Formulation and Assessment of Liposomes Entrapped Natural Substance for Mammary Cancer Treatment

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Abstract

Breast/mammary cancer is the most common cancer in women due to abandoned breast tissue growth. Many therapies and treatments are available for breast cancer treatment. However, each therapy or treatment has its side effect. The natural compound has uncovered a significant influence on the development of contemporary therapy with reduced side effects. But it lags behind in the race due to lesser bioavailability and indecorous delivered dosage form. In this research efforts made to develop a novel targeted dose delivery system for breast cancer. The breast cancer cell has shown higher expression of HIF-1α, TOP-II α, Proteine Kinase C, and MMP-2 receptors. By reviewing the literature followed by molecular modeling with these receptors, seven natural compounds were identified which have significant effect on above receptors: sesamin, aloe-emodin, gallic acid, catechin, Butein, 6-gingerol, and curcumin, respectively. Hence Liposomes were prepared with different formulations to entrap all the above active compounds. Then formulated liposomes were characterized, and their stabilities were also evaluated for 60 days at room temperature and 2-8 °C. Among all formulations, liposomes with GCDCA (1.8 mM), lecithin (62.12 mM), and cholesterol (25.88 mM) have proven to be the most stable, robust, and effective formulation using breast cancer MCF-7 cell line.

1. Introduction

Breast cancer is the most common invasive cancer in women and the second leading cause of cancer death, with 2.3 million women diagnosed with breast cancer and 6,85,000 deaths globally. From this, around 1.39 million cases were reported in India [1]. Many effective treatments are available for breast cancer [2,3], but each therapy has its side effect [4]. General chemotherapeutics agents are now developing drug resistance in cancer patients [5].

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The natural compound has shown promising effects for cancer treatment [6], but it strides behind due to lesser bioavailability of natural compound and indecorous delivered dosage form. The current research work made efforts to provide multi-targeted, nano-chemo prevention treatment for breast cancer by making liposomes from multiple natural compounds.

In cancer, multiple receptors may exploit unconscientiously for hyper or hypo expression of their role due to endogenous or exogenous factors; from earlier research in breast cancer unconscientiously exploiting receptors identified as HIF-1α [7], TOP-II α [8], Proteine Kinase C [9], and MMP-2 [10].

Nature is a treasure of many unsung marvelous remedies; to date, many times, we got marvelous medicine from nature for life intimidating diseases. We have dug out some of the natural compounds with exaggerated drift from this huge treasure over the receptors mentioned above. We have selected sesamin, aloe-emodin, gallic acid, catechin, 6-gingerol, and curcumin, which have shown exaggerated effects on breast cancer tissue individually, but one combined formulation with all the below components is not available.

2. Objective

From the literature, all of these compounds are proven effective against breast cancer; an example study conducted by Siao shows that seasamin may cause apoptosis and cell cycle arrest in human breast cancer MCF-7 cells [11]. Huang and his group suppressed breast cancer cell proliferation through ERα inhibition [12]. Gallic acid is also proven for its cytotoxic activity against MCF-7 human breast cancer cells [13]. A study by Xiang demonstrated the suppressive effects of tea catechins on breast cancer [14]. As per work done by Wang, Butein inhibits testosterone-induced proliferation in breast cancer cells [15]. Research by Lee has exhibited that 6-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells [16]; curcumin is also the best anti-cancer natural drug against many types of cancers [17].

From the above literature survey, it is clear that some of the compounds might have been studied for their effect on breast cancer in an individual or formulated form. However, collectively all compounds together were never tested and tried for any of the cancer studies. Hence in this study, to increase the bioavailability and get the cumulative pharmacological effect of all the selected natural compounds, Liposome with entrapped natural substances were formulated and evaluated for their action against mammary cancer.

3. Materials and Methodology

Active compound seasamin, aloe-emodin, gallic acid, catechin, Butein, 6-gingerol, and curcumin were extracted from crude and confirmed its purity, sodium chloride, potassium chloride, disodium hydrogen phosphate, monobasic potassium phosphate, and chloroform were purchased from SDFCL, Spectroguard, soya-lecithin, sodium

glycohenodeoxycholate procure from SRL, MCF-7 cell line was purchased from NCCS, Pune and RPMI_1640 medium were procured from Thermo fisher scientific.

High-performance liquid chromatography was used of Shimandzu LC2010 CHT make, Malvern pan analytical zeta sizer was used for particle size measurement and zeta potential measurement, Hitachi Transmission Electron Microscope H-9500 for TEM images, Remi ultracentrifuge was also used, and rotary evaporator used was a generic model.

3.1. *Method*

3.1.1. *Formula optimization*

To optimize the liposome preparation formula, 5 formulations were made with different compositions, as shown below table 1, and the final formula was selected based on characterization and stability data.

Table 1. Formula optimization trials for Liposomes preparation.

Formulation number	Total drug content (mM)	GCDCA content (mM)	Lecithin content (mM)	Cholesterol content (mM)
1	7.9	1.8	62.12	25.88
2	7.9	1.35	62.12	25.88
3	7.9	2.25	62.12	25.88
4	7.9	1.8	55.91	32.09
5	7.9	1.8	68.33	19.67

3.1.2. *Method of preparation for Liposomes (optimized formula)*

Preparation of phosphate buffer saline (pH 7.4): The following components were sequentially added in sufficient quantity to obtain desired pH and concentration of each salt, i.e., 800 mL of distilled water, 8 g of NaCl, 200 mg of KCl, 1.44 g of Na_2HPO_4, 245 mg of KH_2PO_4 then adjusted solution to desired pH (7.4), finally added distilled water up to a volume of 1 L. The solution was stored at 2°-8°C and used within a week of preparation.

Preparation of hydration solution: Sodiumglycochenodeoxycholte salt (GCDCA) was dissolved in PBS buffer pH 7.4 to the obtained concentration of 1.8 mM of salt (GCDCA-PBS Buffer). Then the equivalent amount of drug was dissolved into GCDCA-PBS Buffer to obtain a solution having sesamin 15 μM, gallic acid 210 μM, catechin 440 μM, Butein 15 μM, and gingerol 30 μM. The solution is stored in 2-8°C and used within a week of preparation

Preparation of phospholipid Bilayer thin film: Phospholipid bilayer thin film was formed through the thin-film dispersion method. Concisely Soy Lecithin (LC), cholesterol (4:1), curcumin (CUR-3.24 μmoles), and emodin (EMO-4.35 μmoles) were dissolved in a 100 mL mixture of chloroform and ethanol (ratio by volume; 2:1 v/v).

This solution is subjected to evaporation under reduced pressure in a 250 mL round bottom flask with glass beads to obtain a thin film of CUR-EMO-LC. Then the film was then dried in a vacuum oven for at least 12-18 h to remove traces of organic solvent.

Preparation of Liposomes: After that, the phospholipid bilayer thin film was hydrated with a hydration solution. The formed dispersion was then left to settle for about 3 h to facilitate maximum swelling of the film to obtain vesicular suspension of lipids and then sonicate it for 15 min. The liposomal suspension was centrifuged at 12 000 rpm for 5 min to separate unloaded drugs, and the supernatant was collected, which contained drug-loaded liposomes. The empty Liposomes were prepared in the same way, just without an active component.

3.1.3. *Study on physicochemical properties*

Drug loading and EE%: The content of active component encapsulated in Liposomes was determined by HPLC using UV-Vis spectrophotometer detector at 430 nm (for Aloe Emodin and Curcumin) and 280 nm for (sesamin, gallic acid, catechin, Butein, and 6-gingerol).

A C18 reserved phase analytical column (3.5 μm, 4.6 mm×150 mm) was applied in HPLC analyses. The mobile phase-A consisted of methanol:water: orthophosphoric acid (35:65:1 %v/v), and mobile phase-B consisted of methanol:water: orthophosphoric acid (65:35:1 %v/v). The mobile phase was pumped at the rate of 1.0 mL/min through gradient flow as shown in Table 2. The gradient implemented is as follows.

Table 2. Mobile phase gradient for HPLC analysis of liposomes.

Time (Min)	0	8	12	13	15
% Mobile Phase-A	80	80	40	80	80
% Mobile Phase-B	20	20	60	20	20

The column temperature was set at 35 °C. The prepared liposomal solution was diluted to a certain concentration with anhydrous ethanol before determination. The drug loading (DL%) and entrapment efficiency (EE%) was calculated by the following equations [18].

$$DL\% = \frac{W_{active}}{W_{liposome}} \times 100\,\%$$

$$EE\% = \frac{W_{active}}{W_{active'}} \times 100\,\%$$

Where, W_{active}, $W_{active'}$ and $W_{liposome}$ are the weight of the drug in liposomes, the weight of feeding drug and the total weight of feeding liposome, respectively.

In vitro release of active component: The release behaviors of main components from liposomal systems were studied with physiological saline containing 1 % tween 80 (w/v) as a release medium using the dialysis method. Under the premise of sink condition, an appropriate volume of Liposomes and active component solution (dissolved in ethanol) as control, with an equal content of active components, were placed into a pre-swollen

dialysis membrane bag. The dialysis bag was tied and placed into an Erlenmeyer flask containing 50 mL of release medium. The temperature and stirring speed were set at 37 °C and 120 rpm separately.

At each pre-set time point, 1 mL of the medium was withdrawn from the flask, and 1 mL of fresh medium was added. The samples taken out were filtered through a 0.22 µm filter membrane, and the filter liquor was measured by high-performance liquid chromatography (HPLC) at 280 nm and 430 nm. The same chromatographic condition shown in % drug loading and % entrapment efficacy was used to determine active drug release. The cumulative release percentage of the drug was calculated. Every release experiment was repeated three times.

Particle size and zeta potential: Liposomes' particle size and zeta potential were measured using Malvern Pan Analytical Zeta-Sizer at 25 °C [19].

Micro-morphology: The morphology of liposomes was observed by transmission electron microscopy (TEM). A drop of the formulation was placed on a copper grid and stained with a phosphotungstic acid solution (2 %, w/v) for 15 s. Then, the excess solution was absorbed, and the sample was dried in air and examined under TEM [20].

Cell cytotoxicity assay: MCF-7 cells were adopted to evaluate the in vitro cytotoxicity of active components with the MTT method. Cells were cultured in the RPMI-1640 medium supplemented with 10 % PBS. All the cells were grown at 37 °C in a 5% CO_2 atmosphere in a humidified incubator and subcultured once every two days.

MCF-7 cell lines were seeded in 96-well culture plates at the density of 5×10^3 per well. After 24 h of incubation, cells were handled with active drug liposomes of different concentrations, blank liposomes, and active drug solution dissolved in dimethyl sulfoxide (DMSO). According to references, the final DMSO concentration was below 0.2 %, and the concentrations of cumulative treatment agents ranged from 39.30 to 196.48 µM.

Media was withdrawn from the wells after incubation for 48 hrs, and phosphate buffer saline was added to wash the well. Next, each well was replenished with a fresh culture medium with 20 µL of MTT (5 mg/mL) in it. The culture plates were incubated for 4 h at 37 °C and centrifuged for 10 min at 3000 rpm. Subsequently, the medium was moved out, and 150 µL of DMSO was added to dissolve the formazan crystals produced inside cells.

The absorbance of cells was measured by multi-well scanning spectrophotometer Model 680 (Bio-Rad, Hercules, CA) at 570 nm and 630 nm [21]. Each concentration was set up in six replicates, and the experiment was measured in triplicate. The formula of cell inhibition percentage was as follows:

$$\text{Cell inhibition \%} = 1 - \frac{\text{Absorbance}_{\text{experiment}}}{\text{Absorbance}_{\text{control}}} \times 100 \text{ \%}$$

Where, $\text{Absorbance}_{\text{experimental}}$ and $\text{Absorbance}_{\text{control}}$ are the absorbances of cells interacted with liposomes or drug and cells cultured with no liposomes or drug, respectively.

4. Results and Discussion

4.1. *Drug loading and entrapment efficacy*

The total drug loaded per unit weight of the Liposome is measured as Loading Capacity or % drug loading. It represents the percentage mass of liposomes with encapsulated drug, whereas Encapsulation efficiency or Entrapment efficacy represents the percentage of drug that is successfully entrapped into the Liposome. Drug loading and percentage entrapment efficacy for all five formulations were determined and tabulated below in table 3 with average value and their % RSD.

Table 3. Result for % drug loading and % entrapment efficacy for different five formulations.

Formulation -1 initial results (N=3) (From.-1)			Formulation-2 initial results (N=3) (From.-2)		
Parameter	Avg (%)	% RSD	Parameter	Avg (%)	% RSD
% DL	92.21	0.67	% DL	82.24	4.92
% EE	92.91	0.41	% EE	77.49	5.16
% Assay (cumulative)	99.75	0.1	% Assay (cumulative)	98.90	0.22
Formulation-3 initial results (N=3) (From.-3)			Formulation-4 initial results (N=3) (From.-4)		
Parameter	Avg (%)	% RSD	Parameter	Avg (%)	% RSD
% DL	91.86	1.81	% DL	91.35	2.98
% EE	92.50	1.02	% EE	89.46	3.88
% Assay (cumulative)	99.10	0.65	% Assay (cumulative)	98.08	0.65
Formulation -5 initial results (N=3) (From.-5)					
Parameter	Avg (%)	% RSD			
% DL	90.56	1.16			
% EE	89.90	1.43			
% Assay (Cumulative)	97.47	0.61			

Formulations -1, 3, and 5 have shown good entrapment efficacy and good percentage loading capacity from all the above formulations. All three formulations were designed with different amounts of GCDCA, lecithin, and cholesterol. The outcome of % EE and % DL shows that formulations with GCDCA (1.8 mM to 2.25 mM), lecithin (62.12 mM to 68.33 mM), and cholesterol (19.67 mM to 25.88 mM) had better encapsulation capacity (i.e., about more than 7.2 mM) for total drug content.

Similarly, the previously effective incorporation of tacrolimus in liposomes was also done using soy lecithin and cholesterol, showing effective results against atopic dermatitis [22]. GCDCA was used as a surfactant for liposomes is useful to prevent it from aggregation. Due to the elastic nature of the lipid surface, the optimum concentration of surfactant needs to identify, and in the current study, it was found within the range of 1.8 to 2.25 mM for GCDCA. Lecithin and cholesterol were used to do outer packing for liposomes; through different trials, the optimum concentration required for lecithin and cholesterol is in the range of 62.12 to 68.33 mM, 19.67 to 25.88 mM, respectively.

4.2. *In vitro release of the active component*

The release behavior of drug components from the liposomal system was studied with physiological saline containing 1 % tween 80 (w/v) as release medium using dialysis method, and results are given in Fig. 1.
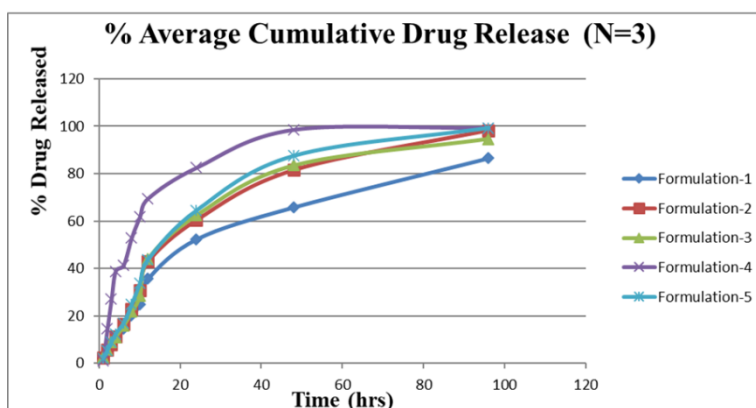

Fig. 1. Percent average cumulative drug release for five Liposome formulations.

The drug holding capacity of liposomes is an important characteristic to achieve the desired pharmacological effect; the drug holding capacity can be assessed from the release pattern of the liposomes. From our above trials, the optimum release was achieved with formulation-1, whereas formulation-4 has shown very spontaneous release, and formulation-2, 3, and 5 have given intermediate release of the drugs. Spontaneous drugs release might be helpful where swift action is also commanded. It might be due to improper encapsulation of the drug; in this contemporary study, we need the comprehensive release of the drug due to the engrossment of multiple drugs component. Impulsive release of multiple drugs component may attest as a frontrunner towards scarcer pharmacological effect. The stability of the Liposome is also an important characteristic needs to be determined apart from percentage drug loading capacity, entrapment efficacy, and percentage drug release.

In the previous study of Nguyen *et al.,* paclitaxel was efficiently encapsulated in soy lecithin liposomes shows 94.2 ± 3.2 % drug loading efficiency and slow-release up to 96 h compared to free paclitaxel [23].

4.3. *Stability of liposomes*

The stability of all five liposome formulations was determined for 2 months duration by storing liposomes at room temperature and 2-8 °C in the refrigerator; for stability confirmation % assay for drug content was determined at a different time interval, the results obtained from this study are summarized in Figs. 2-3.
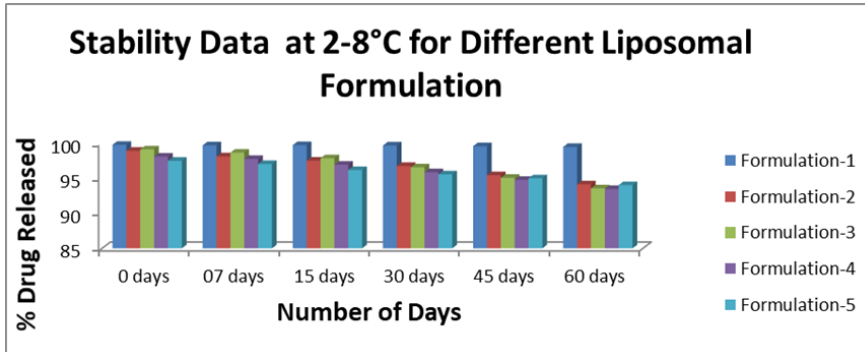
Fig. 2. Stability results up to 60 days for five liposome formulations (at 2-8 °C).
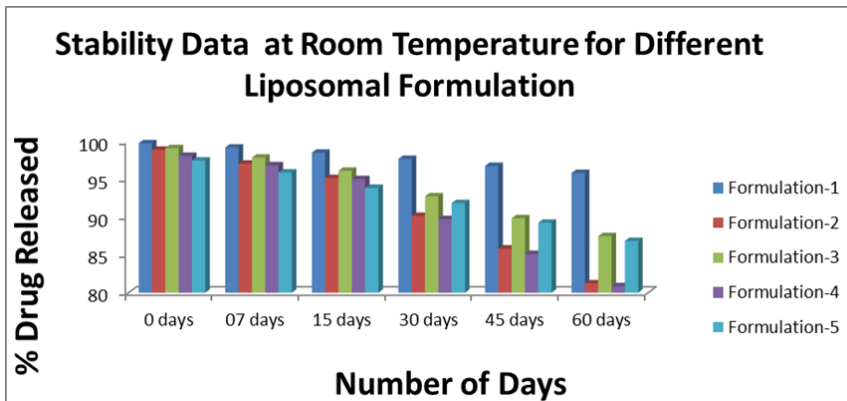

Fig. 3. Stability results up to 60 days for five liposome formulations (at RT).

During storage of liposomes, encapsulated total drugs content must remain stable to triumphanticipated pharmacological effect. Total drugs content stability emulates the stability of the liposomes, as properly encapsulated drugs will have slower degradation than in the free drug. Stability data spectacles that formulation-1 is a more stable and robust formulation than the other 4 formulations. During storage of liposomes, virtuous encapsulation of drugs will thwart drugs from hydrolysis (like acid, base, or oxidation hydrolysis). In contrast, ailing encapsulation denatured lipid bilayer certainly and leashes drugs towards degradation and concocts potency lost. As the remaining formulation is not optimum stable in either refrigerated or nominal environment conditions, the further analysis continued using only formulation-1.

4.4. *Particle size distribution and zeta potential*

Particle size and zeta potential of optimized Liposome's formulation-1 were measured using Malvern Pan Analytical Zeta-Sizer, and the result is comprised in the below Table 4.

Table 4. Particle size and zeta potential results.

Sl. No.	Sample name	Particle size (nm)	PDI	Zeta potential (mV)
1	Empty Liposome without drug sample _1	60.5	0.120	-20.04
2	Empty Liposome without drug sample _2	64.9	0.118	-19.48
3	Empty Liposome without drug Sample _3	71.6	0.105	-20.74
4	Liposome with active drug components_1	79.9	0.131	-22.54
5	Liposome with active drug components_2	71.1	0.119	-28.78
6	Liposome with active drug components_3	75.5	0.125	-26.17

The particle size distribution results indicate that formulation-1 has a good distribution with a narrow polydispersity index (PDI). The results of zeta potential also confirm the excellent stability of the formulation-1, with a better surface charge which will prevent agglomeration of the liposomes. The obtained parameters results also support previous stability data of formulation-1, where it has shown significantly better stability than other formulations. Proper encapsulation of active components with a good distribution pattern and stable surface charge boosted formulation-1 towards the most promising formulation.

4.5. *Micro-morphology (TEM)*

Micro-morphology for TEM was determined by Hitachi Transmission Electron Microscope H-9500 for empty Liposomes without drug samples (Placebo) and Liposomes with Active drug component (drug product) for optimized drug formulation (i.e., formulation-1), and the same was also summarized with Fig 4.
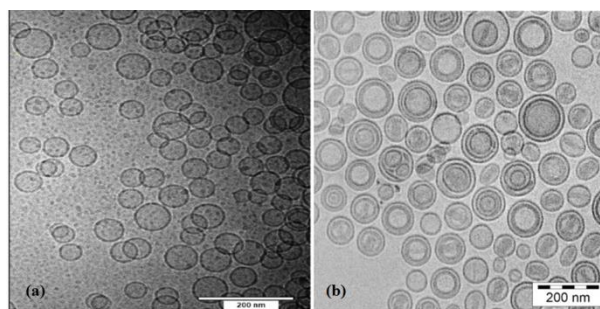

Fig. 4. Micro-morphology by TEM results for (a) TEM images of placebo (with size) and (b) TEM images of the drug product (size).

The transmission electron microscopy results show that the drug product as multilamellar vesicles (MLV) distribution, and the placebo possesses a small unilamellar vesicles (SUV) structure. Proper encapsulation of natural compounds is also confirmed by the TEM results.

4.6. *Cell cytotoxicity assay*

MCF-7 cells were used to evaluate the in vitro cell cytotoxicity of active components with the MTT method with a concentration range of active drugs ranging from 39.30 µM to 196.48 µM for optimized liposomal formulation (i.e., formulation-1), and results were demonstrated in the below Table 5.

Table 5. MTT assay results for Liposome.

Percentage of Liposome dilution	Concentration of total drug in µM	% Cell inhibition
Level-1 (25%)	196.48	100
Level-2 (20%)	157.18	80.3
Level-3 (15%)	117.89	60.2
Level-4 (10%)	78.59	40.1
Level-5 (5%)	39.30	20.1
Correlation coefficient (R^2)	1.0000	
slope	0.50897	
Intercept	0.1	
IC_{50} for Liposome	97.97 µM	

Good inhibitory action against MCF-7 breast cancer cell line was demonstrated by the formulation -1 and obtained IC_{50} value of 97.97 µM of IC_{50} value for total drug content, which is cumulative of seasamine 1.87 µM, gallic acid 26.18 µM, catechin 54.85 µM, Butein 1.87 µM, gingerol 3.74 µM, curcumin 4.04 µM, and emodin 5.42 µM.

In a similar study, curcumin nanoliposomal was formulated by Hasan *et al*. [24] showed a significant inhibitory effect on breast cancer MCF-7 cells and improved curcumin bioavailability, which further leads to cell cycle arrest and apoptosis of breast cancer cells. Akin study of Dhule *et al*. [25] shows significant anti-cancer activity against MCF-7 with the IC_{50} of 11.5±1.1 µg/mL for curcumin-loaded-liposomes against free curcumin IC_{50} (20±1.8 µg/mL).

5. Conclusion

Breast cancer is the prominently occurring cancer in women; researches show higher expression of HIF-1α, TOP-II α, protein kinase C, and MMP-2 receptors in breast cancer patients. Hence based on molecular modeling, effective natural compounds were identified, showing a significant anti-cancer effect. These compounds were seasamin, Aloe-emodin, gallic acid, catechin, Butein, 6-gingerol, and curcumin. Five different formulations of liposomes entrapped with the above natural compounds were prepared with different concentrations of GCDCA (1.35 to 2.25 mM), cholesterol (19.67 to 32.09 mM), and lecithin (55.91 to 68.33 mM). From the analytical results of all five liposome

formulations, it was concluded formulation-1 having GCDCA (1.8 mM), lecithin (62.12 mM), and cholesterol 25.88 (mM) can occupy total drug content of 7.2 mM, and is the stable and robust formulation and also release optimum amount drugs. Hence further assessment of formulation-1 was performed for particle size distribution, zeta potential, micro-morphology (TEM), and cell cytotoxicity assay (MTT). These further assessments show formulation-1 also has a good particle size distribution profile with a narrow polydispersity index of 0.125 and good surface charges rages from -22.54 to -26.17, which can help prevent agglomeration of liposomes and helps to achieve better stability. The result of Transmission electron microscopy shows that liposomes had good micromorphological characteristics with multi-laminar vesicles. Cell cytotoxicity assay results show that formulation-1 are also having desired cumulative inhibitory action against MCF-7 breast cancer cell line also obtained, i.e., 97.97 μM of IC_{50} value for total drug content, for individual drug (in combined formulation) obtained IC_{50} values are seasamine 1.87 μM, gallic acid 26.18 μM, catechin 54.85 μM, Butein 1.87 μM, gingerol 3.74 μM, curcumin 4.04 μM and emodin 5.42μM which cumulatively make 97.97 μM total IC_{50} of prepared liposomes of formulation-1. Promising results of formulation-1 opens the doors for future studies for this; further from formulated liposomes with formulation-1, any stable topical or parenteral drug delivery product can be made, which may provide ease of drug delivery to a breast cancer patient with improvising bioavailability.

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