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Fabrication and Evaluation of Multiple drugs-Loaded Liposome for the Management of HR positive Breast Cancer

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Target delivery of multiple therapeutic agents selectively to cancer cells remains a challenge. This necessitates the development of multifunctional drug delivery platform

Objectives: The aim of this study is to construct a novel drug delivery system that can entrap and selectively deliver two anticancer drugs.

Methods: Fourier transform infrared spectroscopy (FTIR) was used to study the interactions between the pure drugs and the excipients while the liposomes were prepared by film hydration technique. Cryo-Transmission electron microscopy was use to study the morphology and the degree of entrapment, while the zeta potential, size distribution and particle determinations were obtained using a Zetasizer Nano ZS90. The *in-vitro* release and release kinetics were determined by the use of dialysis membrane and UV spectroscopy.

Results: There were no chemical interaction between the drugs and the excipients. The incorporation of drugs in a single liposome structure was successful and exhibited synergy as evident from the MTT viable cell assay. Liposomes obtained were nanosized and negatively charged smooth surfaces with degrees of entrapment above 80%. All had PDI values less than 0.25 indicating homogenous dispersion with particle size ranked, FD2≥FD1≥ FD3 ≥ FD4 ≥ FD5. The release profiles of all the formulations were biphasic with an initial bust followed by sustained release. The mechanism of drug release which best fitted the Korsmeyer model was essentially a combination of diffusion and erosion.

Conclusion: FD2 had the highest degree of entrapment, achieved sustained drug release and was able to synergistically kill MCF-7 breast cancer cell line *in-vitro*. It may be suitable for HR breast cancer treatment due to its good formulation parameters.

Keywords: Liposomes, breast cancer, fulvestrant, doxorubicin, chemotherapy

INTRODUCTION

Breast usually starts from the inner lines of the duct or lobules, (ductal carcinoma or lobular carcinoma), from where it may spread to other parts of the body, (Osborne and Boolbol 2014). It is the most common cancer in women worldwide, of which more than 1.7 million new cases are diagnosed among women worldwide in 2012. Even though the incidence rates of the disease differs around the world, it is the fifth causes of death worldwide, with developed countries having higher incidence rates compared to developing countries, (National Cancer Institute 2016; Dang *et al.*, 2008).

Even though there are various treatment options available, the use of chemotherapeutic agents remains a valuable treatment option. Chemotherapeutic

agents are administered to kill the malignant cells, prevent recurrence of cancer, prevent spreading (adjuvant chemotherapy) and to shrink large tumor prior to removal by surgical procedure. (neo-adjuvant chemotherapy). The choice of chemotherapeutic agents depends largely on the breast cancer stages, types, patient tolerability and age, (Alzouebi *et al.*, 2012).

Co-administration (combination therapy) of these chemotherapeutic agents is not uncommon in clinical practices, (Citron, *et al.*, 2003) with dosage regiments adjusted individually and rationally. Combination therapy is a promising approach in the treatment of cancer with added advantage of exploiting synergistic effects of the co-administered agents. In order to reduce nursing time, therapy cost and discourages poly pharmacy; a novel drug delivery system capable of incorporating two or more chemotherapeutic agents is desirable. Doxorubicin, an anthracycline is one of the prominent chemotherapeutic agents used to treat breast cancer. When used as monotherapy its given at a dose of 60–75mg/m^2 IV every 21 days, and as combination therapy: it's usually dose is 40–60mg/m^2IV every 21 to 28 days (Ni *et al.*, 2017). Fulvestrant is a novel endocrine therapy for hormonal responsive breast cancer, with a unique structure and mode of action. It binds competitively to the oestrogen receptor (ER), with high affinity, and down regulates the ER by functional blockade. Fulvestrant unlike tamoxifen is unique as it shows no agonist characteristics. It shows no cross-resistance when used in combination with other chemotherapeutic agents (Johnston and Cheung 2010).

It has been shown that an appropriate combination of these two chemotherapeutic agents can improve the therapeutic outcome and patient compliance due to reduced dose and decreased development of drug resistance, (Ikeda *et al.*, 2011; Sonia *et al.*, 2014). Also co-administration of fulvestrant and doxorubicin was shown to potentiates doxorubicin-induced cytotoxicity, apoptosis and G2/M arrest with up regulation of cyclin B1. It functioned as a substrate for P-glycoprotein (P-gp) without affecting its expression. Fulvestrant not only restored the intracellular accumulation of doxorubicin but also re-localized it to the nuclei in Bats-72 and Bads-200 cells, which may be another potential mechanism for the reversal of P-gp mediated doxorubicin résistance, (Yuan *et al.*, 2016). A combination of Fulvestrant and doxorubicin chemotherapy is effective for patients with advanced breast cancer and therefore formulation development of both agents in a single liposomal vehicle is most desirable.

Liposomes are nanocarriers using lipids as the drug vehicle which offers a number of desirable features, such as, low toxicity, biodegradable particulate matrix, nontoxic degradation products, high capacity to incorporate lipophilic and hydrophilic drugs, controlled release of the incorporated drug, and easy scale-up at low cost, (Liu *et al.*, 2016; Tsouris *et al.*, 2014; Gandhi, *et al.*, 2014). Liposomes are spherical shaped, colloidal lipid base nano-formulations. They are bilayer of which outer lipid surrounds a central aqueous space, (Rivera,2003).

METHODS

Materials

Fulvestrant and doxorubicin were purchased from Sigma-Aldrich (St Louis, MO, USA). Injectable soya lecithin and cholesterol were obtained from Shanghai Taiwei Pharmaceutical Co, Ltd (Shanghai, People's Republic of China). MCF-7 breast cancer cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All other reagents were of analytical grade.

Methods

Drug/Excipient interaction studies

Doxorubicin (DX), fulvestrant (FT), chloroform (CH), lecithin (LC), were mixed separately and with infrared (IR) grade potassium bromide (KBr) in the ratio of 1:100. Corresponding pellets were prepared by applying 5.5 metric ton pressure with a hydraulic press. The pellets were scanned in an inert atmosphere over a wave number range of 4000–400 cm^{-1} in a Magna IR 750 series II FTIR instrument (Jasco, FTIR 4200, Japan) , (Mukherjee *et al.*, 2007; Oyeniyi and Biswajit 2017). Same procedure was repeated for and a mixture of the drugs, CH, and LC.

Table 1: Batch formulations of FT-DX liposomes

Materials	FD_1	FD_2	FD_3	FD_4	FD_5
FT (mg)	0.5	0.5	0.5	Nil	1.0
DX (mg)	0.5	0.5	0.5	1.0	Nil
CH (mg)	1.0	2.0	1.0	2.0	2.0
LC (%)	1.0	1.0	2.0	1.0	1.0

Preparation of liposomes

Batches of liposomes were prepared by lipid layer hydration method. Fulvestrant, Cholestrol, and lecithin (Table 1) were dissolved in 100mL of chloroform in a 250 mL round bottom flasks. They were mixed vigorously by shaking. The mixture was placed in a rotary vacuum evaporator fitted with an A3S aspirator (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) with a circulating bath (Spac-N Service, Kolkata, India) and rotated at 150 rpm at 37°C in a water bath to evaporate the solvent, while forming film within the flask. The flask was thereafter kept in a vacuum desiccator overnight for complete removal of residual organic solvent. Doxorubicin was dissolved in 20ml of PBS and added to the flask containing the lipid film which was thereafter hydrated at 60°C in a water bath fitted with a rotary vacuum evaporator operating at 100 rpm until the lipid film completely dispersed in the aqueous phase. The dispersion was thereafter sonicated in a bath sonicator, (Trans-o-Sonic, Mumbai, India) operating at 30 ± 3 KHz and 60ºC.The preparation was kept at room temperature for about one hour for vesicle formation before storing overnight at 4°C. The preparation was thereafter centrifuged at 16000 rpm for one hour and the sample lyophilized. Fluorescent liposomes were prepared by the above procedure, except fluorescein isothiocyanate (FITC) was dissolved in the organic phase, (Baillie *et al.,* 1985; Oyeniyi and Biswajit 2017; Manconi *et al.,* 2002)

Morphology and degree of drug encapsulation studies

Specimens for cryo-Transmission electron microscope (cryo-TEM) were prepared using carbon coated copper grids (400 mesh, Agar Grids). The grids were hydrophilized through treatment by glow discharge. The blotting procedure and the quenching of specimens were performed using an improved version of the controlled environment vitrification system. The liposomal dispersions were vitrified on different carbon grids; these were subsequently transferred to the cryo-TEM machine fitted with a vironova analyzing software (VAS). This allows semi-automated particle detection and classification analysis during investigation. The vitrification was accomplished by blotting a very thin film on a carbon-coated grid, (Bellare *et al.,* 1988; Glaser *et al.,* 2007). Best possible images of the liposomes were captured while the degree of drug encapsulation was determined by classifying the liposome cryo-TEM images based on electron density visualized as intensities of gray to identify filled versus empty particles. Results of the classification are presented in a bar chat.

Zeta potential, PDI, and size determination

The hydrodynamic diameter, polydispersity index (PDI), and zeta potentials were measured using a zetasizer nano ZS fitted with DTS software (Malvern Instrument Limited, UK). The lyophilized formulations (10µg) were placed in a 2 mL eppendrof tube, suspended in PBS and thereafter introduced into the instrument, (Malvern Zetasizer Nano – ZS90). The results were then read and recorded.

Drug release studies

A known weight of the liposome was placed in a dialysis membrane (MWCO 20 kDa ; Spectrum Labs, Rancho Dominguez, CA) with both ends tightly bound with threads. The dialysis sac was thereafter suspended with the aid of a glass rod clamped to a restort stand. It is however critical to ensure that portion containing the sample is adequately dipped in a 250 mL conical flask containing 100 mL of PBS pH 7.4 maintained at 37 °C under constant stirring. At predetermined time intervals, 1 mL of the solution was removed with the aid of a micropipette and 1mL of fresh PBS was added in each case. Using a ultraviolet-visible (UV/VIS) spectrometer (Beckman 220 Instruments, Fullerton, CA, USA), operating at485 nm, the amount of for doxorubicin was determined. However, for Fulvestrant, the wave length was set to520 nm and PBS replaced with methanol. Cumulative drug release was calculated in

each case and expressed as a percentage of initial drug encapsulated in the liposomes, (Oyeniyi & Biswajit 2017). In order to investigate the drug release kinetics, the drug release data were fitted into four mathematical models, zero, first order, Higuchi and Korsmeyer.

***In-Vitro* Cytotoxicity testing (MTT)**

MCF-7 cells were cultured in Dulbecco's modified eagle's medium (DMEM) without phenol red and supplemented with 10% fetal bovine serum. The cell culture medium was maintained at 37°C in a humidified incubator containing 5% CO_2 atmosphere. Trypsinized confluent cell monolayers were grown (75%–80%) and the cells in the exponentially growing phase were used for cytotoxicity experiments. Specifically the cells were plated at a density of 5×103 cells/well (optimal seeding density) in 96 well plates and kept at 37°C in 5% CO_2 atmosphere in a CO_2 incubator (Model MCO-15AC; Sanyo Electric Biomedical Co. Ltd., Osaka, Japan). After 12 hours of incubation, the medium in the wells was replaced with fresh medium containing prepared liposome formulations. After 48 hours, MTT dye solution was added to each well and the incubation was continued for another 4 hours. The medium in each well containing unbound MTT and death cells was removed by suction. The formazan crystals were solubilized with 100 µL dimethylsulfoxide, and the solution vigorously mixed to dissolve the reacted dye. The absorbance of each well was determined by reading the, optical density (OD) values at 595nm using DMSO as a blank. A plot of cell viability against the concentration was constructed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined from graphically.

Cell viability (%) = Mean OD/Control OD x 100% …… (5)

Statistical analysis

Data are presented as the average mean ± standard deviation. The significance of the difference between treatment groups was evaluated using unpaired Student's two-tailed t-test. $P \leq 0.05$ was considered statically significant.

RESULTS AND DISCUSSION

FTIR evaluation of the various components of a pharmaceutical formulation is one of the very important pre-formulation studies which provide useful information on the physico-chemical properties, stability of the drug in the dosage form and the drug release pattern. The FTIR results of the pure drugs, pure excipient and mixture of drugs and excipients were as presented in figures 2 -5, which shows some physical interactions between the drugs and the excipients. These observed drugs-excipients interactions are beneficial for the formation of a stable and a well shaped liposome, (Gokhale, *et al.*, 1996; Lia, *et al.*, 2008) .

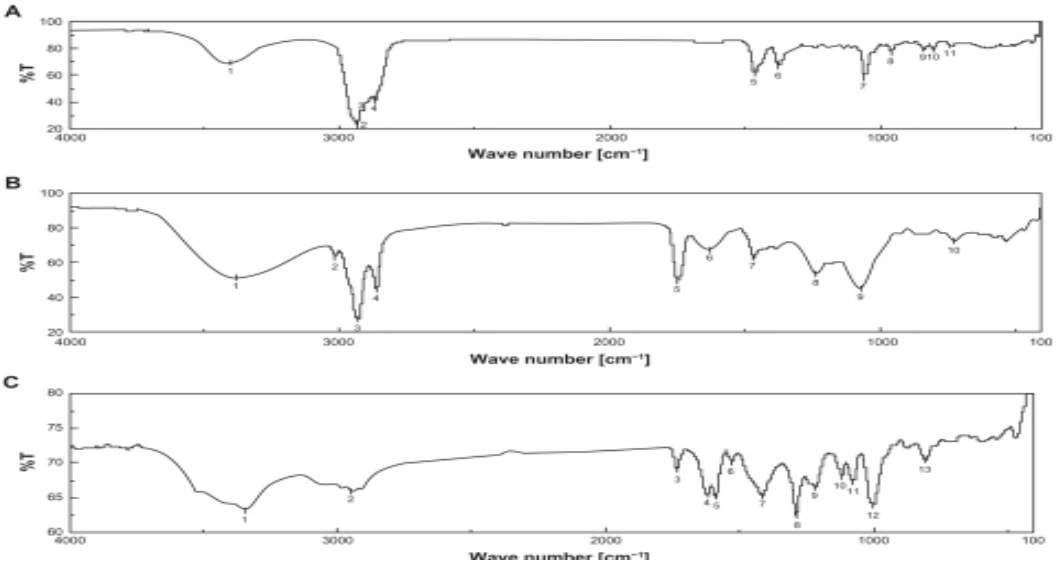

Figure 2: Fourier transform infrared spectroscopy (FTIR) spectra of (A) Cholesterol; (B) Lecithin; (C) Doxorubicin.

Comparative assessment of the FTIR spectra of drugs and excipients mixture shows that all the important peaks of fulvestrant and doxorubicin were present but shifted (figures 3 & 5). The peaks shifting observed at 940 to 920 cm^{-1} was due to alkane, 3,406 to 3,402 cm^{-1} due to benzene ring and its substitutes, while those at 3,435 cm^{-1} were due to OH group. The shifting of such peaks might have taken place due to the weak physical interactions such as formation of weak hydrogen bonding, van der Waals' force of attraction, dipole–dipole interactions, etc, which are beneficial for the formation of liposome spherical structures, (Li *et al.*, 2011.

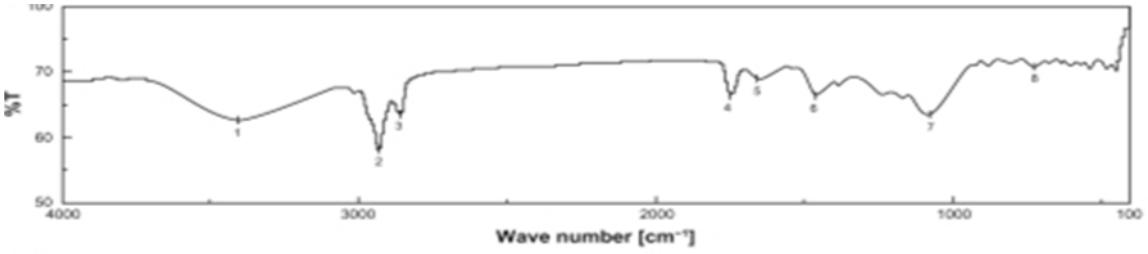

Figure 3: Fourier transform infrared spectroscopy (FTIR) spectra of Cholesterol; Lecithin; Doxorubicin mixture

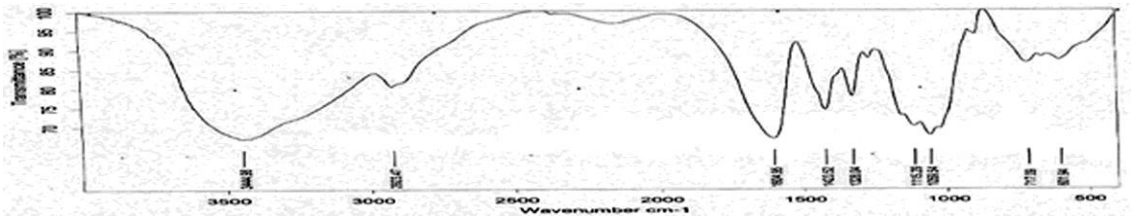

Figure 4: Fourier transform infrared spectroscopy (FTIR) spectra of Fulvestrant

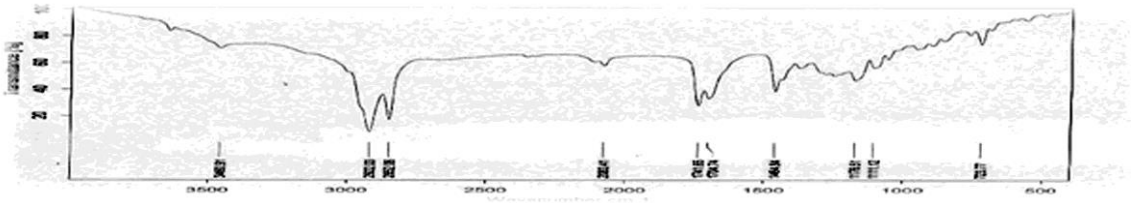

Figure 5: Fourier transform infrared spectroscopy (FTIR) spectra of Fulvestrant, Cholestrol and Lecithin mixture.

Cryogenic transmission electron microscopy (cryo-TEM) is an indispensable tool for the characterization of nanopharmaceuticals. It is applicable in studying the size, shape and internal structure of nanoparticles as well as the overall colloidal composition of the corresponding dispersions, (Kuntsche *et al.*, 2011). Figure 6 (a-e) shows that there was a clear distribution of both small and large (nano size range) vesicles that are smooth and spherically shaped.

To evaluate the degree of entrapment using the cryo-TEM, liposomes detected are classified based on electron density visualized as intensities of gray to identify filled (red color) versus empty (blue color) liposome particles, (Figure7). The degree of entrapment is one of the key parameters needed to quantify the therapeutic activity of the drug since it is directly proportional to the therapeutic action of the entrapped drugs, (Kamba *et al.,* 2013; Naruphontjirakul, *et al.,* 2011). All the formulations had degrees of entrapment above 80 %, (Figure 8) in a ranking order of FD2 ≥FD1 ≥ FD3 ≥FD4 ≥FD5, ($p \leq 0.05$)

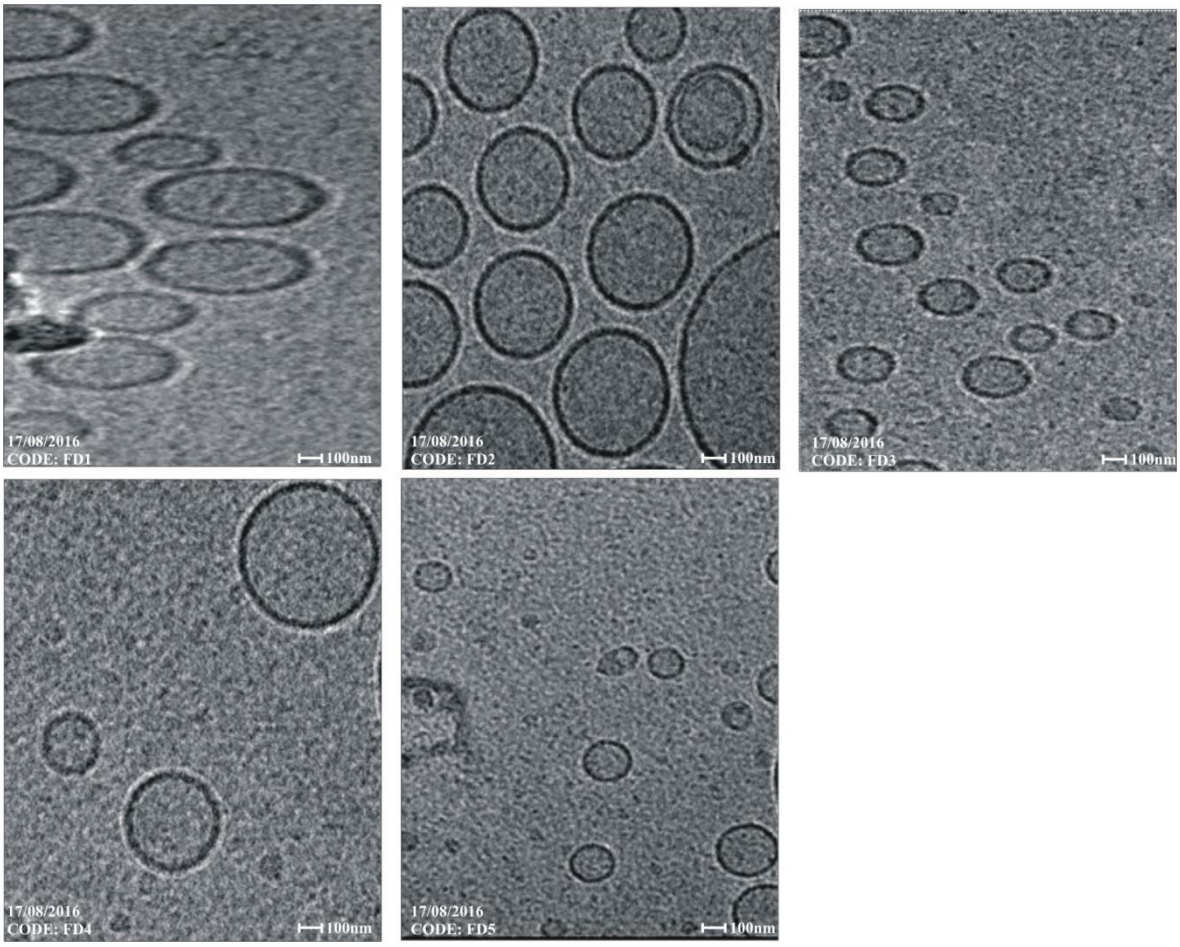

Figure 6: Cryogenic transmission electron microscopy (Cryo-TEM) Fulvestrant-Doxorubicin liposomes of FD1, FD2, FD3, FD4 and FD5

The zeta potential values provide an understanding of the surface charges acquired by the liposomes. These values are important for determining the circulation time, potential immune response *in- vivo* and drug stability. A zeta potential of ≤ -30 mV is ideal for stability of therapeutic agents, (Honary and Zahir 2013). The zeta potentials of the formulations are all negative values ranging from -54.5 to -66.0 mV. These negative charges may be due to ionization of free groups present on the surface of liposomes suggesting that all the formulations are stable in the colloidal state and may be stored in a liquid form. FD2 is the most negatively charged and would therefore be expected to be the most stable amongst all the formulations.

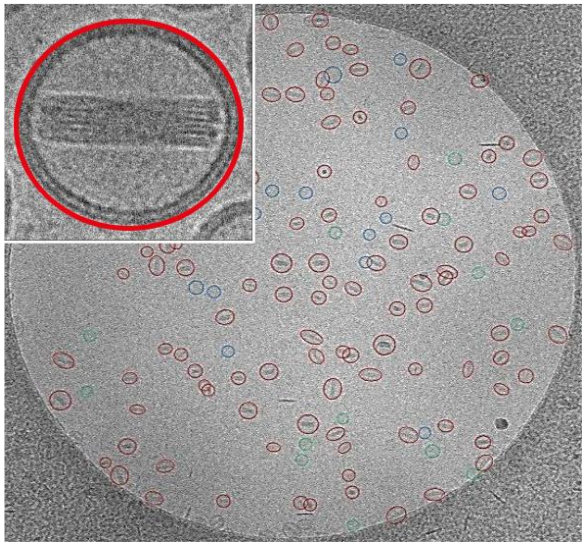

Figure 7: Sample cryogenic transmission electron microscopy analysis of filled (red color) versus empty (blue) liposomes

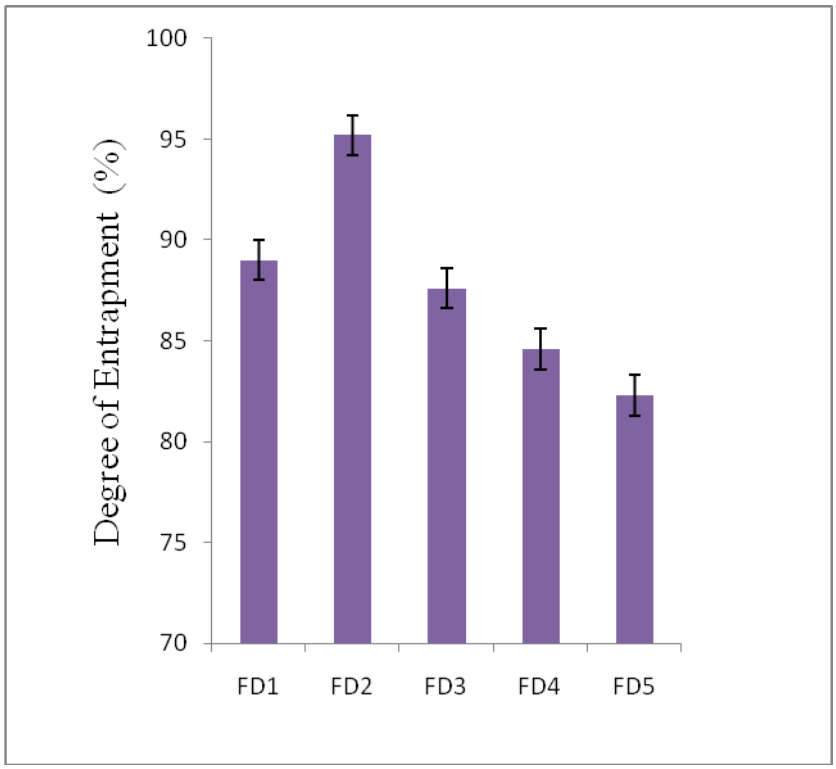

Figure 8: Degree of entrapment of five batches of Fulvestrant/doxorubicin loaded liposomes

The size and size distribution patterns of liposomes depend largely on its production technique and as such the use of ultra-sonication homogenization to reduce the size of the liposomes to nano size range is a common practice in pharmaceutical industries. Bath ultra-sonication method, as compared to probe

sonication method, has been reported to be more popular and efficient for this purpose, (Santra *et al.*, 2010; Martin 1998). Liposomes as drug carriers are required to have an average particle size below 100 nm and a poly dispersible index (PDI) of 0.1, (Cabral *et al.*, 2011). The size is important for the effective bio-distribution within the vasculature and for receptor targeting, while the PDI is an indication of the homogeneity of the sample. Liposomes with mean particle size below 100nm could passively target tumor tissues more efficiently than larger ones, likely due to enhanced permeation and retention, (Atsumura 1986; Torchilin 2005; Tang *et al.*, 2014).

All the five batches of liposomes produced met the requirements with the following average hydrodynamic diameter and PDI values: FD_1 53.3 nm, 0.046; FD_2 51.2 nm, 0.047; FD_3 51.2 nm, 0.047; FD_4 48.6nm, 0.074 and FD_5 43.5 nm, 0.090. Formulation FD_2 with the lowest PDI and mean particle size (table2) is the most homogeneous (mono-dispersed) formulation and would be expected to possess excellent vascular bio-distribution.

Table 2: Some physicochemical properties of FT-DX liposomes

Batch	ZP	PDI	Av.PS	% degree of encapsulation
FD_1	-60.2	0.046	53.3	89.0
FD_2	-66.0	0.040	58.6	95.2
FD_3	-60.5	0.047	51.2	87.6
FD_4	-55.0	0.074	48.6	84.6
FD_5	-54.5	0.090	47.5	82.3

The MTT assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water. Viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formation which precipitates in the cellular cytosol and can be dissolved after cell lysis, whereas cells being dead following a toxic damage, cannot transform MTT. This formation production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity. The reaction is mediated by dehydrogenases enzymes associated with the endoplasmatic reticulum and the mitochondria, (Fotakis and Timbrell, 2006). After 48 hours of incubation, cell viability as determined by the MTT assay revealed that all the (FD1-FD5) formulations reduced the number of viable cells significantly $p \leq 0.05$ and inhibit the proliferation of the breast cancer cells (Fig. 9). However formulations containing two cytotoxic agents FD1-3 show higher cytotoxic effect compared to those with single agent FD4 and FD5. These observed differences are significant, $p \leq 0.05$ and may be due to synergetic effects of the two cytotoxic agents. FD2 with the highest percentage viable cell reduction may be related to its observed formulation properties such as optimal large nano sized particles, higher degree of drug entrapment and monodispersed (homogenous). This finding is in agreement with earlier reports that nano sized liposomes with high degree of entrapment and large particle size may reduce wastage of the active ingredients as they are able to incorporate more of the active drugs within their structure, (Ashley *et al.*, 2016).

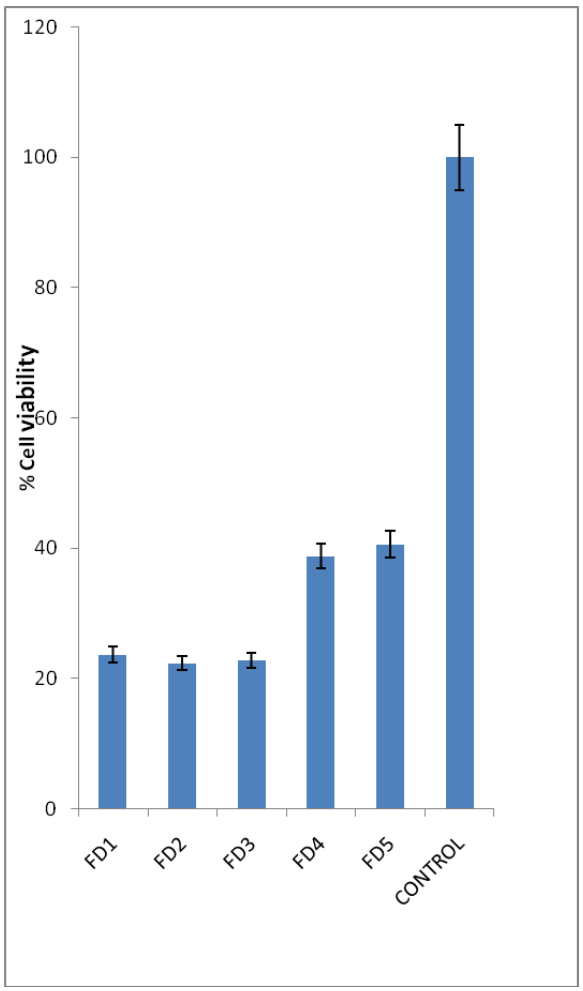

Figure 9: Percentage MCF-7 cell viability after 48 hrs

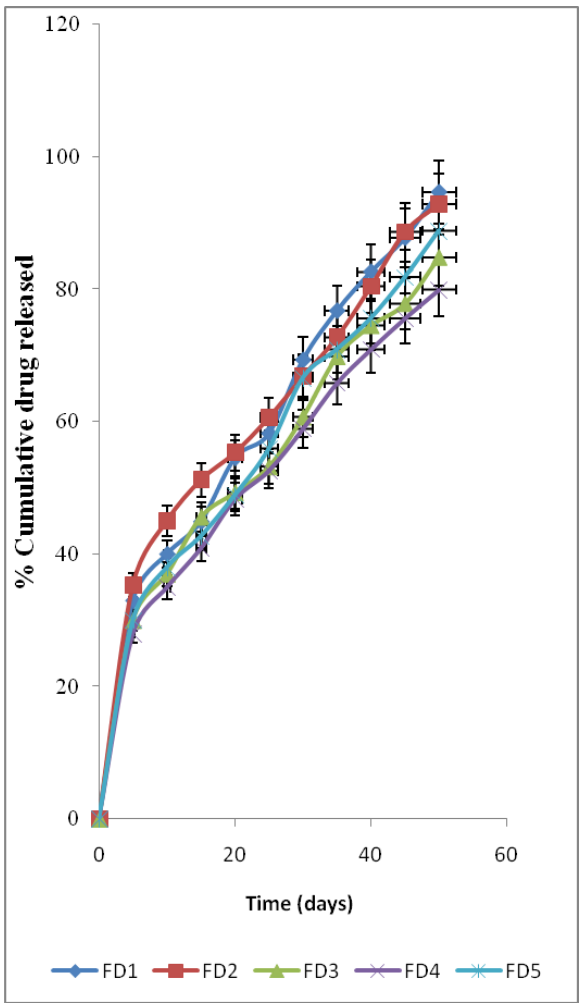

Figure 10: Percentage cumulative release of fulvestra

In all the formulations, there was initial burst release of the drugs, followed by sustained release (Fig.10 & 11). No dose dumping was observed in any of the formulations. The initial burst release of the drug is advantageous to initiate therapeutic activity. The use of a rigid cholesterol nucleus together with acyl chain of phospholipids are known to reduce the freedom of motion of the acyl chain which ultimately causes the membrane to condense, decrease its fluidity and act as a barrier to the entrapped drugs and ultimately retard the drug release, (G´omez-Gaete *et al.,* 2009; Jain, 2000). The cumulated drug release in all the formulations was well above 80%. FD2 with larger particles had slower drug release possibly due to the longer diffusion pathway that the drug would need to travel before being released. The cumulative release of the fulvestrant was observed to be higher compared to doxorubicin within the first 10 days, which may be due to its lipophilicity resulting in the dispersion of fulvestrant in the lipid outer layer of the liposomes.

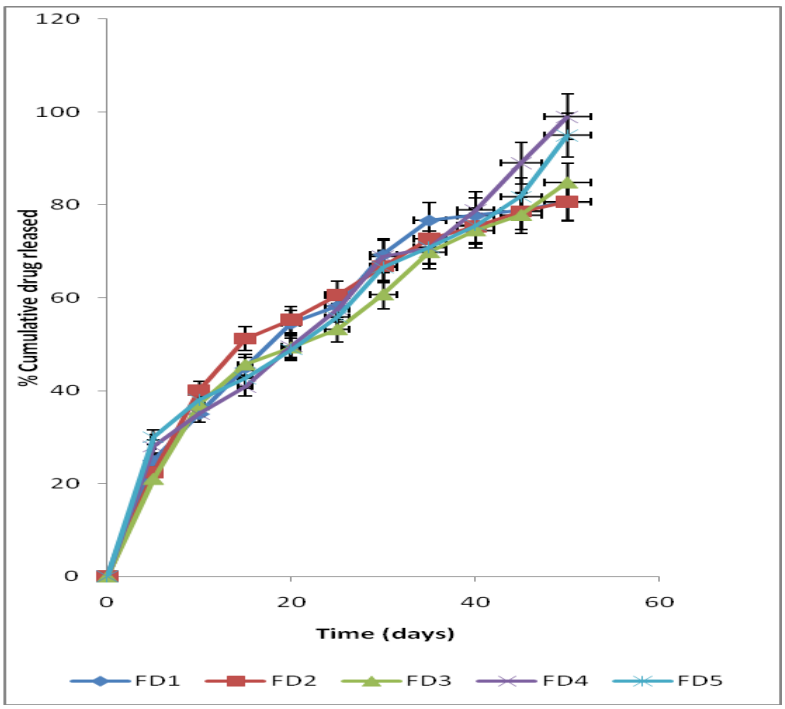

Figure 11: Percentage cumulative release of doxorubicin

Table 4: Correlation co-efficiencies (R^2) for Fulvestrant/ Doxorubicin releases (n= 6)

	Fulvestrant				Doxorubicin			
Code	**Zero order**	**First order**	**Higuchi**	**Korsmeyer**	**Zero order**	**First order**	**Higuchi**	**Korsmeyer**
FD1	0.826	0.880	0.903	0.956	0.851	0.888	0.899	0.961
FD2	0.822	0.887	0.919	0.991	0.859	0.891	0.952	0.967
FD3	0.832	0.867	0.968	0.996	0.861	0.895	0.965	0.977
FD4	0.837	0.876	0.889	0.891	0.866	0.895	0.966	0.977
FD5	0.833	0.873	0.899	0.899	0.868	0.899	0.967	0.995

Formulations were thereafter subjected to four different release kinetic mathematical models to determine the release mechanism.

Korsmeyer release model: $Mt/M\infty = K_k T^n$ ……(1)

Where $M_t/M\infty$ is the fraction of drug released at each time internal (T), K_k is kinetic constant and n is diffusion exponent indicative of the mechanism of transport of drug through the polymer, (Korsmeyer *et al.*, 1983)

Higuchi release model: $Mt/M\infty = K_h T^{1/2}$ …… (2)

Where $M_t/M\infty$ is the fraction of drug released at each time internal (T), and K_h represents the Higuchi release kinetic constant.

First-order release model: $Mt/M\infty = 1-e^{-K_1 T}$…… (3)

Where $M_t/M\infty$ is the fraction of drug released at each time interval (T), and K_1 represents the first-order release kinetic constant, (Ahuja *et al.,* 2007; Quiten *et al.*, 2009).

Zero-order release model: $M_t = K_0 T$……. (4)

Where M_t is the fraction of drug released at each time point (T), and K_0 represents the zero-order release kinetic constant, (Quinten *et al.,* 2009).

Table 4 shows that all the formulations essentially follow the Korsmeyer model (highest value of R^2). This implies that the mechanisms of release of the two drugs were the same, and could be both fickian and non-fickian, i.e. a combination of diffusion and non diffusion processes simultaneously occurring within the liposomes, (Korsmeyer *et al.*, 1983; Oyeniyi & Biswajit 2017).

Conclusion

In this study, we demonstrated the preparation and evaluation of double loaded liposomes. Our study demonstrated for the first time, the possibility of incorporating fulvestrant and doxorubicin in a single liposomal formulation for synergy and to improve therapeutic activity.

We have successfully incorporated multiple drugs in the liposomal bilayer by taking advantage of their disparate physical-chemical properties. This combination therapy might be useful in the treatment of hormonal positive breast cancer that may require multiple drugs administration. This can significantly advance treatment of hormonal positive breast cancer.

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