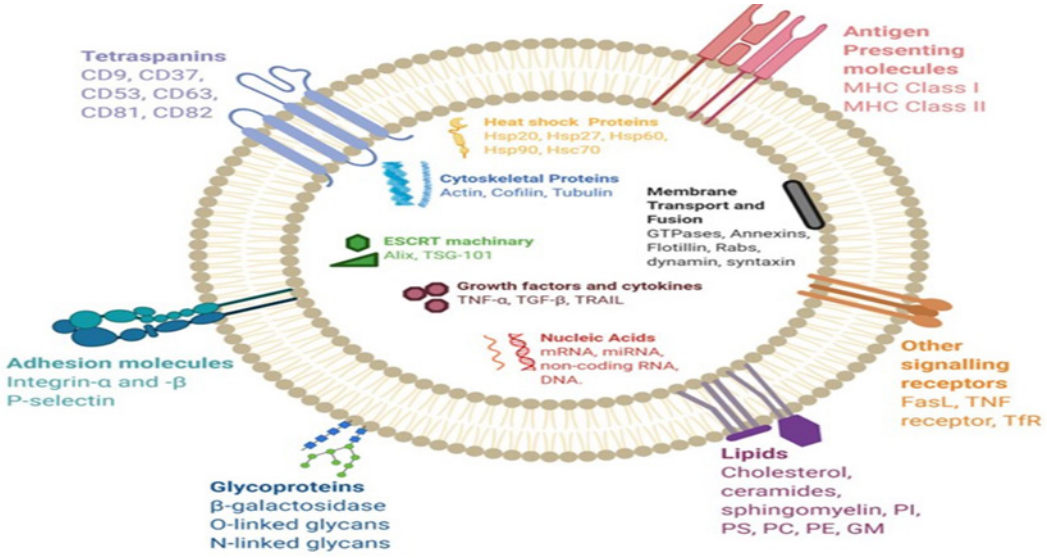

Figure 1: Structure of Exosomes.

which is achieved by ultrasonication of a mixture of drug solution and exosomes for 3 cycles followed by incubation.

Determination of Encapsulation Efficiency

Drug entrapment following loading is calculated by determining the concentration of unentrapped drug in the preparation after incubation.[31-33] After incubation, 1 mL of the supernatant was collected before and after centrifugation at 5500 rpm for 15 min and diluted to 5 mL with methanol. The absorbance was calculated at a wavelength of 265 nm for Quercetin and 269 for 5-FU using UV spectrophotometer.

The encapsulation of the drugs inside the exosomes were determined by the formula:

Encapsulation efficiency={1-(unencapsulated drug/Total drug)}*100

In vitro Diffusion Studies

Diffusion studies were conducted by using the Franz diffusion cell apparatus.[34-36] The diffusion studies were carried out using a dialysis membrane. To about 2 mL of drug loaded exosomes and 2 mL of buffer solution was added and placed in donor compartment. 100 mL of buffer solution was kept in receptor compartment. At regular intervals of time, the aliquots of 2 mL of the sample were withdrawn from the receiver compartment. Fresh medium was replaced in the donor compartment. The drug content was estimated by measuring absorbance using a UV-Spectrophotometer.

Flux of the diffusion study was calculated by the following formula:

$$Flux(J) = \frac{Q}{A * t}$$

Where *J*=Flux of the release,

Q=Amount of drug released per hour,

t=Time (Hr),

A=Area of the diffusion membrane.

$$permeability\ constant, Kp = \frac{Q}{A * t(C0 - Ci)}$$

Where,

$C_{0=}$Concentration of the compound on the donor side,

$C_{i=}$Concentration of the compound on the receptor side.

In vitro Cytotoxicity Studies

The ability of the exosomal formulation to suppress the growth of tumor cells is determined *in vitro* by MTT assay against the cancer cell lines.[16,21,35,37-40] The MTT test is a colorimetric method that calculates cytotoxicity and cell proliferation by detecting the conversion of yellow-colored, water-soluble tetrazolium dye MTT to insoluble formazan crystals. In this study, the effect of exosomal formulations on the viability of colon cancer cell lines HCT 116 cell lines was studied. Briefly, to the cell lines maintained in DMEM medium with glucose and Fetal Bovine Serum (FBS) supplements, the test formulations were added and incubated in the atmosphere of 5% CO_2, 18-20% O_2 at 37°C temperature in the CO_2 incubator and observed for cell viability using the formula

$$\%\ cell\ viability = \frac{Absorbance of treated cells}{absorbance\ of\ untreated\ cells} * 100$$

The IC_{50} value was determined by using a linear regression equation i.e. y=mx+c.

Statistical Analyses

All the experiments were conducted in triplicates and the information was shown as the mean±Standard Deviation (SD). *p* below 0.05 was deemed significant.

RESULTS AND DISCUSSION

Analytical Method

The λ_{max} for 5-FU and Quercetin was observed at 269 nm and 265 nm respectively. A linear relationship was observed between absorbance and concentration in the range of 2 µg/mL to 10 µg/mL. From Calibration data, the regression equation was found to be y=0.092x+0.011 for 5FU with a correlation coefficient of 0.97 and y=0.0543x-0.005 with a correlation coefficient of 0.996 for Quercetin. The results are shown in Table 1 and Figures 2 and 3, respectively.

Isolation of exosomes

Exosomes are separated from milk from cows (Figure 4). Usually, it's a dense mixture suspended in PBS and freeze-dried. The yield of the exosomes was found to be 0.2% w/v.

Characterization

Particle Size

The particle size of the pure exosomes obtained from lyophilization was determined by Zeta sizer as in Figure 5 and found to be 125.7 nm with a Z average value of 112.3 nm and PI value of 0.176. The particle size of the drug-loaded exosomes containing 5 Fluoro Uracil and Quercetin was also determined to be 157.8 nm with

Table 1: Calibration data of 5-FU and Quercetin.

Concentration (µg/mL)	Absorbance (Avg±S.D.) (*n*=3)	
	5-Fluoro Uracil	Quercetin
0	0	0
2	0.096±0.041	0.091±0.008
4	0.185±0.037	0.235±0.017
6	0.287±0.076	0.316±0.038
8	0.472±0.006	0.421±0.030
10	0.646±0.051	0.541±0.053

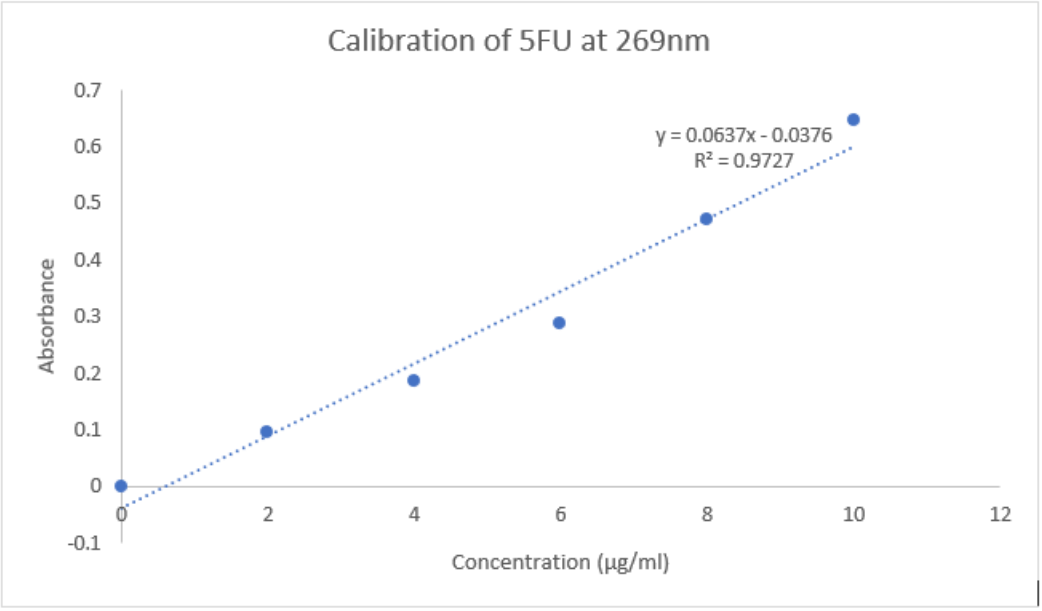

Figure 2: Standard Curve for 5 FU.

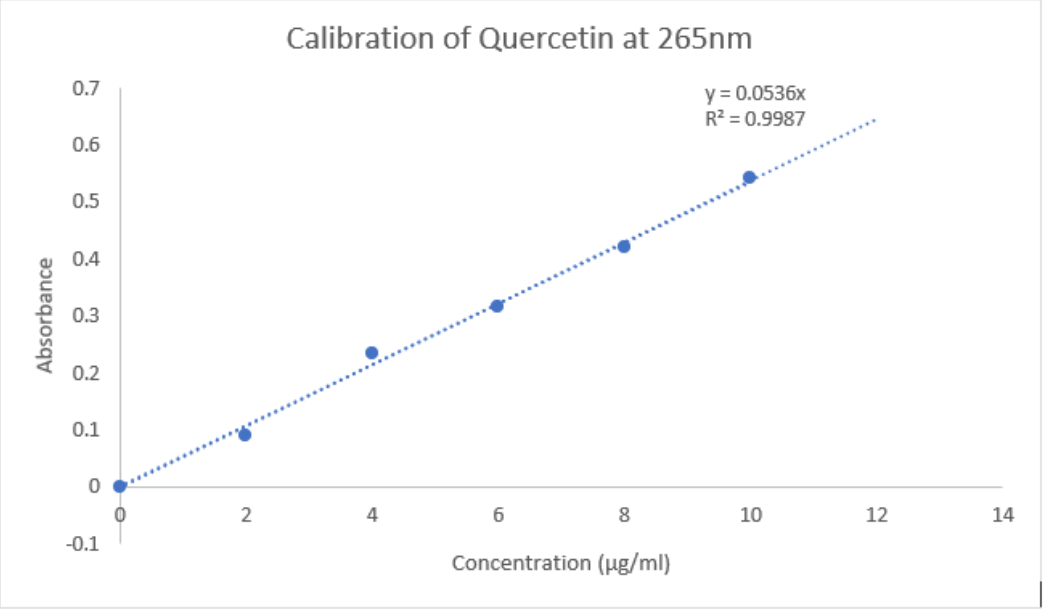

Figure 3: Standard Curve for Quercetin.

a Z average value of 223.2 nm and a PI value of 1.207 for 5FU and 142.4 nm with a Z average value of 124.9 nm and PI value of 0.241 for Quercetin respectively. Further, the mean particle size of (5-FU+QCT) combination loaded exosomes was found to be 115.8 nm with a Z average value of 172.0 nm and PI value of 0.040.

Scanning Electron Microscopy

The morphological characterization of the exosomes shown in Figure 6 was determined by images from Scanning Electron Microscopy, where exosomes of particle size between 135 and 175 nm for pure exosomes increased in size after drug loading.

Fourier Transform Infrared Spectroscopy (FTIR)

The interaction between 5FU and quercetin was evaluated by FTIR spectroscopy. Figure 7 explains the FTIR spectra of various compounds such as pure exosomes, pure drugs quercetin and 5FU and drug-loaded exosomes. The FTIR spectra of pure exosomes is complex and showed absorption peaks in the range of 4000 to 500 cm^{-1} owing to complex macromolecules such as amino acids, lipids, nucleic acids etc., The IR spectrum of exosomes is typified by spectrum components at ~2955 cm^{-1} (CH_3 asymmetric stretching), 2.934 cm^{-1} (CH_2 antisymmetric stretching), and a broad band at, as illustrated in the spectral

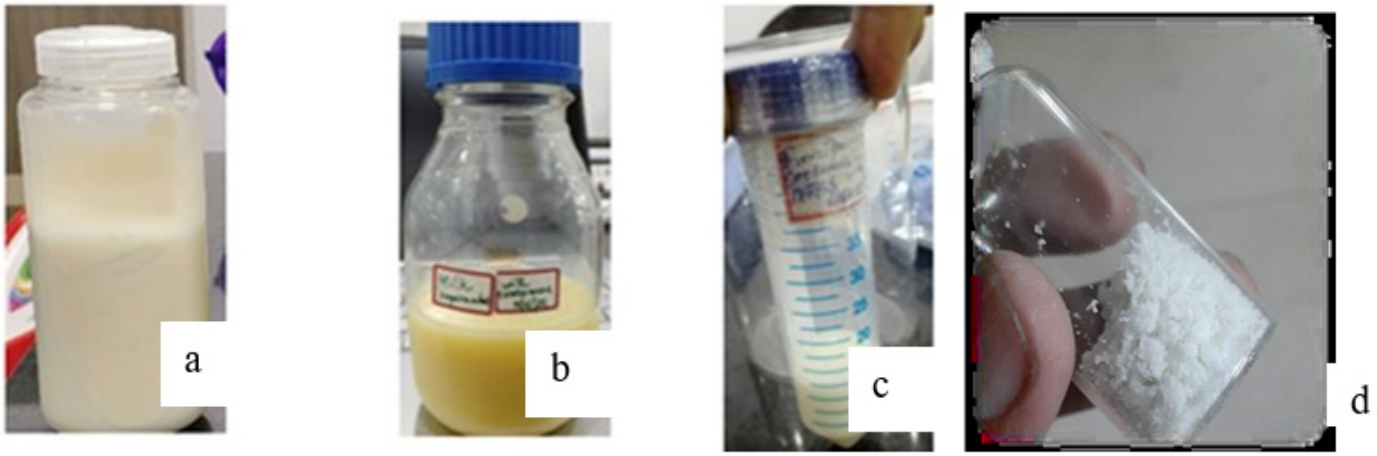

Figure 4: Isolation of exosomes from bovine milk; a. bovine milk; b. final supernatant; c. exosomes in PBS; d. lyophilized exosomes.

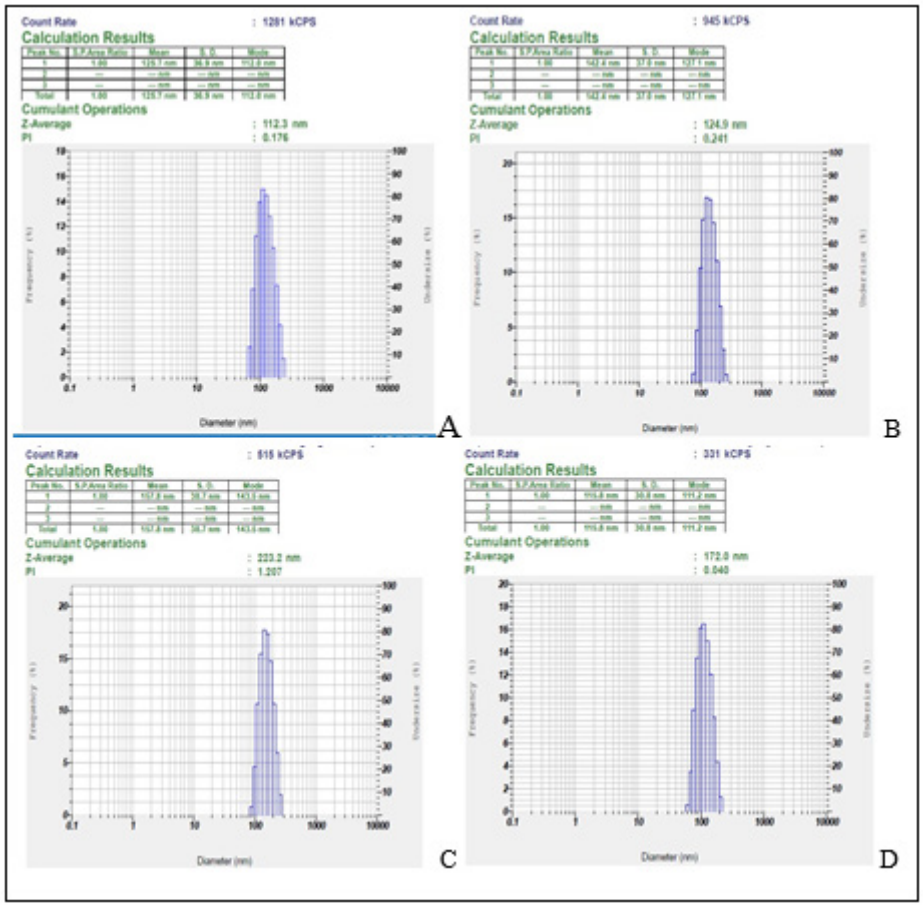

Figure 5: Particle size of exosomes; A-pure exosomes; B-Quercetin (QCT) loaded exosomes; C-5FU loaded exosomes; D- 5FU+QCT combination loaded exosomes.

magnification. Furthermore, the spectra of quercetin showed characteristic bands at 3406 and 3283cm^{-1} Figure 7(a) displays the FTIR spectrum of pure quercetin with distinctive bands.[41] The phenol function exhibited OH bending at 1379 cm^{-1}, whereas OH group stretching was observed at 3406 and 3283 cm^{-1}. At 1666 cm^{-1}, the C=O aryl ketonic stretch absorption was visible. Stretch bands containing C=C aromatic rings were seen at 1610, 1560, and 1510 cm^{-1}. Aromatic hydrocarbons exhibited an in-plane bending band of C-H at 1317 cm^{-1}, as well as out-of-plane bending bands at 933 820, 679, and 600 cm^{-1}. The C-O stretching in the aryl ether ring, the C-O stretching in phenol, and the C-CO-C stretch and bending in ketone were responsible for the bands at 1263, 1200, and 1165 cm^{-1}, respectively and 5FU displays the typical bands, which are allocated to conjugated (at 1655, 1670 cm^{-1}) and non-conjugated (at 1695 cm^{-1}) C=O Stretch, in the range of 1700

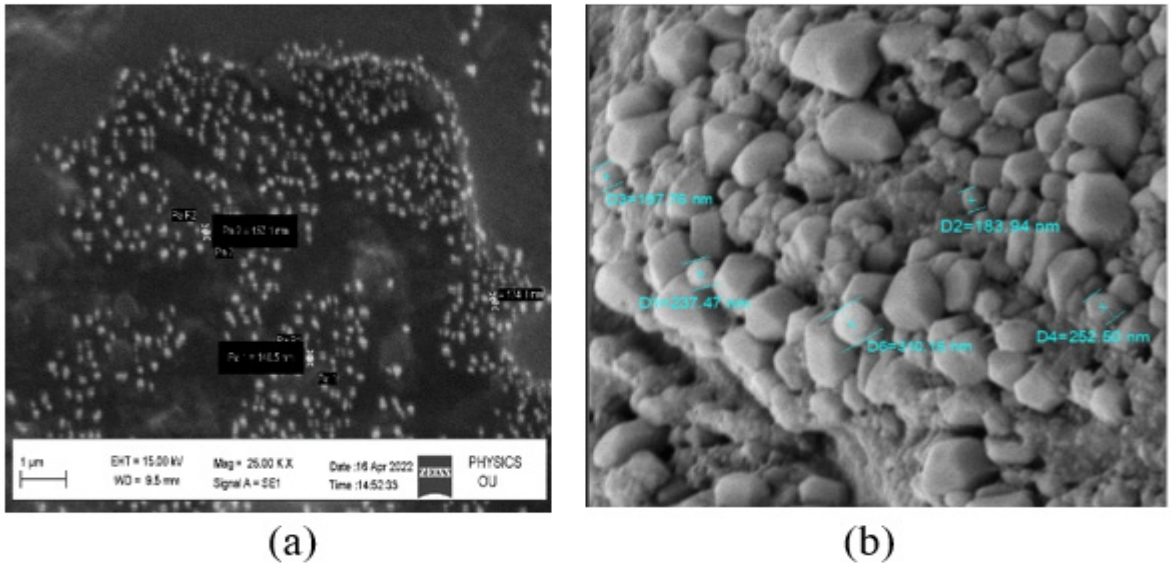

Figure 6: A-SEM Images of Pure Exosome; B-SEM Images of Quercetin Loaded Exosome.

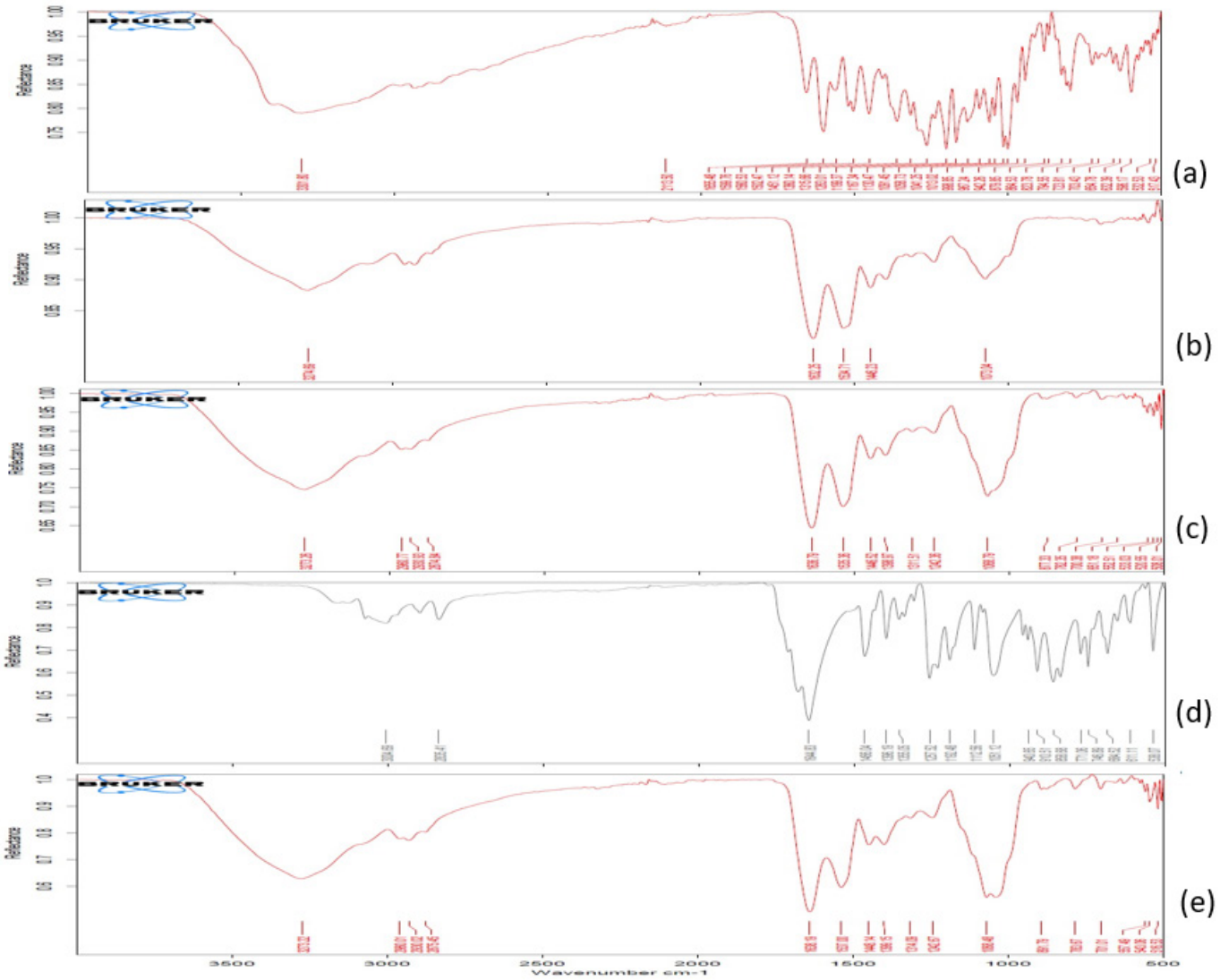

Figure 7: FTIR images of pure drugs and exosomal formulations; a-pure Quercetin(QCT); b-pure exosomes (Exo); c-Exo-QCT; d-pure 5FU; e-Exo (5FU+QCT).

to 1650 cm^{-1}. These suggest that there is absence of interactions in the combination therapy of exosome loaded drugs.

Calculation of Drug Loading Efficiency

The result of entrapment efficiency of the drug-loaded exosomes as in Table 2 and Figure 8 was determined by UV spectroscopy for different approaches practiced. For Quercetin-Exo formulation, the drug loading efficiency was found to be 81.29%, 94.92%, and 66.92% in the Passive Loading method, Freeze-Thaw method, and ultrasonication method, respectively. For 5-FU formulations, the drug loading efficiency was found to be 88.49%, 81.75%, and 89.79% in Passive-Loading, Freeze-Thaw method, and

Table 2: Drug Loading Efficiency of Exosome formulations.

Loading Method	Concentration of Drug (µg/mL)	Drug Loading Efficiency (% w/w)			
		Monotherapy		Combination therapy	
		Exo QCT	Exo 5FU	QCT	5FU
Passive Loading	100	81.29	88.49	-	-
Freeze- Thawing		94.92	81.75	66.4	71.2
Ultrasonication		66.92	89.79	-	-

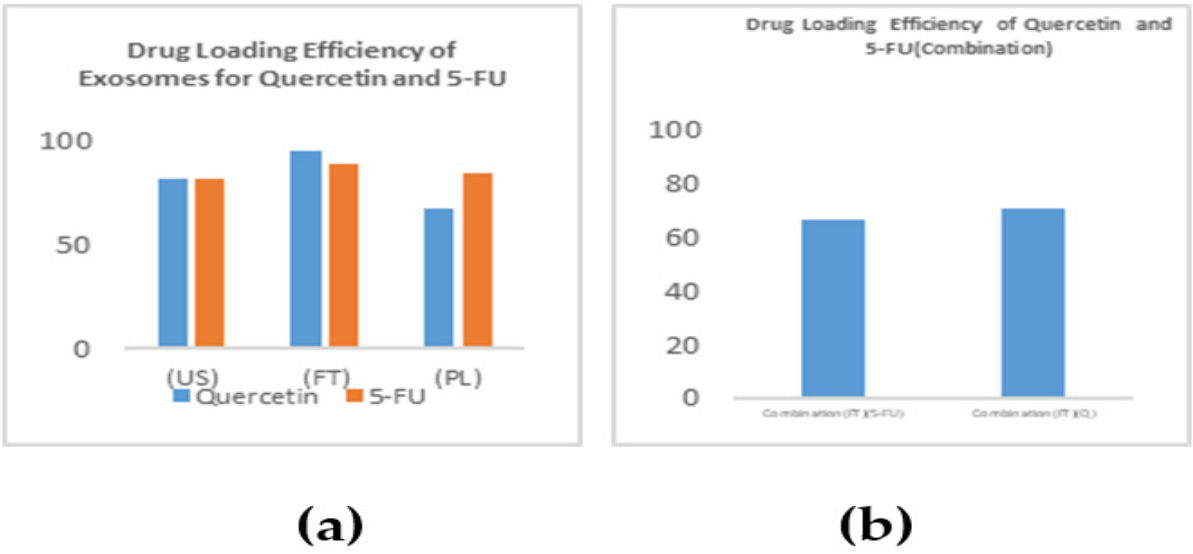

Figure 8: Drug Loading Efficiency of Exosomes for Quercetin and 5-FU; a-loading methods; b-combination.

Table 3: Cumulative % of Drug Release.

Time (Hr)	Percentage Drug release			
	Pure QCT	Exo-QCT-PL	Exo-QCT-FT	Exo-QCT-US
1	5.91	4	7.76	5.91
2	6.17	4.8	10.30	9.24
3	7.76	9.24	11.44	9.61
4	10.35	10.35	13.36	12.56
5	12.19	11.46	22.55	14.41
6	13.38	18.85	39.18	17.74
7	14.78	20.7	46.58	19.22
8	15.89	23.65	51.01	20.70
9	17.74	24.76	58.04	23.29
10	18.85	25.5	59.51	24.02
11	20.33	26.24	64.68	24.38
24	32.532	46.8	72.828	34.75

Ultrasonication method, respectively. In the case of combination drugs, the loading efficiency for Quercetin was 66.4%, and for 5-FU, it was 71.2% by freeze and thaw method.

Diffusion studies

The drug release patterns were gradual and showed an enhancement in concentration after loading into exosomes and shown in Tables 3-5 and Figures 9 and 10. The percentage of drug release after 24 hr for pure Quercetin was found to be 32.53% when compared to its release from exosomes as 72.82%, 34.75%, and 46.8% by freeze and thawing, Ultrasonication, and passive methods of drug loading, respectively. In the case of combination drugs, the release was 53.97% for Quercetin and 42.72% for 5-FU. The flux of the release is almost constant for 24 hr and the permeability constant was found to be 0.138.

Table 4: Drug Release of Combination Drugs.

Time (hr)	Cumulative % of Drug Release	
	Combination (Quercetin)	Combination (5-FU)
1	8.13	10.52
2	11.82	12.38
3	17	14.24
4	19.22	18
5	25.13	19.19
6	28.83	21.67
7	31.05	24.14
8	34.01	26.62
9	36.91	30.34
10	39.18	34.05
24	54	42.72

In vitro Cytotoxicity Studies

The *in vitro* cytotoxicity studies were conducted on colon cancer cell lines HCT 116 by MTT colorimetric assay. Different concentrations of the exosomal formulations were studied for the activity against colon cancer cells. Table 6 and Figure 11 indicate the efficiency of exosomal formulations against the HCT 116 cells.

DISCUSSION

Yield

The yield of exosomes from cow milk was found to be 2 mg/mL.

Particle Size Analysis

Pure Exosomes exhibited discrete particle size distribution with mean particles of 125.7 nm. Quercetin-loaded Exosomes exhibited discrete particle size distribution with mean particles of 142.4 nm. 5-FU loaded Exosomes exhibited discrete particle size distribution with mean particles of 157.8 nm. Combination-loaded Exosomes exhibited discrete particle size distribution with mean particles of 115.8 nm.

Scanning Electron Microscopy

The pictures obtained after SEM showed Loaded Exosomes as fine discrete particles with a size range of around 183 to 197 nm.

FTIR Spectroscopy

FTIR studies have been performed for the exosome pure, pure quercetin, pure 5-FU and Quercetin loaded exosomes and 5-FU+Quercetin loaded Exosomes. The FTIR spectra of pure exosome showed Alcoholic O-H at 3274.69 cm^{-1}, Carbonyl C=O 1632.25 cm^{-1}, Aromatic C=C at 1534.71 cm^{-1} and C-O stretching at 1073.04 cm^{-1}. The FTIR spectra of pure quercetin showed CH_2=CH_2 at 1655 cm^{-1}, Methyl Benzene at 1058.73 cm^{-1}

Table 5: Drug Release Flux Data.

Time (hr)	Flux				
	EXO-QCT(FT)	EXO-QCT(PL)	EXO-QCT(US)	Combination (5-FU)	Combination (QCT)
1	38.62	13.78	45.97	26.18	20.23
2	12.64	4	16.55	4.62	9.19
3	5.65	22.09	1.85	4.62	12.87
4	9.55	5.52	14.67	9.24	5.51
5	45.73	5.52	9.23	3.08	14.71
6	82.78	36.78	16.56	6.16	9.19
7	36.8	9.19	7.35	6.16	5.51
8	22.07	14.71	7.36	6.16	7.35
9	35	5.5	12.84	9.24	7.35
10	7.35	3.68	3.67	9.24	5.51
11	25.6	3.68	1.75	8.79	4.06
24	3.3	8.53	4.30	1.79	2.60

and carbonyl R-CH_3 at 1451.2 cm^{-1}, C=N-OH at 3301.80 cm^{-1} and Straight Chain Alkanes at 538.07 cm^{-1}. The FTIR spectra of quercetin loaded exosome showed CH_2=CH_2 at 1636.79 cm^{-1}, R-CH_3 at 1446.52 cm^{-1}, Aromatic C=C at 1535.36 cm^{-1}, C=O stretch at 1066.79 cm-1, C=N-OH at 3273.26 cm^{-1}. The FTIR spectra of 5-FU+quercetin loaded exosomes showed CH_2=CH_2 at 1638.19 cm^{-1}, R-CH_3 at 1448.14 cm^{-1}, C=C(metal) at 1537 cm^{-1}, $CO(CH_3)_2$ at 891.79 cm^{-1}, C=O stretch at 1066.79 cm^{-1}, Methyl Benzene at 1068.48 cm^{-1}.

Drug Loading Efficiency

Drug loading efficiency following loading is calculated by determining the concentration of unentrapped drugs in the preparation after incubation. After incubation, 1 mL of the supernatant was collected before and after centrifugation at 5500rpm for 15 min and diluted to 5 mL with methanol and the absorbance was calculated at wavelength of 265nm for quercetin and 269 nm 5-FU using UV spectrophotometer. The drug loading was done in 3 different methods (passive loading, freeze thaw and sonication) for weith quercetin. For Passive Loading, drug loading efficiency for Quercetin, 5-FU were found out to be 81.29, 88.49%w/w respectively. For Freeze Thaw, drug loading efficiency for quercetin, 5-FU were found out to be 94.92, 81.75%w/w respectively. For Ultrasonication, drug loading efficiency for quercetin, 5-FU were found out to be 66.92, 89.79%w/w respectively. In totality, Freeze-thaw method was found to be the most efficient method of drug loading, followed by passive loading and finally, sonication.

Drug Diffusion Studies

Drug diffusion studies indicated that the release of the drug from the exosomes was gradual and complete in case of quercetin and

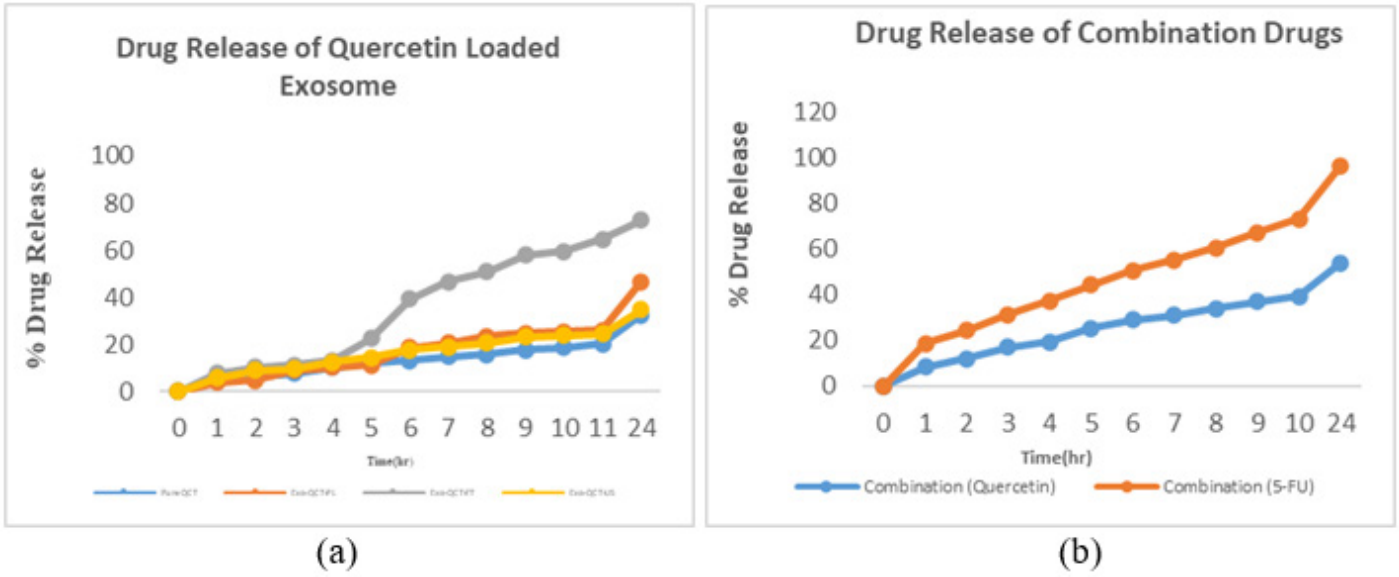

Figure 9: Drug Release Data of Formulations; a-Quercetin Exosomes; b- Combination of Quercetin and 5FU in Exosomes.

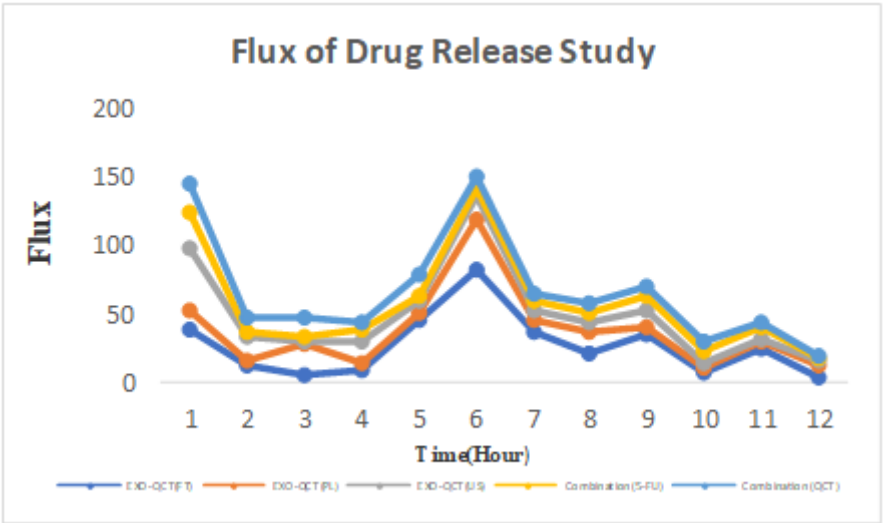

Figure 10: Flux of Drug Release Study.

Table 6: % cell viability of exosomal formulations.

Concentration (µg/mL)	% Cell Viability
	Exo-Quercetin
1.56	93.81
3.125	73.68
6.25	47.38
12.5	26.72
25	7.68
Concentration (µg/mL)	**% Cell Viability**
	Exo-5-Fluoro Uracil
6.25	80.33
12.5	57.27
25	42.08
50	21.55
100	6.49

combination of Quercetin and 5-FU. The studies were conducted over a period of 24 hr and the release was obtained over period of 24 hr. The pure Quercetin showed a release of 32.352%w/w after 24 hr, and the passive-loaded quercetin showed 46.8% w/w after 24 hr. Freeze-thaw loaded quercetin showed 72.82 w/w after 24 hr. Sonication-loaded quercetin showed 34.75 w/w after 24 hr. In the combination of 5-FU and Quercetin, Quercetin showed a drug release of 53.97% in 24 hr, and 5-FU showed a drug release of 42.72% in 24 hr.

In vitro Cytotoxicity Studies

The Observations from the data of the cytotoxicity study against HCT-116 cell lines showed effective cytotoxic properties with the IC_{50} concentration at 6.22 µg/mL after the incubation period of 24 hr, respectively. SDS with 1 mg/mL was used as a standard control for the study.

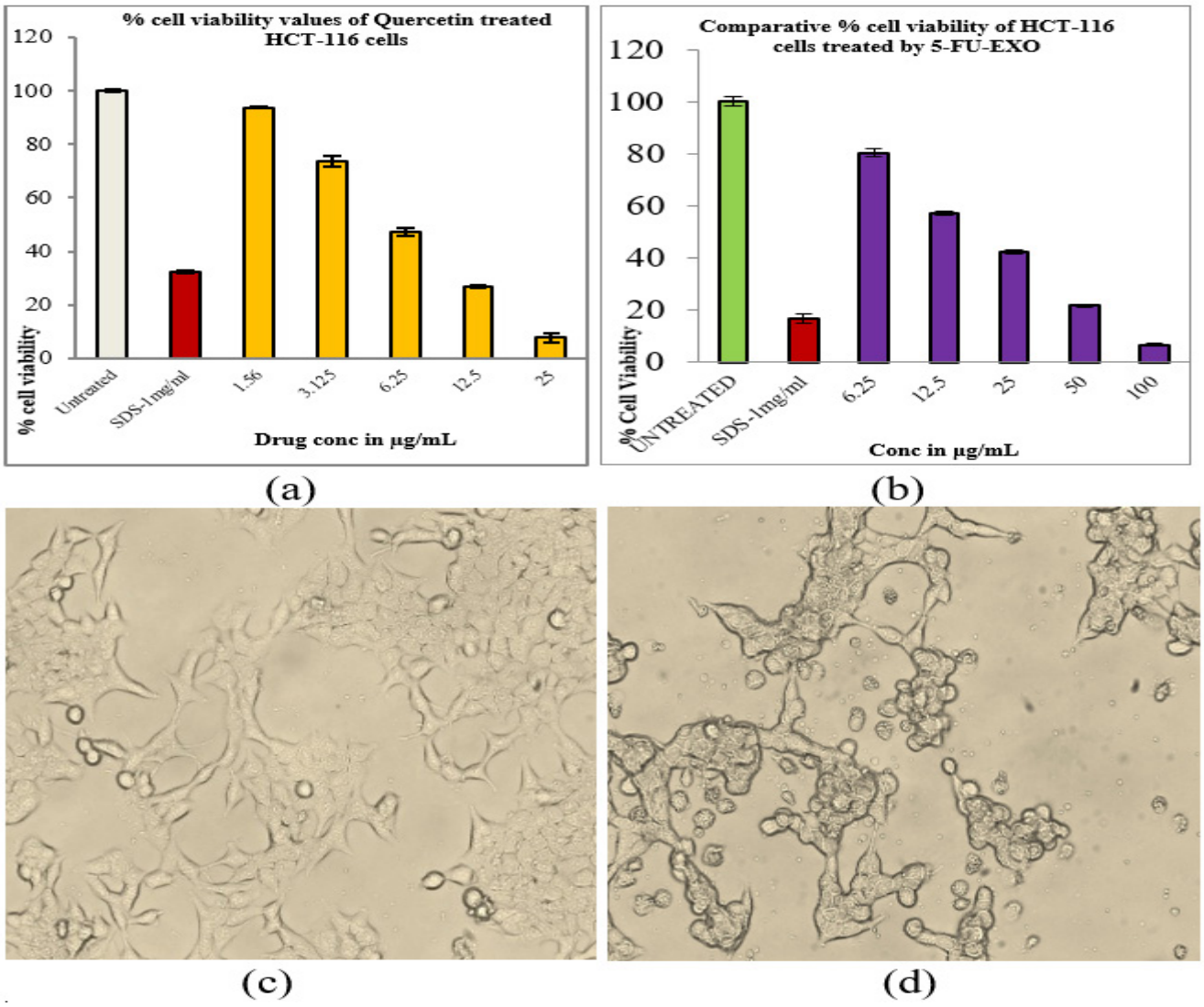

Figure 11: % cell viability values of HCT-116 cell lines treated with different formulations by MTT study. a-quercetin; b-5-Fluoro Uracil; c-HCT 116 cells untreated; d-HCT 116 cells at IC_{50} conc'n of quercetin.

CONCLUSION

The present study indicated the feasibility of isolating exosomes from cow milk exploited in drug delivery systems as they remain unaffected by stomach acids. The yield was found to be 2 mg/mL. The exosomes were discrete, uniform and stable with a size range of 110 to 142 nm size. Selected drugs Quercetin and 5-FU and Combination were loaded in exosomes to determine the combinatorial effect by incubation, sonication, and freeze-thaw methods. Among the three, the freeze-thaw method showed promising drug loading of above 90% and also the highest drug release, which is 72.82%. FTIR study showed that there is no interaction or very little interaction as almost all the functional groups are the same in the case of pure drug and the formulation. The *in vitro* drug diffusion study showed gradual and complete for Quercetin and Combination drugs. These results indicated the applicability of exosomes for the loading of Quercetin and Combination drugs. The flux of the release study is almost constant, and the permeability constant was found to be 0.138. The cytotoxicity study showed that Exo-Quercetin has an IC_{50} value of 6.22 µg/mL, which is much less than pure Quercetin IC_{50} value in HCT-116, i.e. 17.97 µg/mL. Furthermore, the combination therapy of drugs loaded at IC_{50} concentrations showed an enhanced activity with cell viability of 7.09, indicating a synergistic effect of quercetin and 5 fluoro uracil.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

SEM: Scanning Electron Microscopy; **FTIR:** Fourier Transform Infrared Spectroscopy; **SD:** Standard Deviation; **5FU:** 5-Fluoro Uracil; **Exo:** Exosomes; **HCT:** Human colorectal carcinoma; **NCM:** Normal Human Colon Mucosal Epithelial Cell Line; **DMEM:** Dulbecco's Modified Eagle Medium.

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