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Original Article

Design and Characterization of Letrozole Ethosomes for Improved Topical Treatment of Breast Cancer

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ABSTRACT

Background: Breast cancer is the leading and most frequent cancer among women worldwide, with rapidly growing new cases diagnosed, surpassing lung cancer. The current study aims to design, optimize and characterize Letrozole (LTZ) Loaded Ethosomes (LTZ-ETH) for the treatment of breast cancer. **Materials and Methods:** Ethosomes were optimized using a two-factor, three-level (32) factorial design technique. The ethosomes were characterized by vesicle size, zeta potential and entrapment efficiency. The optimized LTZ-ETH was tested for surface morphology and *in vitro* cytotoxicity. **Results:** The optimized LTZ-ETH has flawed round-shaped unilamellar structures with an average vesicle size of 218.6±5 nm and an entrapment efficiency of 92.45±3.42%. Optimized LTZ-ETH demonstrated significant ($p<0.01$) *in vitro* cytotoxicity (IC_{50}: 23.27±1.48 µg/mL) than LTZ (58.70±2.46 µg/mL) against MCF-7 Cells. Compared to LTZ, LTZ-ETH treatment caused apoptosis of large proportion of cancer cells. The above results could be correlated to the increased cell uptake of LTZ-ETH as shown by *in vitro* cell uptake study. Furthermore, the *in vitro* skin permeation study results revealed enhanced penetration of LTZ-ETH into the deeper layers of the skin. **Conclusion:** The study results revealed that LTZ-ETH could be used as a potential alternative treatment approach to conventional chemotherapy. However; further *in vivo* animal studies are required to establish its efficacy in the treatment of breast cancer.

Keywords: Apoptosis, Breast Cancer, Ethosomes, Factorial Design, Letrozole, Optimization.

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INTRODUCTION

Background

Globally burden of the rapidly increasing cancer epidemic has significantly escalated and patients afflicted by this disease are in dire need of an optimal therapy that can effectively eradicate cancer. With over 1.7 million new cases reported, breast cancer is the predominant form of cancer among women globally and ranks as the second most prevalent disease overall.[1] This accounts for around 12% of the total number of new cancer cases, which amounts to 14.1 million. Exclusively, breast cancer constitutes a quarter of all cancer cases and 15% of all cancer-related fatalities in females. Nevertheless, breast cancer is not exclusive to females. Male breast carcinoma represents around 0.8%-1% of the total cases of breast cancer.[2,3] The management of breast cancer may involve surgical intervention, radiation, chemotherapy and hormone therapy.[4] The choice of treatment is determined by the prognosis, stage of the disease, available treatment options, potential side effects and the patient's preferences.[5] After receiving more therapy, women may encounter bowel disruption, neutropenia, radiation dermatitis and weariness.[6,7]

A variety of therapies, most commonly a mix of hormone therapy, radiation, chemotherapy, surgery and targeted therapy, may be suggested. But even with this advancement, there are still a lot of psychological, physical and social adverse effects associated with breast cancer and its therapies. Breast cancer treatments still revolve around surgery, including radical mastectomy and lumpectomy linked or not with axillary dissection.[8] Within 30 days after a radical mastectomy and a lumpectomy with axillary dissection, wound infection is the most frequent complication experienced immediately following breast surgery. After lumpectomy and axillary dissection, cardiac and pulmonary problems are rare following mastectomy.[9]

Additional adverse effects that can be caused by axillary dissection include lymphedema, numbness, loss of strength and reduced amplitude of arm movement,[10-12] all of which can diminish the

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quality of life. Radiation therapy is associated with a number of side effects, including erythema (which affects more than half of women), dry or wet peeling, fatigue, edema of the subcutaneous fat, hyperpigmentation, myelosuppression, pain, anxiety, depression and a diminished quality of life.[13-17] Asthenia is one of the many side effects that chemotherapy can induce. Yes, asthenia is experienced by 70 to 100% of patients throughout chemotherapy,[18] and it can also be experienced following chemotherapy.[19,20] Fatigue seems to be constant in intensity during treatment,[21-24] and 60% of patients report moderate to severe fatigue.[25,26] Recent reports suggest that hormonal fluctuations caused by administration of Letrozole (LTZ) may cause mental depression.[27] The drug is prescribed in a low oral dose due to its adverse effects, which include arthralgia, bone fragility, hypercholesterolemia and other health problems. Low plasma levels of the medicine and certain side effects may reduce the total therapeutic benefit,[28] these are caused by LTZ's vast volume of distribution.

The development of parenteral LTZ infusions has the potential to significantly reduce unwanted effects while boosting medication localization at the cancer site. However, the limited aqueous solubility of letrozole complicates the development of such alternative LTZ formulations. Because of its great efficiency, transdermal drug delivery systems have recently received a lot of attention for treating breast cancer instead of the oral route.[29] As a better option, transdermal method has been proposed to achieve steady levels of plasma for an extended length of time, which may also be favourable because it requires less frequent dosing regimens.[30] Transdermal drug delivery offers the advantage of increasing patient compliance and eliminating the first pass impact of oral administration.[31] Transdermal delivery provides numerous advantages, including regulated, consistent drug administration, elimination of pulsed entry into systemic circulation and continuous input of medications with short biological half-lives, which frequently result in unwanted side effects. TDDS overcomes hepatic first pass metabolism, improves therapeutic efficiency and maintains a stable plasma level of the medication.[32] Colloidal particle carriers, such as noisome, are commonly used in medication delivery systems.

Advancement like ethosomes created from noisome gives upper hand over them. These are drug reservoirs and changing their composition controls their rate of release.[33] The lipid vesicles include hydrophobic pharmaceuticals in lipid domain and hydrophilic drugs (via encapsulation). Lipid vesicles are applied in several drug delivery systems including controlled release, drug targeting and permeation improvement of pharmaceuticals as they are so very good at expressing a wide range of drugs.[34] Several investigations have shown LTZ as a special moiety suitable for treating breast cancer. Some of these studies have made advantage of several creative drug delivery techniques. Olate-conjugated polymer: lipid hybrid nanoparticles,[35] Poly (D, L-Lactide) nanoparticles,[36] Liposomes,[37] pronisomes[38] of LTZ for treatment of breast cancer the extant research just notes Not any research where LTZ has been administered trans dermally with ethosomes acting as carrier systems. Nonetheless, it has not yet been shown if LTZ loaded ethosomes are suitable new drug delivery vehicles for treating breast cancer. Therefore, the present work aims to create, optimize and characterize an ethosome formulation of LTZ for efficient treatment of breast cancer.

MATERIALS AND METHODS

Materials

Letrozole was procured from Clyearsynth India Pvt. Ltd., Phospholipid 90G (PL90G) was gifted from Lipoid GmBH, Germany. Ethanol, rhodamine 6G and cholesterol were obtained from SD Fine-Chem Ltd., Mumbai. HPLC grade ethanol was obtained from Fisher Scientific, India. All other chemicals used in the current research work were of analytical grade.

Preparation and optimization of LTZ-ETH

The Classical cold method was used to generate LTZ-loaded ethosomal formulation (LTZ-ETH) formulations with a few minor modifications. Required amount of ethanol was used to dissolve Phospholipon 90G and LTZ and the mixture was stirred with a magnetic stirrer set at 700 rpm (IKA, Germany RCT Basic) and 30°C±1°C. Phosphate buffer saline (PBS, pH 6.4) was gradually added to the ethanolic solution at a uniform rate of 200 µL/min while being continuously mixed. The PBS was preheated to 30°C±1°C. The temperature was reserved at 30°C for the experiment. After another 10 min of stirring, the ethosomal solution was kept at 4°C overnight to allow for swelling. A ultrasonicator (Rivotek™) was used to probe-sonicate the ethosomal suspension for five min at 4°C in order to produce smaller vesicles. To create homogeneous ethosomes, sonicated vesicles were extruded 10 times through 0.45 and 0.20 µm Mini smart sterile syringe filters (Sartorius, AG, Germany) and kept at 4°C until further analysis. Comparably, a similar process was used to create the blank ethosomes (Blank-ETH), however this time; no LTZ was added throughout the preparation phase. For *in vitro* skin penetration as well as cellular absorption experiments, Rhodamine 6G (Rh6G-ETH, 0.03% w/v) loaded ethosomes were also synthesized using the same technique, but Rh6G was used in place of LTZ. Details of batch mentioned in Table 1.[37]

Experimental design

The ethosomal formulations were optimized using a 32 factorial design 2-factor, 3-level for LTZ ethosomes. The 2 factors were assessed at 3 distinct levels (low, medium and high) and using the Software: Design Expert software, experimental trials were conducted utilizing all nine possible combinations (Version 7.0.0, Stat-Ease Inc., USA). The dependent variables for ethosomes were vesicle size and percent entrapment efficiency (Y1) and (Y2), respectively, whereas the independent factors were the proportion

of phospholipid and ethanol" (X1) and (X2), respectively. For the synthesis of the ethosomes, the same amount of LTZ (5 mg) was used in each of the 9 batches. Table 1 displays the independent as well as dependent variables that were employed in the 32 factorial design technique for the LTZ-ETH formulation.

Characterization of Ethosomes

Vesicle size and PDI

The vesicle size of LTZ-ETH was determined using a Malvern Zetasizer. The vesicle size distributions of each sample were investigated in triplicate and the mean value was calculated.[39]

Zeta potential

Zeta potential of LTZ-ETH was evaluated using the Malvern Zetasizer, UK. Three different samples were tested at 25°C using a cell drive that was kept at 150 mV. Zeta potential was determined by utilizing the dielectric constant and viscosity of the dispersion medium to convert electrophoretic mobility.[38]

Surface Morphology by TEM

The carbon-coated copper grid having mesh size of 300 was used to deposit 5 µL of ethosomes on the grid and then fix the grid. The grid was then freeze-dried before being stained with uranyl acetate stain at a conc. of 1% for 3-5 min with a TEM (JEOL-JEM 1400 USA).[39]

Entrapment Efficiency

The amount of LTZ entrapped inside the vesicular system was determined by the ultracentrifugation technique. An aliquot of the ethosomal dispersion was placed in the centrifuge tubes and centrifuged at 40 000 rpm for 3 hr at 4°C using a Sorvall TM MTX 150 Micro Ultracentrifuge (Thermo scientific, Mumbai, India). Following centrifugation, the free LTZ present in the supernatant was separated from the pellet, appropriately diluted and quantified for LTZ content by using UV visible spectrophotometer (Shimadzu-1900) at 243 nm. To assess the total LTZ present in the prepared ethosomal dispersion, it was lysed with methanol and subjected to sonication. The solution was filtered through 0.45 mm filters, diluted and analyzed by UV visible spectrophotometer. The percentage Entrapment Efficiency (% EE) was determined as follows:

$$\% EE=(T-S)/T\times100 \quad \text{Eq. 1}$$

Where T is the total amount of LTZ in the emulsion; S is the amount of LTZ present in supernatant only and T-S is the amount of LTZ present inside the emulsion droplets.

In vitro anticancer activity

Cytotoxicity

The LTZ and LTZ-ETH effects on survivability of MCF-7 carcinoma cell lines were investigated using an MTT dye reduction test. Cells were placed in a 96-well plate and left overnight at 37°C. The cells were incubated for 48 hr at different concentrations (0.325 to 100 µg/mL) of the LTZ and LTZ-ETH. Following the withdrawal of test solutions, 100 µL of MTT (6 mg/10 mL in PBS) was added to every well. The plates were then incubated for 4 hr in a similar condition. After eliminating trash, 100 µL of DMSO was injected to dissolve the formazan crystals produced by living cells. The solution absorbance was assessed at 570 nm using a microplate reader. The concentrations of test samples required to kill 50% of cells (IC_{50} values) were determined using dose-effect relationship curves.[40]

Apoptosis by DAPI

In a 24-well plate, $1x10^4$ cells/well were introduced, cultured overnight at 37°C in a CO_2 incubator and then treated with chemicals at a concentration of around 50 µg/mL. The cells were then incubated for 24 hr. Following a PBS wash, the cells were fixed for 30 min with paraformaldehyde (4%) and examined after being incubated for 20 L with DAPI (0.1 g/mL) in the dark. By selecting microscopic fields at random, the cells that had undergone apoptosis were enumerated and the percentage of apoptotic cells was estimated.[41,42]

Test for Live and Dead Cells

MCF-7 cells were seeded into each well of a 24-well plate (5×10^4 cells per well), which was subsequently exposed to IC_{50} concentrations of LTZ-ETH for 48 hr. After the treatment period, the cells were rinsed in PBS and fixed in 4 percent PFA solutions for 20 min. The frozen cells from each treatment were then stained for 30 min with AO (5 g/mL of each) and EB (Ethidium Bromide). Cells were viewed under a fluorescent microscope at a 10x magnification after incubation.[43]

In vitro Cellular Uptake

Using a fluorescence microscope, the qualitative analysis of ethosome absorption by cells was carried out. MCF-7 cells were initially cultivated for 24 hr in 24-well microplates. After 24 hr, the cells were exposed to Rh6G-ETH ("Rhodamine-Loaded Ethosomal) and Rh6G for different amounts of time (1, 3 and 6 hr). After the culture media was withdrawn and the cells were preserved with PFA ("Paraformaldehyde") for 20 min, the cells were carefully washed twice with PBS. After then, the cells were looked at under a fluorescent microscope. To assess the absorption of LTZ ETH formulations with cells, MCF-7 cells were seeded on twenty-four well plates at a density of $5x10^4$ cells/well and cultured in 1 mL of growth medium. Following a 24-hr incubation period at 37°C, the linked cells were given equal doses of LTZ and kept at that temperature in a 5 percent CO_2 incubator. Before being three times rinsed with buffer and lysed with methanol, the cells were incubated for 1, 3 and 6 hr.[44]

In vitro skin permeation study

Fluorescence microscopy was utilized to determine the ethosomes' capacity to penetrate the skin layers. A homogenous application of plain Rh6G and Rh6G-loaded ethosomes was made to the goat ear skin and placed on the Franz diffusion cell assembly. The skin has been removed from cell and properly rinsed with water to eliminate any remaining formulation after the 1h treatment. The treated portion of the skin specimens was removed and fixed within buffered formalin at a 10% concentration. Following fixation, skin specimens were embedded in paraffin wax and sectioned perpendicular to the surface at 4.5µm thickness with a microtome. The acquired thin tissue ribbons or sections were placed on the glass slides to be detected using a fluorescence microscope (Olympus, Japan) for the existence of fluorescence within the skin layers.[44]

FTIR spectroscopy

FTIR spectrophotometer (Bruker, Alpha-II) was used to record the FTIR spectra of LTZ, PL90G, carbopol-934 and LTZ-ETH along with ethosomal formulation excipients in a range of 4000 to 650 cm^{-1} frequency.[45]

Differential Scanning Calorimetry (DSC)

LTZ, Phospolipon-G90 and LTZ-ETH were used in the DSC. The samples were placed on a DSC reference dish and DSC thermograms were recorded using a Differential Scanning Calorimeter (Mettler Toledo® USA) in a nitrogen environment at a warming rate of 10°C/min.[46]

Stability Studies

A stability study of lyophilized LTZ-ETH was carried out at 4±1°C and 25±1°C temperature for 90 days. Samples were analyzed for physical parameters like zeta potential and vesicle size after specific time intervals (30, 60 and 90 days).[47]

Statistical analysis

The data are expressed as the mean ± Standard Deviation (SD) derived from three independent experiments to ensure reliability and reproducibility. Statistical analysis was performed using GraphPad Prism software version 8 (GraphPad Software, Inc., La Jolla, CA, USA). The obtained results were subjected to one-way Analysis of Variance (ANOVA) to determine statistical significance. A *p*-value of less than 0.05 ($p<0.05$) was considered indicative of a statistically significant difference, highlighting the effects observed in the experiments.

RESULTS

Experimental design for the preparation of LTZ-ETH

Impact of independent factors on the LTZ-ETH vesicle size (Y1)

The LTZ-ETH formulations' vesicle sizes are displayed in Table. LTZ-ETH 3 had the smallest vesicle size measured (142 nm), whereas LTZ-ETH 7 had the largest vesicle size measured (247 nm). The following quadratic equation explains how independent variables affect vesicle size.

Y1=+190.27+32.52X1-19.85 X2+ 0.17X1X2+3.85 X1 2+1.05X 2 2

Table 1: Full factorial Design matrix summarizing the levels, factors and responses of 09 runs for optimization of LTZ loaded Ethosomes.

Batch code	Factor X_1	Factor X_2	Y_1	PDI	Y_2
LTZ ETH-1	-1	-1	182.2±2.94	0.314±0.28	61.50±5.24
LTZ ETH-2	-1	0	161.8±2.48	0.247±0.27	72.12±4.27
LTZ ETH-3	-1	1	142.9±1.85	0.152±0.19	74.69±3.82
LTZ ETH-4	0	-1	211.8±3.14	0.194±0.23	78.86±4.38
LTZ ETH-5	0	0	190.5±2.95	0.268±0.27	86.35±2.84
LTZ ETH-6	0	1	170.6±2.57	0.165±0.24	88.44±3.71
LTZ ETH-7	-1	-1	247.2±4.26	0.372±0.37	89.92±4.24
LTZ ETH-8	-1	0	226.2±3.63	0.367±0.32	90.92±2.57
LTZ ETH-9	-1	1	218.6±3.72	0.195±0.20	92.45±3.42
Factor (Independent variables)			Actual levels		
			Low (-1)	Medium (0)	High (+1)
Factor X_1: Phospholipon-90G (% w/v).			1	2	3
Factor X_2: Ethanol (% v/v).			20	30	40
Dependent variables or Responses.			Constraint		
Response 1: Y_1: Vesicle size (nm) (%).			Minimize		
Response 2: Y_2: Entrapment Efficiency (%).			Maximize		

Impact of independent factors on Entrapment Efficiency (EE) (Y_2) of LTZ-ETH.

Where Y_1 is the vesicle size, X_1 is the % of PLG and X_2 is the % of ethanol. According to the equation, ethanol has a negative impact on vesicle size while phospholipid has a beneficial impact. This indicates that the size of the vesicles in letrozole ethosomes rose as the % of phospholipid increased while it reduced as the % of ethanol increased. The high coefficient value of X1 indicates that, when compared to other factors, phospholipid had a more dramatic impact on the vesicle size of letrozole ethosomes. The model is significant according to the Model F-value of 2484.36. An excellent fit is shown by the Predicted R^2 value of 0.9971, which is in good agreement with the Adjusted R^2 value of 0.9994. Significant ($p>0.05$) behavior is defined as a p value (significance probability value) less than 0.05. The bold-faced values are not significant. Indicators of the impact of independent variables on vesicle size include 2D contour plots and 3D response surface graphs (Figure 1A). These Figures and plots make it abundantly clear that while vesicle size declined with an increase in ethanol concentration from 20% to 40%, it grew significantly with an increase in phospholipid % from 1 to 3%. To determine the variables that have the greatest impact on the response, a perturbation graph was created (Figure 1B). Factor A for vesicle size exhibits a sharp slope, while Factor B exhibits a small bend. It suggests that % of phospholipid was a significant factor.[48,49]

Table 1 displays the findings of the LTZ-ETH formulations' entrapment effectiveness. LTZ-ETH 9 achieved the highest entrapment efficiency (92.45%), whereas LTZ-ETH 1 achieved the lowest value (61.50%). The following quadratic equation can be used to show how independent factors affect the percent EE.

Y2=+85.99+10.83 X1+4.22 X2-2.67 X1X2-4.28 X1 2-2.15 X2 2.

Where X1 is the proportion of phospholipids, X2 is the % of ethanol and Y2 is EE. The equation shows that it is evident that % of lipid has a positive effect on the vesicle size and % of ethanol has a negative effect. This indicates that the % EE of ethosomes increased with an increase in % of phospholipid, but it decreased with a rise in % ethanol. The high coefficient value of X2 indicates that ethanol's impact on the EE of letrozole ethosomes was more pronounced than that of phospholipid. The model is significant ($p>0.05$), according to the Model F-value of 71.55 (p 0.0026). A "Model F-Value" this large could only occur owing to noise in 0.26% of cases. Adjusted R^2 value of 0.9778 and the predicted R^2 value of 0.9002 are reasonably congruent. Figure 1C show the 3D-response surface graphs (showing the impact of independent factors on the %EE). These graphs demonstrated that letrozole entrapment efficiency in ethosomes increased when phospholipid content rose from 1% to 3%. On the other hand, it was observed that the proportion of ethanol dramatically increased when the percentage rose from 20% to 40%. However, a further rise in ethanol concentration to 50% led to a marked decline in the % of EE. To identify the variables that have the greatest impact on entrapment efficiency, a perturbation graph was created (Figure 1D). In terms of entrapment effectiveness, component A exhibits a little bend, while factor B exhibits a steep slope. It suggests that the % of ethanol was one of the most crucial variables affecting the effectiveness of drug entrapment.[50,51]

Optimization of LTZ-ETH

The LTZ-Ethosomes (LTZ-ETH) were optimized using a design space and numerical optimization method. This was done by imposing restrictions on the independent variables, specifically reducing the vesicle size and maximizing the percentage of entrapment efficiency. The software provided the optimal recipe, which included 3% phospholipid and 36.7% ethanol. This formula yielded a desirability value that was quite near to unity (0.992). The overlay plot displayed the enhanced formulation alongside their expected response values (Figure 1E). The LTZ-ETH was refined, manufactured and analysed for the reactions. Table 2 presents the response values for the optimized formulation, along with the percentage prediction error calculated by comparing the anticipated and observed values. The prediction error percentage values, which ranged from 1.28 to 1.57, were found to be within the agreed range. This confirms the success of the Quality by Design (QbD) approach that was utilized to optimize LTZ-ETH.

Vesicle Size and Zeta Potential

Development of tiny ethosomes requires large volumes of ethanol. Similar to this, the amount of PL 90G had an impact on the LTZ's particle size.[52,53] Ethosomes' zeta potential was essentially value-neutral. Because of how much ethanol is present, the vesicles' surface has a slight negative charge. According to the explanation above, the formulation LTZ-ETH 9 with the largest amounts of ethanol (40%) and P 90G (3%), had ethosomes with an optimal size of 218.6±5.72 nm (Figure 2A) and zeta potential 27.3±1.45 mV (Figure 2B).

Surface Morphology

Surface morphology of the optimized LTZ-ETH was investigated by TEM. In the TEM analysis, LTZ-ETH appeared as unilamellar vesicles nearly round shape and in nanometer size range (Figure 2C-D).

Table 2: Validation of optimized formulation of LTZ-ETH.

Response	Predicted value	Observed value	Prediction error (%)
Vesicle size (nm)	213.86	210.54	1.57
Entrapment Efficiency (%)	92.60	91.41	1.28

Characterization of optimized LTZ-ETH.

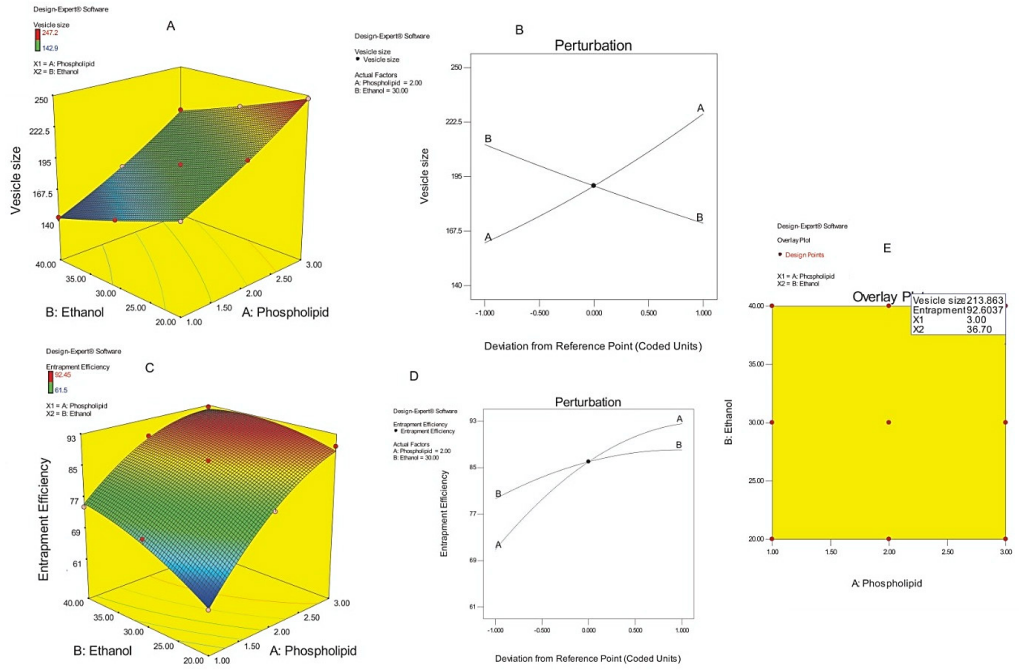

Figure 1: 3D-Response surface (A, C) and perturbation plots (B, D) showing the effect of amount of phospolipon G90 and amount of ethanol on vesicle size and zeta potential of LTZ-ETH respectively. E) The overlay plot displaying the optimized LTZ-ETH formulation in the design space and their predicted response values.

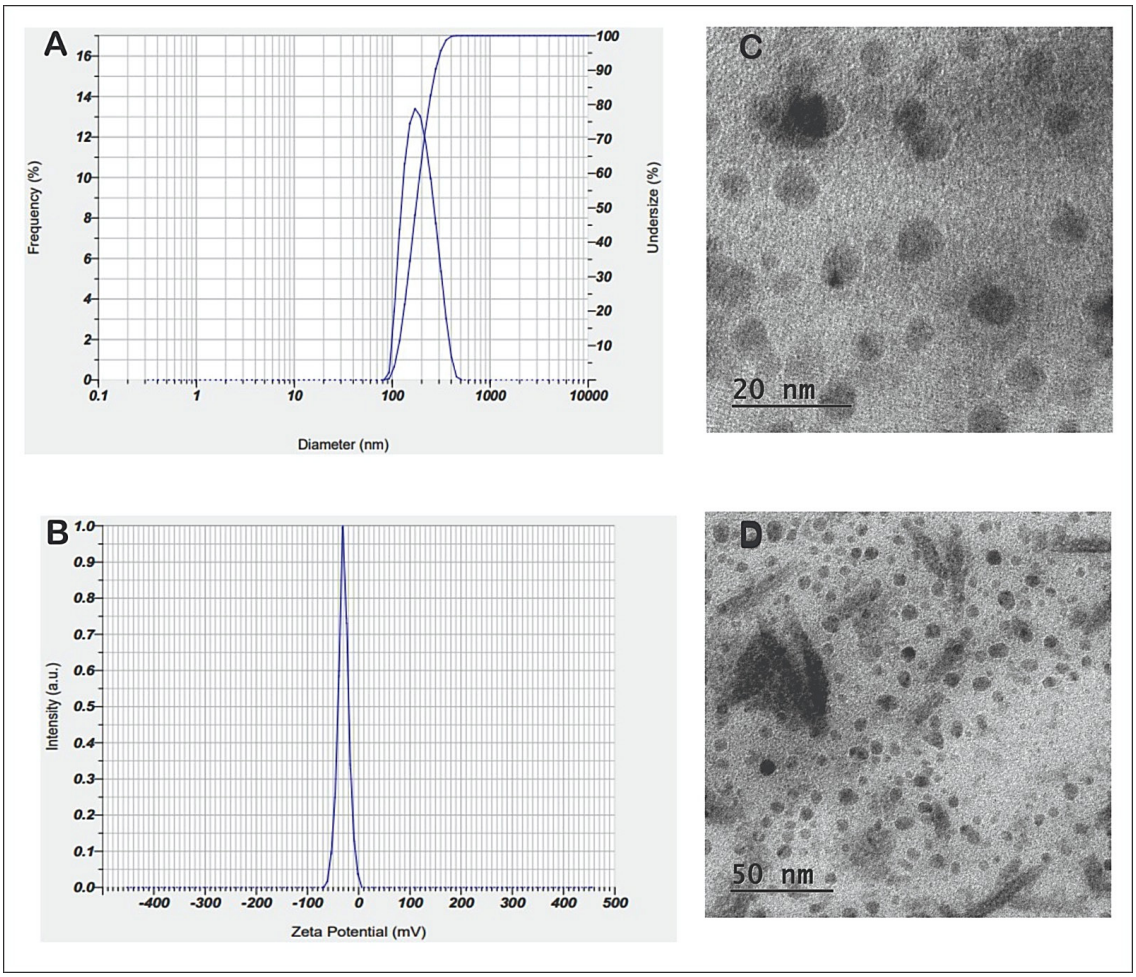

Figure 2: A) Globule size B) Zeta Potential and C) Surface Morphology of Optimized LTZ-ETH formulation.

In vitro Anticancer Activity

Cytotoxicity Study

The *in vitro* cytotoxicity of LTZ ETH was studied against MCF-7 (Breast cancer) cells using MTT assay and compared with LTZ. The % viability MCF-7 cells with respect to formulation concentration after 48 hr incubation showed concentration-dependent cytotoxicity.[54,55] The LTZ ETH exhibited significant ($p<0.01$) cytotoxicity (low IC_{50}: 23.27±1.48 µg/mL) than LTZ (58.70±2.46 µg/mL) against MCF-7 cells respectively after 48 hr of incubation (Figure 3).

Apoptosis by DAPI

DAPI staining technique was utilized to check programmed cell death for 48 hr in MFC-7 breast cancer cells after treatment with LTZ and LTZ-ETH. Nuclear morphological changes in negative control cells, LTZ and LTZ-ETH treated cells are shown in Figure 4. Negative control (untreated cells) (Figure 4D) showed weak homogenous blue staining along with intact normal nuclei whereas, in the cells treated with LTZ (Figure 4E) and LTZ-ETH (Figure 4F) circle represents chromatin condensation and nuclear shrinkage, square represents nuclear blebbing, nuclear fragmentation and arrow represents apoptotic bodies.[54,55]

Live and dead cell assay

The untreated cell groups appeared green in color with similar intensity (Figure 4A) whereas cells treated with LTZ (Figure 4B) and LTZ-ETH (Figure 4C) revealed a strong orange-red color, indicating considerable cell death. The death of the cell plasma membrane caused by LTZ at the conclusion of the treatment period could be the cause of the cell death.[56,57]

In vitro Cellular Uptake

Fluorescent dye Rhodamine-6G was introduced into ethosomes to observe intracellular uptake. Figure 5A-C and Figure D-F displays images of MCF-7 cells after 1, 3 and 6 hr of Rh6G and Rh6G-ETH treatment respectively. Up to 3 hr after Rh6G treatment, the cells exhibited no fluorescence. However, cells exposed to Rh6G-ETH showed a gradual rise in intensity of fluorescence over 6 hr. At all the periods, the fluorescence concentration of the Rh6G-ETH treated cell is much higher than Rh6G, indicating greater cellular absorption of Rh6G-ETH into the cancer cells. These findings verify the efficient internalization of LTZ ETH by MCF-7 cells as compared to LTZ.[58,59]

In vitro skin permeation study

Figure 6 displays images taken under a rat skin's fluorescence microscope after 6 hr of treatment with Rh6G-ETH and Rh6G solution (control). Only the superficial SC layer displayed fluorescence when the Rh6G solution was used (Figure 6A). In case of Rh6G-ETH, the fluorescence throughout the top skin layers relatively consistent bright fluorescence during the SC, dermis and epidermis was observed after the treatment using Rh6G-ETH (Figure 6B).

FTIR spectroscopy

We conducted this FTIR study to get some hands-on experience with Fourier-transform infrared spectroscopy using a variety of materials. Infrared spectroscopy was used to examine LTZ (Figure 7A), Phospolipon-G90 (Figure 7B) and LTZ-ETH (Figure 7C) in order to determine their purity and to identify any potential structural modifications to the drug sample. FTIR of letrozole shows specific peaks related to specific structural features as follows, -CN at 2257.69, C-H stretch at 3112.27, C=C at 1606.38, Para substitution at 821.80, C=N at 3297.14 and CN stretch at 1424.94 cm^{-1}. The values are near or equal to values mentioned in standard structure of letrozole. LTZ-ETH shows characteristic peaks, CN at 2228.61, C-H stretch at 3111.93, C=C at 1601.72, Para substitution at 867.6, C=N at 3432.53 and CN stretch at 1367.60 cm^{-1}. It demonstrated that the mixture preserves the LTZ's fundamental peaks.[60,61]

Differential Scanning Calorimetry (DSC)

Figure 8 shows the DSC thermograms of LTZ (Figure 8A), Phospholipon G-90 (Figure 8B) and LTZ-ETH (Figure 8C). It is clear that the DSC thermogram of LTZ and Phospolipon G-90 exhibits a single sharp characteristic, melting peak at 185.08°C and 160.90° which is in agreement with that reported previously. While DSC thermogram of LTZ-ETH showed melting peak at 161.54°C which is nearby equal to phospholipon-90G it was proved that the LTZ is molecularly dispersed in to lipid matrix of phospholipon.[62,63]

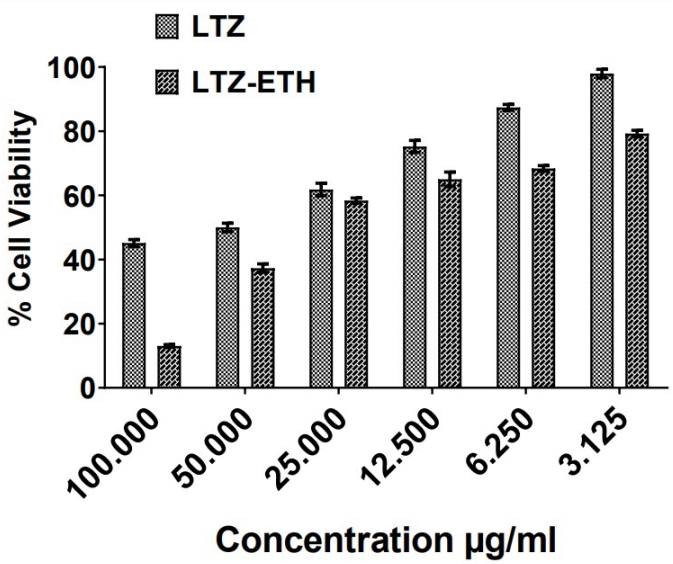

Figure 3: Cytotoxicity of LTZ and LTZ-ETH on MCF-7 breast cancer cell line (MTT assay). Data is expressed as mean±SEM (i=3). The significance difference indicated as *$p<0.05$ as LTZ compared to LTZ-ETH.

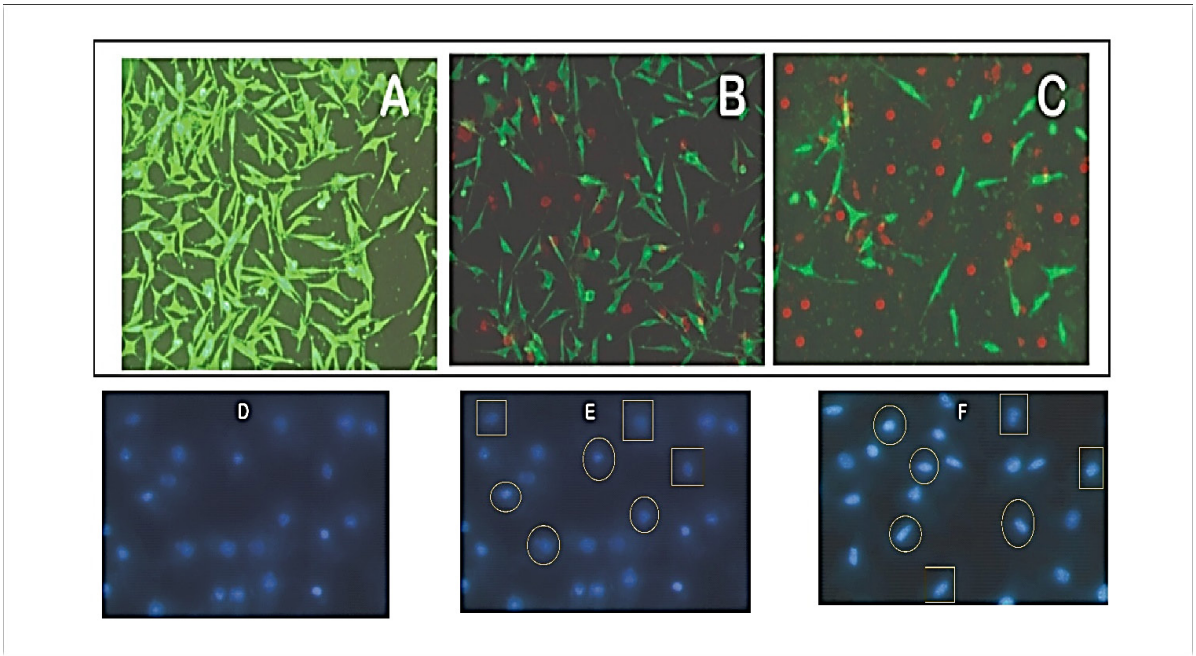

Figure 4: Live and dead cell assay of MCF-7 cells using AO and EB double staining. A) Untreated control cells, B) Cells treated with LTZ C) Optimized LTZ-ETH treated cells for 48 hr. The live cells demonstrated green fluorescence and dead cells exhibited dark-orange red fluorescence. Untreated cells are shown as controls: DAPI Apoptosis D) Normal Control E) LTZ Treated F) LTZ-ETH treated. Circle represents chromatin condensation and nuclear shrinkage, square represents nuclear blebbing and nuclear fragmentation.

Table 3: Accelerated Stability Study Data of optimized LTZ-ETH for 3 Months.

Parameter	Initial	One Month	Two Months	Three Months
Vesicle Size (nm)	215.4±2	225.4±3	228.7±4	235.5±4
Zeta Potential (mV)	-28.8±2.42	-27.6±2.14	-27.1±2.58	-26.4±2.95

All values represent mean±standard deviation (n=3).

Stability Study

Studies on the LTZ ETH stability demonstrated that the ZP, vesicle size, or outward appearance did not change significantly after being held at 4±1°C for three months. The ZP of LTZ-ETH significantly decreased and the vesicle size significantly increased at 25±1°C. The partial degradation of the phospholipids that make up the bilayer, which results in packing flaws in the membrane and makes the vesicles leaky, is what causes the greater amount of drug leakage at higher temperatures. In order to maintain the original vesicle size, reduce drug loss and increase in vesicle size, it can be advised to store ethosomal at a lower temperature. Our findings concur with those that have already been published.[64,65] Results are displayed in Table 3.

DISCUSSION

Globally there is rapid rise of cancer cases and persons suffering from it badly need complete cure from it.[1] This represents about 14.1 million of all new cancer cases. Breast cancer alone accounts for 25% of all cancer cases and 15% of all cancer deaths among females. However, breast cancer is not limited to females. Carcinoma of the male breast accounts for 0.8%-1% of all breast cancers.[3,4]

The size of the vesicle is crucial for therapeutic efficacy since it allows for extensive penetration into the epidermal layers. The produced ethosomes have particles with sizes ranging from 142.9 nm to 247.2 nm. The findings of the particle size analysis showed that the amount of ethanol and PBS added to the formulation affected the particle size of ethosomes. When ethosomes were being prepared, ethanol was utilized as a penetration enhancer to break apart the lipid vesicles that were present in the skin and make it easier for the drug to cross the stratum corneum.[64,65] When the amount of LTZ remained constant, the results of the particle size analysis shown in Table demonstrate that increasing the amount of ethanol from 20 to 40% greatly reduced the particle size. A high ethanol concentration will also alter the vesicle's surface properties, which will diminish the particle size. Furthermore, it has been observed that reduced vesicle sizes result in better permeability and bioavailability. Systems that meet the nano formulations requirements have mean vesicle sizes less than 300 nm. The amplitude of the zeta potential shows the vesicular system's potential stability. Vesicle size has significant negative or positive zeta potential, they will repel one another and generate stability in the dispersion.[66,67]

The *in vitro* cytotoxicity of optimized LTZ-ETH was studied against MCF-7 cells showed concentration-dependent cytotoxicity. Apoptosis potential of LTZ-ETH was studied by using the DAPI staining technique. Live and dead cell assay utilizes a combination of 2 highly fluorescent dyes, AO and EB, which mark living and dead cells differently. Living cells having intact cell membranes are penetrated by AO, which results in a strong, consistent green fluorescence. Nevertheless, EB could only penetrate dead cells with impaired cell membranes that glow brightly orange, or red.[68] Figure 4 displays the "fluorescence microscopic" photos of untreated (control group) MCF-7 cells as well as cells that have been given LTZ and LTZ-ETH treatment for 48 hr.

The quantitative and qualitative cellular uptake tests were carried out on MCF-7 cell lines with "fluorescence microscopy" to determine the cause underlying the improved cytotoxicity of LTZ-ETH. The increased intracellular absorption of the "ethosomal formulation" could be owing to its nano size,[69] vesicular composition and deformable nature.[70] Different physicochemical features of nanocarriers, such as particle shape, size and surface charge, were identified as having a substantial impact on the cellular absorption efficiency of nanoparticles.[71] Overall, when compared to free LTZ, LTZ-ETH effectively induces a markedly enhanced cellular uptake and cytotoxic activity in MCF-7 cells.

Since breast cancer arises from the granular tissues that are located in the bottom layers of the lobules, greater drug administration

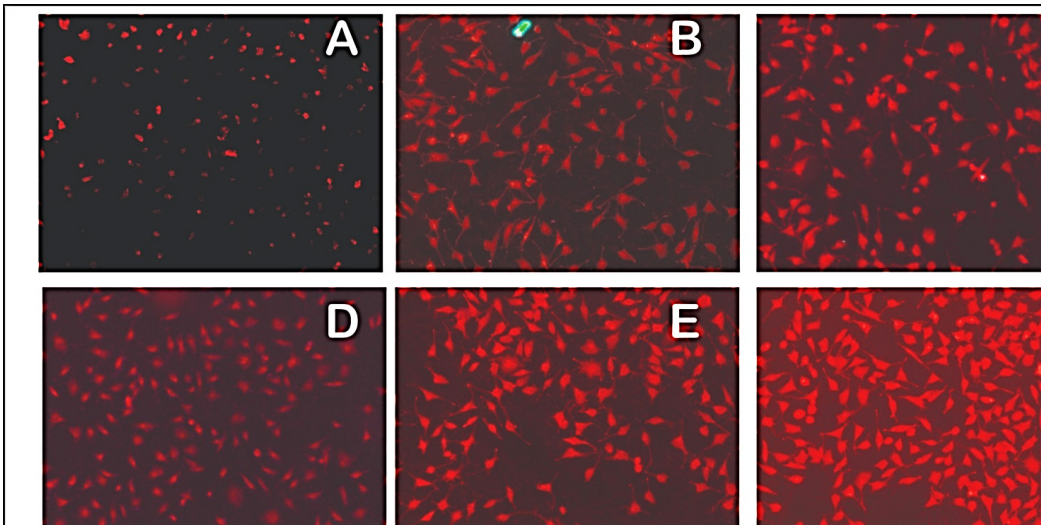

Figure 5: *In vitro* cellular uptake of Rh6G and Rh6G-ETH by MCF-7 cell lines after different Incubation times (1, 3 and 6 hr). In order to observe the intracellular distribution, red fluorescence dye rhodamine 6G was incorporated into nanovesicles.

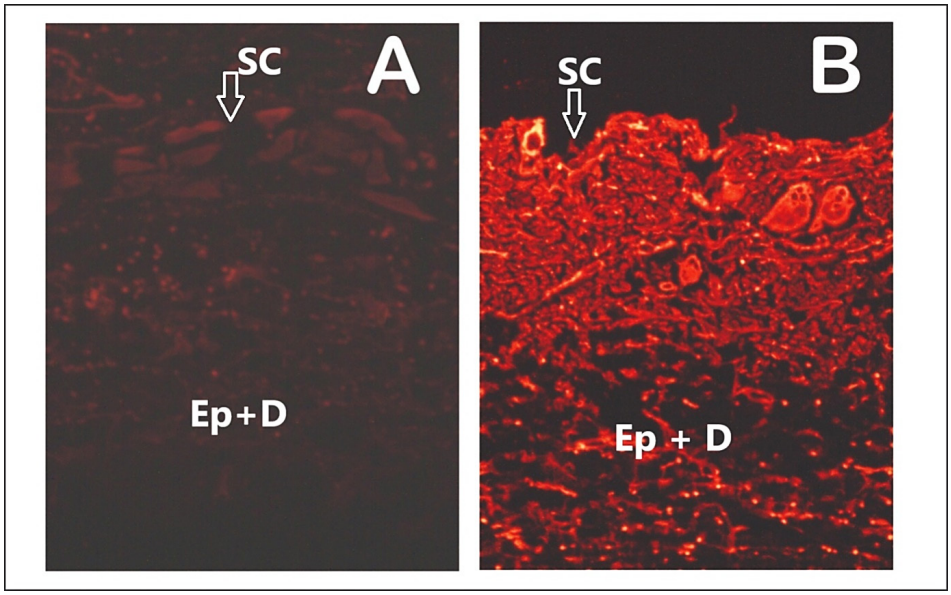

Figure 6: Fluorescence photomicrographs of goat skin after 8h application of A) Rhodamine-6G Solution B) Rhodamine 6G loaded ethosomes. Images display the distribution of fluorescence into different skin layers.

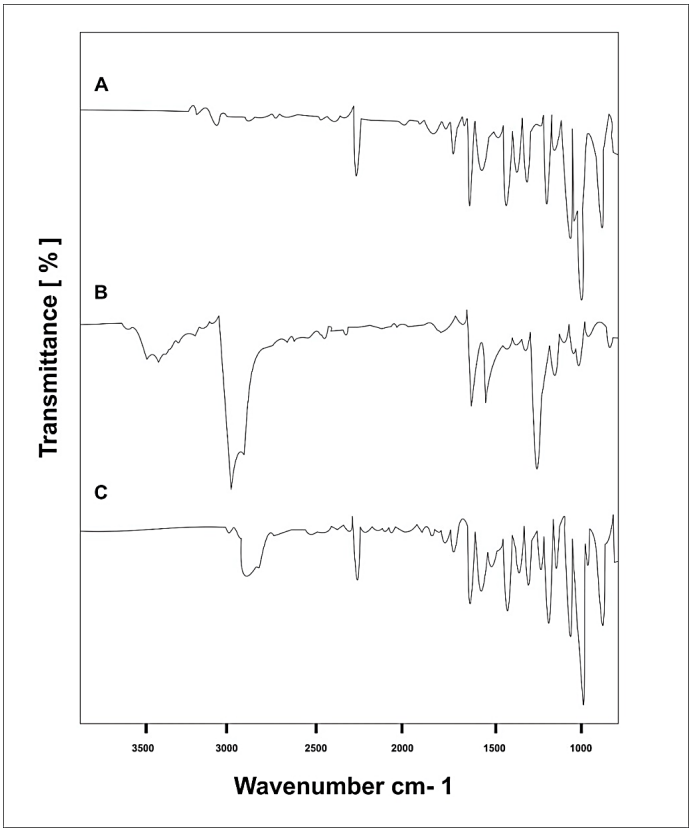

Figure 7: FTIR of A) LTZ B) Phospolipon-90G C) Optimized LTZ-Ethosomes.

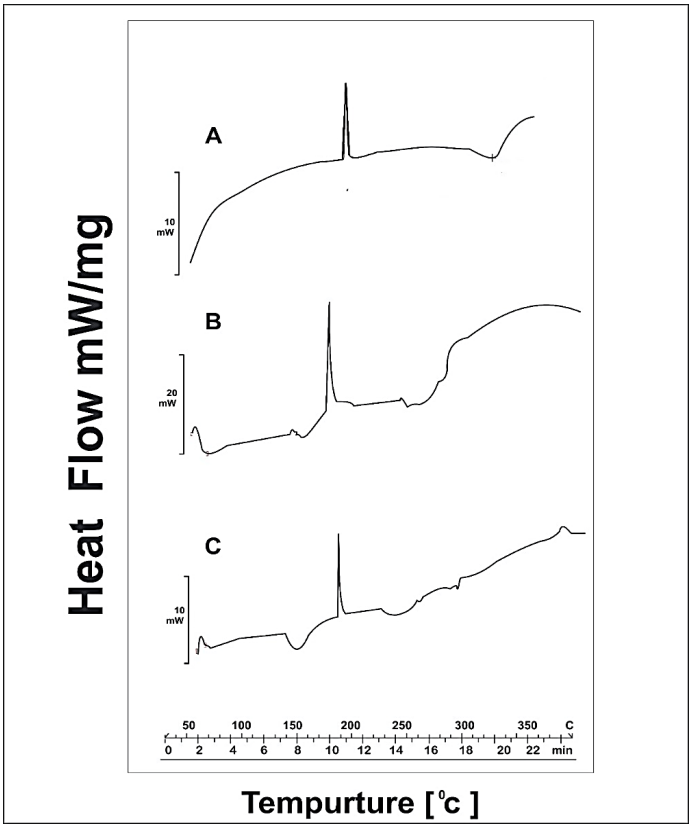

Figure 8: DSC thermogram of A) LTZ B) Phospolipon G90 C) Optimized LTZ-Ethosomes.

into the deeper skin layers is essential for effective treatment of breast cancer.[71-73] The large rise in intensity of fluorescence from deeper skin layers suggests that ethosomes are effectively delivered to these deeper skin layers that they can promote skin penetration and the active agents transport inside the ethosomes deeply into the dermis and epidermis.[74,75] Ethanol is a useful tool for improving skin penetration because it alters the organization of the lipids in the Stratum Corneum (SC) and makes the SC more fluid. Moreover, it fluidizes the phospholipids in the ethosomal structure, producing pliable vesicles that may be able to penetrate the stratum corneum's altered structure and deliver the drug to the skin's deeper layers. Transdermal absorption is the consequence of the medicine being released at different locations along the paths of penetration and the ethosomes fusing with skin lipids. The medication is released from the ethosomes deep inside the layers of the skin.[76-80] The FTIR data indicates that the drug and excipients are compatible and no interaction was observed between LTZ and its formulation excipients.[81,82] Thermogram of LTZ-ETH showed melting peak at 161.54°C which is nearby equal to phospholipon-90G it was proved that the LTZ is molecularly dispersed in to lipid matrix of phospholipon.[83-86]

CONCLUSION

Based on the findings, it was determined that LTZ is appropriate for transdermal distribution in the form of LTZ-ETH. The developed formulation performed well in terms of entrapment efficiency, vesicle size and drug release. In terms of skin permeability and other *in vitro* testing, the results support the superiority of the ethosomal formulation over the LTZ. To summaries, ethosomes can be a better drug carrier for topical administration of letrozole in the treatment of breast cancer and may serve as a competent alternative to present therapy. The outcome from release profiling and anticancer study strongly recommends that developed LTZ-ETH could play a pivotal role for treatment of breast cancer and can serve as competent alternative to current treatments. However, further studies like development of suitable conventional dosage form containing ethosomal dispersion suitable for topical application (for example semisolid dosage forms like ointment, gels, creams etc.,) are required. In addition, *in vivo* investigations of developed dosage form is needed to reveal the effectiveness of topical treatment of breast cancer.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LTZ: Letrozole; **LTZ-ETH:** Letrozole loaded Ethosomes; **ETH:** Ethosomes; **OPT:** Optimized; **OLTZE:** Optimized letrozole loaded Ethosomes; **AO:** Acridine orange; **EB:** Ethidium bromide; **MFC-7:** Breast Cancer Cell lines; **DCFH-DA:** Dichloro-dihydro-fluorescein diacetate; **Rh6G:** Rhodamine 6G; **SC:** *Stratum corneum*; **PL90G:** Phospholipon® 90G.

AUTHORS CONTRIBUTION

STG and SSH: Investigation, Methodology, Writing- original draft, review and editing Visualization, Validation, Conceptualization, Resources, Supervision, Project administration. **ASM and AK:** Methodology, Writing- review and editing, Supervision, Visualization. **SH and VS:** Writing- review and editing, Software, Visualization, Investigation, Methodology. **RK, GK and SS:** Investigation, Methodology Software, review and editing Visualization, **PK and KB:** Writing- review, editing, Conceptualization, Resources, Supervision.

SUMMARY

Letrozole is suitable for transdemal delivery in form of LTZ ethosomes. Drug-polymer compatibility studies by FT-IR and DSC showed no interaction between the LTZ and selected polymers. Particle size studies revealed that mean size of the prepared ethosomes was in the nano size. Increase in lipid concentration in ethosomes leads to increase in particle size, percentage entrapment efficiency. Increased cellular uptake of LTZ-ETH could be used as a potential alternative treatment approach to conventional chemotherapy.

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