

Development of Solid Lipid Nanoparticles with Letrozole for Breast Cancer Treatment: in-vitro and in-vivo evaluation

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Abstract

Breast cancer (BC) is the most common cancer in women worldwide. Letrozole (LTZ) is a third-generation selective nonsteroidal aromatase inhibitor and one of many anticancer drugs. Conventional LTZ therapies for BC are often administered orally or parenterally and frequently result in severe systemic side effects. To improve this situation, efforts have been made for an advanced drug delivery system that accurately releases the drug at the target site and increases therapeutic efficacy with minimal side effects. Here, we report the delivery system based on poloxamer 188 as a platform for LTZ-based solid lipid nanoparticles (SLNs) in cancer cells. The particle size, PDI, ZP, and DEE % of optimally selected LTZ -SLNs (pH 7.4) were 82.16 ± 7.24 nm, 0.287 ± 0.05 , -15.0 ± 8.83 mV, and 57.89 ± 1.22 %, respectively. The SEM of LTZ -SLNs shows hexagonal phase stacking with a nanoflock morphology. The TEM image clearly shows that the introduced LTZ -SLN drugs contain LTZ drugs inside the particles. After 48 hours, it was reported that the percentage of LTZ release was 82.34%. LTZ-SLNs showed significant cytotoxic properties with an IC₅₀ of 2.22ug/ml. The results of histopathological study confirmed that LTZ-SLNs can minimize the cytotoxicity of LTZ. Overall, the prepared nanoformulation offers great potential for BC treatment.

Keywords: Breast cancer (BC); Letrozole; Poloxamer 188; Solid-lipid nanoparticles, Nanoparticles.

INTRODUCTION

Breast cancer is the leading cause of death in women and the second leading cause of death in women. Breast cancer is a serious health problem for women and is the second leading cause of death worldwide[1]. It is the leading cause (30%) of all cancer-related deaths. Compared to men, women account for almost 99% of all BC cases [2]. Urbanization and significant lifestyle changes, especially in emerging countries, are one of the factors contributing to the increase in the number of BC cases [3].

Surgery, radiation, and chemotherapy treat BC. Systemic BC treatment includes chemotherapy. Nonspecificity and poor biodistribution lead to systemic toxicity and adverse effects. However, a major problem is the high toxicity of some anticancer drugs in healthy tissues. In addition, BC cells may develop resistance to treatment [4]. As effective alternatives to chemotherapy drugs, nano-cancer drugs are an excellent alternative to conventional anti-tumor therapies.[5].

Letrozole (LTZ) is a nonsteroidal therapeutic agent that can block excess estrogen production and is used to treat estrogen receptor-positive breast cancer [6]. It is one of the standard drugs for first- or second-line treatment of advanced breast cancer, which is hydrophobic in nature. It is usually administered orally to treat patients [7]. Several studies based on nanoparticle drug delivery have focused on LTZ delivery [8–11]. Due to various adverse effects on the nervous system, chest tenderness, headaches and a short half-life. This drawback has led to the need for an alternative mechanism of targeted drug delivery. Targeted delivery reduces the adverse effects of anticancer drugs, reduces the therapeutic dose, preserves the therapeutic effect, and enhances the pharmacological effect[12]. Uptake of active therapeutics into cancer cells by endocytosis mediated by ligands is useful for cancer therapy[7].

As drug delivery vehicles, solid lipid nanoparticles (SLNs) have the potential to enhance the safety profile and therapeutic effectiveness of traditional cancer chemotherapeutics [13]. SLN is a promising nanocarrier that usually has a solid lipid core and a layer of emulsifier molecules on the outside. The advantages of SLN include ease of preparation, excellent biosafety, and versatility of use in liquid and solid systems [14]. This delivery method has outstanding, unique properties that make it a worthwhile technique for numerous researchers who want to increase bioavailability and treat a particular condition in a more efficient manner [15–19].

The applications of LTZ are limited due to its water resistance, resulting in low bioavailability and toxicity. Targeted delivery of LTZ through the SLN delivery system appears to be the most effective strategy to overcome both this problem and the limitations of LTZ. Considering the above advantages, LTZ was entrapped in biocompatible SLNs and then subjected to a high-pressure cold homogenization procedure to evaluate its *in vitro* and *in vivo* anticancer activities. This was done in comparison to LTZ in its free form. Thus, the aim of the current work was to prepare solid lipid nanoparticles loaded with LTZ.

Material and Method

Letrozole powder (HPLC purity 99.12%) was purchased from Hetero drugs Pvt. Ltd, Hyderabad. Purified glycerol monostearate powder (GMS) and poloxamer 188 were gift samples from Sun Pharma, New Delhi. Dialysis bags (molecular weight of 14,000 Da) were purchased from Sigma-Aldrich. Sodium dihydrogen orthophosphate dehydrate (Merck) and dibasic sodium phosphate dehydrate (Sigma-Aldrich) were used to prepare 0.1 M phosphate buffer of different pH to perform the pH study. All chemicals were of analytical grade and were used without further purification. All aqueous solutions were prepared with deionized water (DI) to eliminate ions (Millipore Water Purification System).

The MDA MB 231 (human breast cancer cell line) was purchased from NCCS, Pune. Cells were maintained in DMEM high glucose media with 10% FBS along with 1% antibiotic-antifungal solution in an atmosphere of 5% CO₂ and 18-20% O₂ at 37 °C in CO₂ incubator and subcultured every 2 days. All chemicals were used as supplied without further purification or chemical modification.

Experimental animals

All studies were done in accordance with guidelines of the institutional Animal Ethics Committee, VIPER, Narsapur, Hyderabad, under approval number (01/IAEC/VIPER/Ph.D/2021-2022). Mice were anesthetized with oxygen 2% isoflurane at a flow rate of 2 ml/min. Human breast cancer xenografts were generated in nude BALB/c mice by injecting 5X 10⁶ of the interest line of the model into the left mammary line of the brain., and tumor growth was monitored twice weekly by caliper measurements. After the end of the two-week period of cell growth, tumors reached about size 100 mm³, *in vivo* tumor regression studies are performed. In each case, the formulations are injected through the tail vein using a 29G needle. In this study, the doses were administered differently according to the preliminary studies (see Table 1).

Table 1. Grouping of animals.

Groups	Subjects	Treatment Given
Group I	Negative control	Normal saline p.o.
Group II	Positive control	Breast cancer induced and normal saline p.o.
Group III	Drug	Breast cancer induced and bare LTZ
Group IV	LTZ-SLNs	Breast cancer induced and LTZ-SLNs

Preparation of poloxamer stabilised Letrozole nanoparticles

Following the method described by Affram et al. 2020, 5 different LTZ-SLN formulations were prepared using the high-pressure cold homogenization process[20]. In short, different amounts of glycerol monostearate (GMS) were mixed with 15 mg LTZ and melted on a hot plate at 75 to 80 °C. Then, using magnetic stirring, the different amounts of solution of the poloxamer-188 dissolve into distilled water and cooled on ice and added to the pre-cooled LTZ melted oil medium and then intermittently homogenized (10,000 rpm). The obtained microparticulate suspensions were finally dialyzed overnight against 1.5 L phosphate buffer (1x PBS, pH 7.4, stirred) at 4 °C, using dialysis bags with a molecular weight cut-off of 14000 Da to remove untrapped LTZ. The final LTZ-SLN nanoparticles were dried for 48 hours using 10% manitol as a cooling agent, and manitol contributed to the dispersion of nanoparticles.

Characterization of prepared nanoparticles

Particle size, Polydispersity Index and zeta potential determination

The nanoparticle size and ZS potential of the formulations were determined using the Malvern ZS zetasizer (Malvern Instrument, Worcestershire, United Kingdom). The average diameter and polydispersion index (PDI) of SLN in homogeneous mixtures were determined using the dynamic light dispersion (DLS) technique. A sample of 10 µl/ml was prepared for particle size evaluation. The dried nanoparticles were dispersed in deionized water and sonicated for at least 5 minutes to ensure that the mixture was well mixed [21].

Estimation of encapsulation efficiency (% EE)

Encapsulation efficiency (EE) is the rate at which the weight of the drug is encapsulated into the carrier system and the total amount of the drug added. The load drug is the rate at which the weight of the drug varies from the total weight of the transport system. For this purpose, 2.5 ml LTZ-SLN and its empty formula were centrifuged for 45 minutes at 10,000 rpm. After centrifugation, a clear supernatant containing a non-capsulated drug was separated into a nanocarrier. The optical density of this phase was measured by a spectrophotometer (UV-160IPC, Shimadzu, Japan) at 240 nm using a standard curve. The % EE remained designed established on Equation (i).

$$\%EE = \frac{(Total\ amount\ of\ LTZ - Amount\ of\ LTZ\ in\ supernatant)}{Total\ amount\ of\ LTZ}$$

Morphology

The surface morphology of prepared LTZ loaded SLNs was determined using Field emission- scanning electron microscope (FE-SEM; ZEISS Ultra plus, Germany), high resolution-transmission electron microscopy (HR-TEM; JEOL 2100 HRTEM, Korea).

In vitro drug release

As reported previously, LTZ release in vitro from LTZ-SLNs was investigated using the technique of lysis bags in phosphate buffer saline (PBS) with a pH of 7.4) and under simulated cancer conditions (pH 5.0) in vitro for 48 hours at 37 °C. The dialysis membrane used has a molecular weight cut-off (MWCO) of 14000 Da. A sample of LTZ-SLNs equivalent to 10 mg LTZ was dissolved in 10 mL distilled water and sealed in a dialysis bag. 50 mL each of PBS pH 7.4 and sodium acetate buffer pH 5.0 were used as the release medium. Three milliliters of the two samples were removed from the release medium at a specified interval of time. Replacement with the same fresh PBS will achieve a standardized volume and sinking condition. The amount of LTZ present in the sample was determined by UV-vis spectrophotometry at 240 nm wavelength. The study was performed in triplicate [21].

In vitro cytotoxicity studies

The MDA MB 231 (Human Breast Adenocarcinoma Cell Line) was purchased by the NCCS in Pune. The cells were maintained in DMEM high glucose media supplemented with 10 % FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO₂, 18-20% O₂ at 37 °C temperature in the CO₂ incubator and sub-cultured for every 2 days. The MTT test is a

colorimetric test used to determine cell proliferation and cell toxicity. Based on the reduction of yellow-coloured water-soluble Tetrazolium dye MTT to Formazan crystals. For this, seed 200µl cell suspension was taken in a 96-well plate at required cell density (20,000 cells per well), without the test agent. It was allowed the cells to grow for about 24 hours. To this, appropriate concentrations of the LTZ-SLNs was added. It was incubated for 24 hrs at 37°C in a 5% CO₂ atmosphere. After the completion of incubation period, plates was takeout from incubator and spent media was removed. After this MTT reagent was added to a final concentration of 0.5mg/mL of total volume. After this, plate was wrapped with aluminium foil to avoid exposure to light. It was incubated for 3 hours. MTT reagent was removed and 100µl of DMSO was added. It was stirred gently to enhance the dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals especially in dense cultures[22]. The absorbance on a spectrophotometer or an ELISA reader at 570nm wavelength was taken. % cell viability is calculated using below formula:

$$\% \text{ cell viability} = \frac{\text{Abs of treated cells}}{\text{Abs of Untreated cells}} \times 100$$

The IC₅₀ value was determined by using linear regression equation i.e. $Y = Mx + C$.

Here, Y = 50, M and C values were derived from the viability graph.

Histopathological analysis

Within thirty minutes after resection, each treated tissue was preserved in neutral-buffered formalin at a concentration of ten percent and then periodically embedded in kerosene. Sections were stained with hematoxylin and eosin. Histological changes in the vehicle control groups[23].

Results and discussion

Formulation optimization

For LTZ-SLNs, the amount of poloxamer-188 (1.0-4.0 w/v%) was varied, while GMS (0.5-3.5%) was maintained. The drug concentration of drug and water was maintained constant. The particle size and % EE was optimised and best was selected as given Table 2. From this, we selected, formulation code F3. It is because it has particle size of 82.16 ± 7.24 with 57.89 ± 1.22 .

Table 2 LTZ solid lipid nanoparticles for subcutaneous delivery

Formulation code	LTZ (mg)	Poloxamer 188 (w/v%)	GMS (w/v%)	Water (mL)	Size (nm)	% EE
F1	15	1.0	0.5	10	200 ± 6.89	17.45 ± 7.09
F2	15	2.0	1.0	10	304 ± 6.85	34.899 ± 0.92
F3	15	2.5	2.0	10	82.16 ± 7.24	57.89 ± 1.22
F4	15	3.0	2.5	10	77 ± 3.44	46.56 ± 5.6
F5	15	4.0	3.5	10	91.34 ± 6.56	53.56 ± 8.9

Size, PDI, and Zeta Potential and %EE of LTZ -SLNs

The particle size, PDI, ZP and DEE% of the optimal selected LTZ -SLNs (pH 7.4) were 82.16 ± 7.24 nm, 0.287 ± 0.05 , -15.0 ± 8.83 mV (Figure 1a & b) and 57.89 ± 1.22 %, respectively. The average size of the final formulations indicates that they are acceptable for better delivery to cancer cells because the small NPs can cross biological membranes more effectively. There is a significant difference in the average hydrodynamic diameter of the formulations with different concentrations of GSM and poloxamer 188. Smaller particles have more surface area, which aids medication absorption[24–26]. The value of the zeta potential of the formulations showed how charged the nanoparticles were. The zeta potential is an attractive factor that can be

used to determine how stable a formulation is. The greater the value of the zeta potential, the more stable the formulation[27].The negative values of the Zeta potential of LTZ-SLN match some previous reports[20].The percentage yield from LTZ-SLNs was found to be 57.89 ± 1.22 %. It was observed that ‘Poloxamer 188’ gave more drug entrapment. However, based on these findings, it can be assumed that "Poloxamer 188-GSM" is better able to achieve a higher dosage with a lower amount of formulation than other formulations.

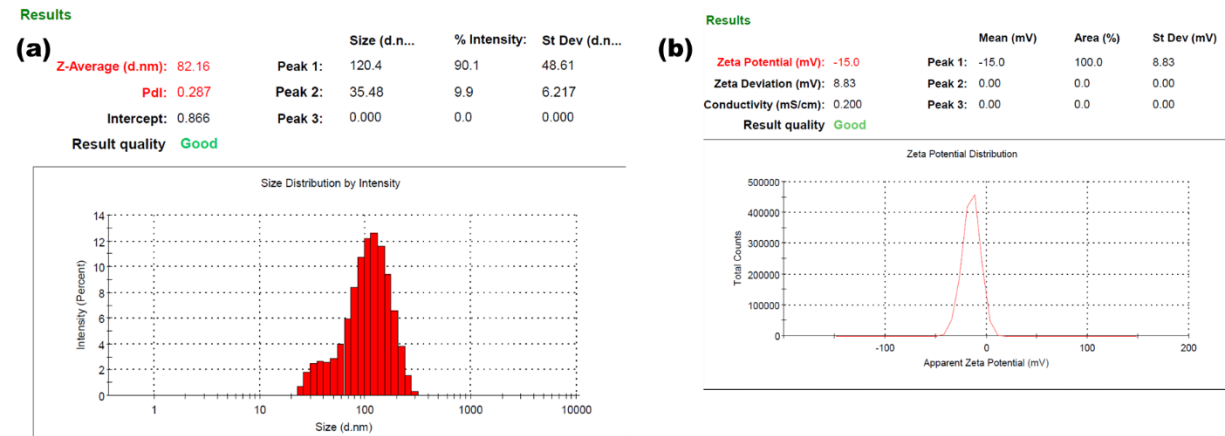


Figure 1. (a) Size distribution histogram of LTZ -SLNs(b) ZP of the prepared LTZ -SLNs

Morphological analysis

The SEM image in Figure 2a shows that LTZ -SLNs has a stacked hexagonal phase with nanoflake-like morphology. To visualize the internal microstructure of LTZ -SLNs, TEM was used. TEM Image (Figure 2b) clearly shows that the inserted LTZ -SLNs have drug LTZs inside the flakes. Consequently, the TEM image showed that the LTZ -SLNs have a size of 200 nm. The large size differences between TEM and DLS can be explained by the formation of spherical aggregates rather than individual particles. In the case of DLS, the scattering of light was evident due to the cluster and not due to a single particle. In the case of DLS, they were not aggregated. In contrast to TEM, the particles were freeze-dried by adding a freezing agent and sonicated before freezing. Excess moisture was also almost negligible with this treatment [28–30].

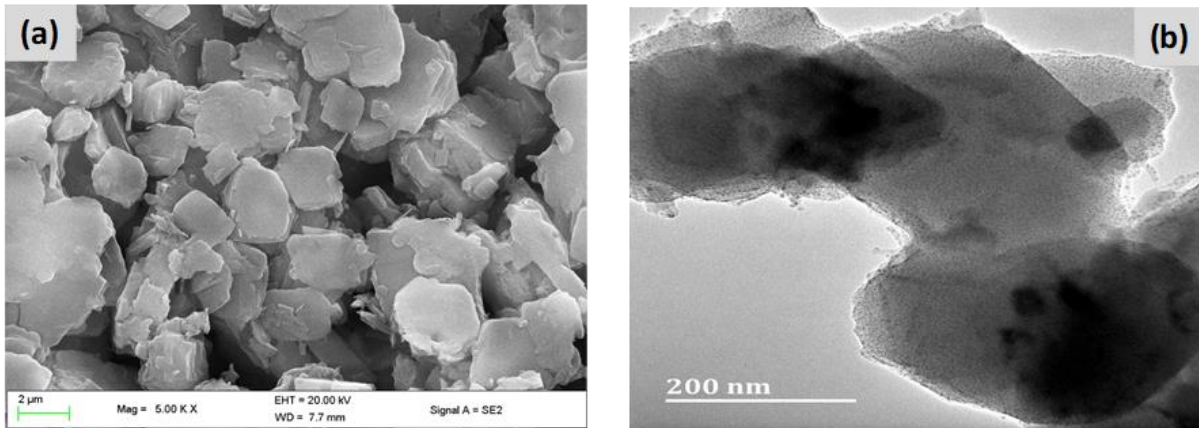


Figure 2.a) FE-SEM image of LTZ -SLNs; b) HR-TEM image of LTZ -SLNs.

In-vitro drug release study

Figure 3 shows the release characteristics of LTZ-SLNs compared to the pure LTZs. The formulations showed an irregular release of LTZs in the first 12 hours, followed by a more uniform release in the next 48 hours (Figure 3). Although the release was irregular, the LTZ SLNs were able to release 63.6% of the LTZ in the first 12 hours, while the other formulations released

less than 50% pure LTZ. After 48 hours, 82.34% LTZ was released. Several factors, such as the type of carrier system and the amount of drug on the surface of the carrier system, could cause the release to be inconsistent. However, the most important thing was to find out how the carrier system releases the drug.

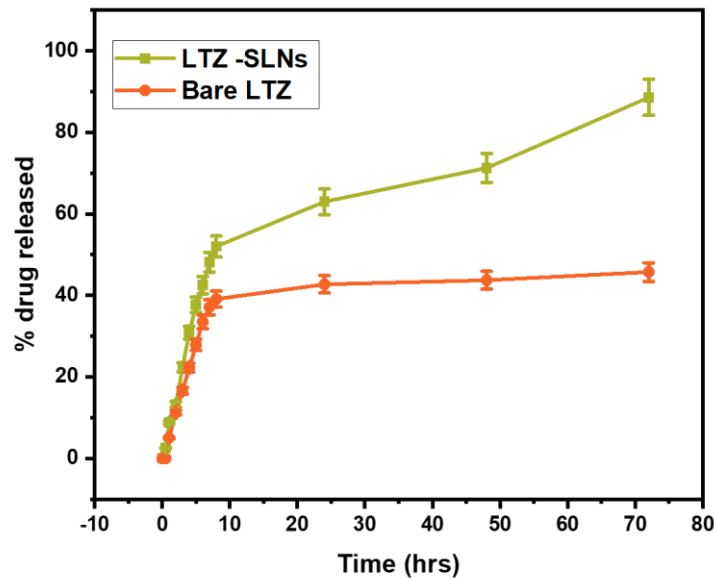


Figure 3. Comparative in vitro cumulative percentage drug release study of LTZ released from LTZ-SLNs and bare LTZ for 72h.

Cytotoxicity assay

Observations from MTT's cell cytotoxicity studies suggest that LTZ-SLNs show a significant cytotoxicity potential against the MDAMB 231 cell line with the IC₅₀ values of 2.22ug/ml. Camptothecin was used as a std control for the study. Hence, LTZ-SLNs were considered as a potent anti-breast cancer nanoformulation agents due to their low IC₅₀ values. LTZ-SLNs were considered as a potent anti-breast cancer agents due to their low IC₅₀ values as shown in Figure 4 and Figure 5.

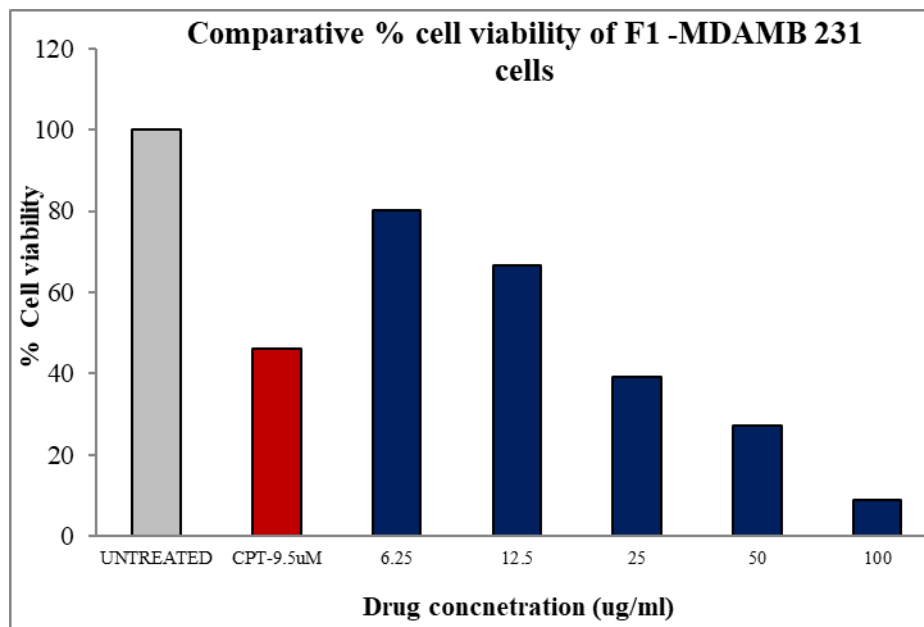


Figure 4. In vitro cytotoxicity and percent cell viability assay against MDA-MB-231 breast cancer cell lines.

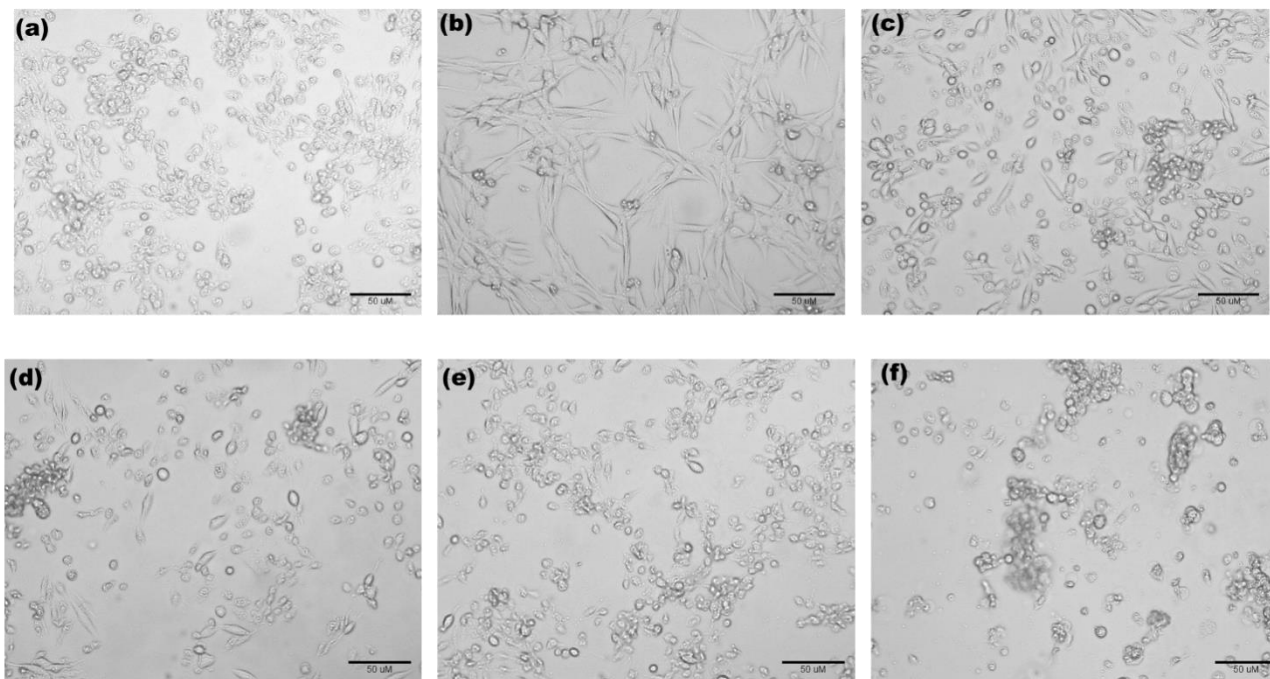


Figure 6. Morphological changes of the human breast cancer cell line MDA-MB-231 after given treatment; (a) Camptothecin-Std (b) 6.25 µg/ml; (c) 12.5 µg/ml; (d) 25 µg/ml; (e) 50 µg/ml µg; (f) 100 µg/ml

In-vivo animal study

After the in vitro administration of nanoparticles, their effects were determined by histopathological changes in the breast tissue. The image shows neutrophil infiltration, ductal in situ cribriform carcinoma, tumorstroma and adenosis, as shown in Figure 7 (a). Figure 7 (b) shows the areas of the adenomatous tissue and the quadruple differentiation. Figure 7 (c) shows adenomatous tissue and squamous differentiation areas, including the formation of the keratin pearl. Figure 7(d) shows invasive tubular carcinoma with moderate mitotic numbers and pleomorphic nuclei.

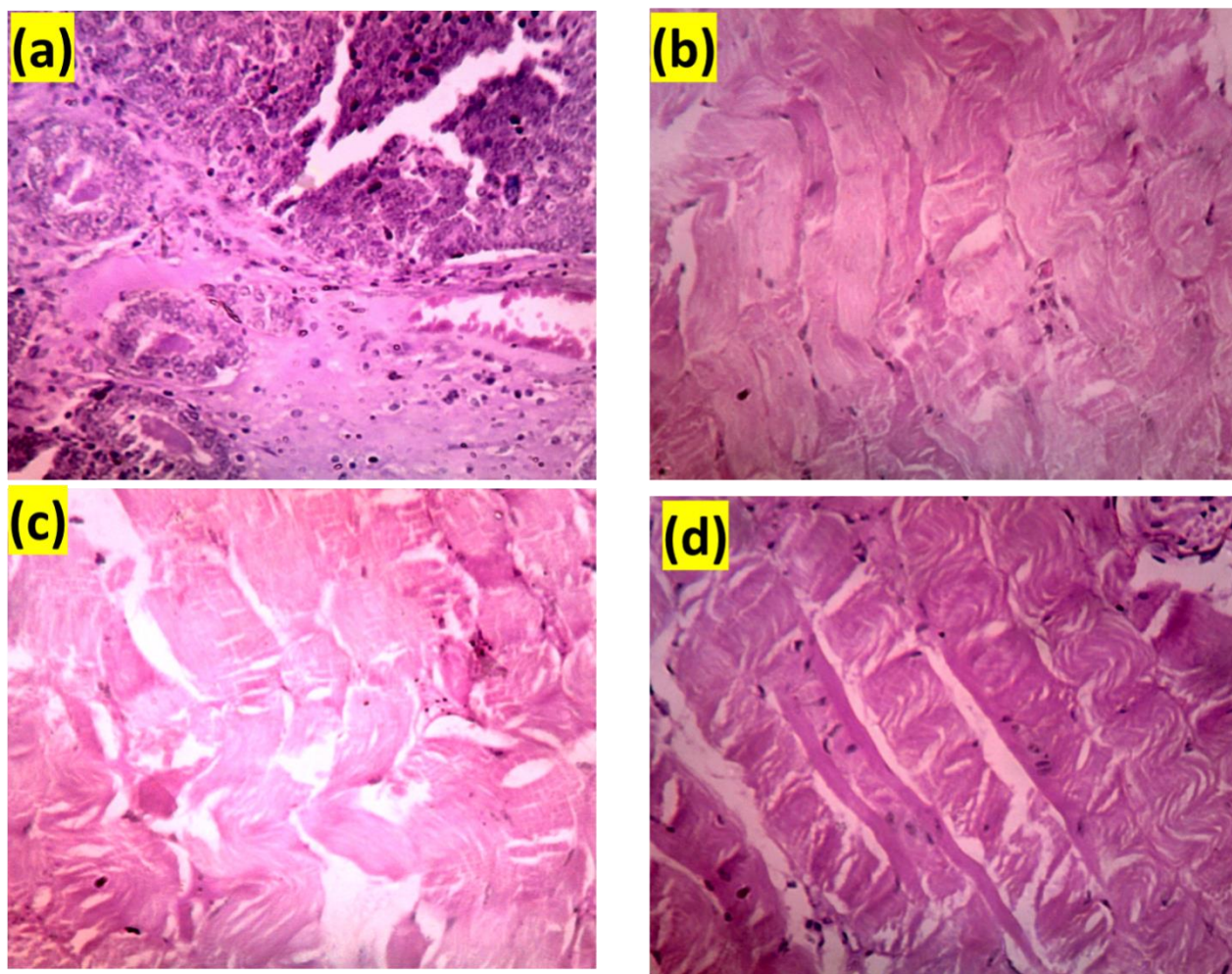


Figure 7. Histopathology of breast after treatment (a)Tumor control; (b)Standard treatment; (c)Treated with bare LTZ; (d) treated with LTZ-SLNs

Conclusion

LTZ is an aromatase inhibitor that inhibits the formation of estrogens from androgens. Nanoparticle-based therapies with local delivery are extremely promising for soft tissue cancers such as connective tissue cancer. However, knowledge of the distribution and fate of nanoparticles in vitro is critical for clinical implementation. Solid lipid nanoparticles improve the solubility, bioavailability, and delivery of anticancer drugs. The aim of this study is to improve the solubility of LTZ by adding SLN formulations to it. The proposed formulation was successfully developed and the research objectives were achieved. The formulated SLNs have a promising result in improving LTZ release and could be demonstrated in future bioavailability studies.

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