Tamoxifen Citrate- loaded synthetic high-density lipoproteins: Assessment of cellular toxicity in breast cancer cells

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Abstract:

Tamoxifen Citrate (TC) is the standard endocrine therapy for estrogen receptor (ER) positive breast cancer. TC is a selective estrogen receptor modulator (SERM) whose estrogenic properties in uterus have been linked to increased side effects like blood clots, endometrial polyps and cancer. Therefore, significant amount of research has been carried out to develop tamoxifen loaded nano-formulations with a preferential accumulation in tumor tissue rather than healthy tissues.

Synthetic high-density lipoproteins (sHDL) are novel nanocarriers with inherent active-targeting ability towards tumor cells through the ligand–receptor interaction between apolipoprotein A-I (Apo A-I) and scavenger receptor class B type I (SR-BI) overexpressed in various malignant cells. The current study was carried out to investigate whether encapsulation of TC in sHDL could improve the cytotoxic effect of TC against malignant cells. For this purpose, the cytotoxicity of TC-sHDL was evaluated in MCF-7 cell line in vitro. MTT assay demonstrated the increased cytotoxicity of TC-sHDL against cancer cells as compared with the cytotoxic effect of the free drug.

Key words: Tamoxifen Citrate, breast cancer, in vitro cytotoxicity

البروتينات الدهنية المصنّعة العالية الكثافة المحمّلة بالتاموكسفين سترات: التقييم المختبري للسمية الخلوية ضد خلايا سرطان الثدي

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****فرع الصيدلانيات ، كلية الصيدلة ، الجامعة المستنصرية ، بغداد ، العراق.***

*****فرع العلوم المختبرية السريرية ، كلية الصيدلة ،الجامعة المستنصرية، بغداد، العراق.***

الخلاصة:

دواء التاموكسفين سترات هو علاج هرموني قياسي لسرطان الثدي الإيجابي لمستقبلات الاستروجين. التاموكسفين هو مستقبل انتقائي لمستقبلات الاستروجين، ارتبط عمله كمنشط جزئي على بطانة الرحم بتزايد الأثار الجانبية كالجلطات الدموية و أورام وسرطان بطانة الرحم .أدى ذلك الى إجراء قدر كبير من البحوث لتطوير صيغ نانوية محمّلة بالدواء ذات تراكم تفضيلي في النسيج السرطاني عوضا عن الأنسجة السليمة.

البروتينات الدهنية المصنّعة العالية الكثافة هي نواقل نانوية حديثة تملك خاصية أصيلة للإستهداف النشط للخلايا السرطانية وذلك للارتباط بين البروتين (apolipoprotein -AI) و مستقبلات (SR--BI) المفرطة التعبير في الخلايا السرطانية.

تهدف الدراسة الحالية الى التحّري اذا كان تحميل التاموكسفين ضمن البروتينات الدهنية المصنعه عالية الكثافة قد يسهم في تحسين السمية الخلوية ضد الخلايا السرطانية , لهذا الغرض تم إجراء التقييم المختبري للسمية الخلوية ضد خط خلايا سرطان الثدي. وقد أظهرت نتائج الدراسة (MTT) إزدياد فعالية السمية الخلوية للصيغ النانوية المحملة بالتاموكسفين مقارنة بالتاموكسفين.

الكلمات المفتاحية: تاموكسفين سترات، سرطان الثدي ، التقييم المختبري للسمية الخلوية.

Introduction

Tamoxifen citrate (TC), whose structure is depicted in figure 1, is the gold standard for the treatment of estrogen receptor (ER) positive breast cancer. Tamoxifen is supplied as oral tablets, marketed by AstraZeneca, UK and the recommended dosage is 10-20 mg daily. Tamoxifen is defined pharmacologically as a selective estrogen receptor modulator (SERM). SERMs are compounds that interact with estrogen receptors, resulting in either estrogen-agonist or estrogen-antagonist effects based on the target tissue and physiological context [1,2]. Tamoxifen competes with estrogen for binding to estrogen receptors alpha (ERα) in mammary glands, thereby inhibiting DNA synthesis, proliferation and induces apoptosis in breast cancer cells. However, its agonist effect in uterus may lead to endometrial changes including hyperplasia, polyps, cancer, and uterine sarcoma [3,4].

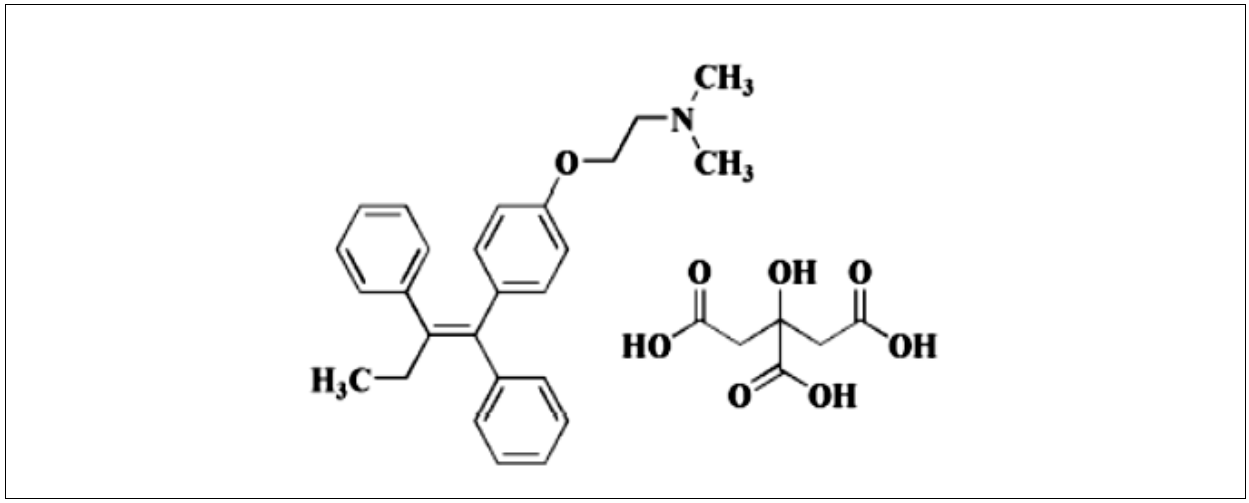


Figure (1): The chemical structure of tamoxifen citrate (5)

Significant body of research has been carried out to develop tamoxifen loaded nano-formulations with a preferential accumulation in tumor tissue rather than healthy tissues. Examples of latest research carried out include development of tamoxifen loaded temperature sensitive liposomes, tamoxifen loaded erythropoietin-coated nanostructured lipid carriers, iRGD-guided tamoxifen polymersomes, tamoxifen-loaded silk fibroin nanoparticles, and tamoxifen loaded hyaluronic acid-coated chitosan nanoparticles [6–9].

Scavenger receptor class B type I (SR-BI) is a high-density lipoprotein (HDL) receptor that mediate the uptake of cholesterol esters from HDL molecules. Overexpression of SR-BI has been observed in several malignant tumors including breast, lymphoma, prostate, adrenocortical, and ovarian cancers in order to obtain extra cholesterol needed to proliferate [10]. ApoA-I, the main protein constituent of HDL particles is known for its ability to specifically target SR-BI receptor. With this understanding in mind, HDL nanoparticles seems to be suitable candidates for targeted delivery of anti-cancer therapy [11].

HDL are naturally occurring nanoparticles known for their key role in the reversal cholesterol transport (RCT) and in the transport of vitamins, steroid hormones, signaling lipids, and micro RNAs [12]. During the last decade, a steadily increasing research have been undertaken to utilize HDL nanoparticles as delivery

systems of chemotherapeutic agents especially hydrophobic molecules as 10-hydroxycamptothecin, docetaxel, paclitaxel, valrubicin, withalongolide, and salinomycin [13–18].

Synthetic HDL are traditionally prepared using full length apoA-I isolated from human plasma or bacterial or mammalian expression systems, or alternatively using synthetic ApoA mimetic peptides to simplify the production steps and costs associated with the full length apoA-I. Mimetic peptides are short synthetic peptides with high lipid binding affinity and structures based on the amphipathic helical structure of the native apo A-I. Yet, they do not necessarily have the exact amino acid sequences[19].

In this study, apoA-I mimetic peptide, 5A, was used. 5A is a bihelical peptide comprises a proline residue connecting two amphipathic helices, each is 18 amino acid long. The molecular formula of 5A is C197H295N47O56, and the molecular weight is 4217.75 g/mol. The chemical structure is depicted in Figure (2).

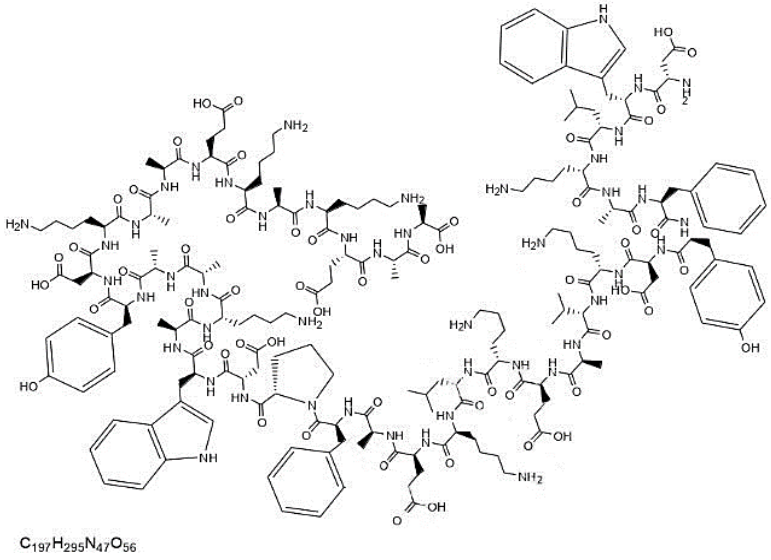


Figure (2): Chemical structure of 5A peptide provided by GenScript USA, Inc.

To the best of our knowledge, no research has been undertaken to prepare sHDL encapsulating tamoxifen citrate. In this study, we evaluate the *in vitro* cytotoxicity of the prepared formula in estrogen receptor (ER) -positive human breast cancer cell line (MCF-7).

Materials and Methods

Materials

Synthesized 5A, WLKAFYDKVAE-KLKEAFPDWAKAAYDKAAEKAKEA A, was purchased from GenScript USA, Inc. Tamoxifen Citrate (TC), and free cholesterol (FC) were purchased from Sigma Aldrich, Germany. Egg yolk lecithin, and cholesteryl oleate (CO) were purchased from Shanghai Tunchem Pharm, China. Human breast cancer cell line (MCF-7) was obtained the American Type Culture Collection (ATCC).

MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) was purchased from Ambeed, USA. Roswell Park Memorial Institute (RPMI 1640) culture medium, trypsin –EDTA, and penicillin-streptomycin were purchased from Euroclone, Italy. Fetal Bovine Serum (FBS) was purchased from Cytiva, Austria. Dimethyl sulfoxide (DMSO)was purchased from Oxford lab fine chem, UK.

Preparation of TC-sHDL

Thin film hydration method was used to prepare TC-sHDL at lipid to peptide ratio (2: 1), and drug to the carrier ratio 0.1 (w/w). Briefly, the anticancer drug TC at quantity of 6.08 mg equivalent to 4 mg free base, 20 mg egg yolk lecithin, 10 mg FC and 10 mg CO were dissolved in chloroform: methanol (1:1) volume ratio. The solvent was dried using rotary evaporator at reduced pressure, and temperature set at 50 ºC, leaving a thin lipid film on the inner walls of the flask, which was then hydrated with phosphate buffer saline PBS (pH 7.4). Thereafter,5A (20 mg) was dissolved in PBS and added into the lipid suspension followed by intermittent sonication of the resulting suspension using ultrasonic processor FS-300 N (XZB Elec, China) for one-minute duration. The mixture was incubated in water bath at 50ºC for 10 minutes and cooled below phase transition temperature of the phospholipid (-5 to -15) ºC for 10 minutes. The temperature was cycled at least three times to form sHDL(20).

Characterization of TC-sHDL

Characterization of size and morphology

The hydrodynamic diameters and size distribution (polydispersity index) of the obtained sHDL were assessed by dynamic light scattering system (DLS) using a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation, USA). Briefly, samples were diluted with equal volume of distilled water, added into a disposable cuvette and analyzed at temperature of 25ºC and angle of detection 90º.The experiment was performed in triplicate and the reported result represent the mean ± standard deviation [21].

Transmission electron microscope (TEM) (Philips, Netherland) was used to observe the morphology of nanoparticles. Sample was deposited onto carbon-coated copper grid, and stained using 1% aqueous solution of phosphotungstic acid. Next, it was allowed to dry at room temperature for 1-2 min, then examined using TEM [22].

Similarly, field emission scanning electron microscope (FESEM) (Inspect TM F50, FEI company, USA) was employed to visualize the morphology and the surface characteristics of nanoparticles. Two drop - sample was deposited onto a glass slide, and allowed to dry at room temperature. Afterwards, the sample was coated with gold under vacuum to make it electrically conductive, then it was examined by FESEM [23].

Zeta potential (ζ) Analysis

Zeta potential (ζ) determination was performed using a zeta plus zeta potential analyzer (Brookhaven Instruments Corporation, USA), under the principle of laser Doppler microelectrophoresis. Each sample was diluted with equal volume of 0.1M KCl solution, then added to a cell containing two gold electrodes. Then, ζ-potential measurement was carried out at 25ºC. The experiment was performed in triplicate and the data reported represent the mean ± standard deviation [24].

Determination of drug encapsulation efficiency (EE) and drug loading (DL)

Samples were added to amicon® ultra-4 centrifugal filter device (molecular weight cut off MWCO 10,000Da) and centrifuged at 3000 rpm to remove any unencapsulated drug. Methanol was added to break sHDL and dissolve the encapsulated drug, which was then quantified by UV spectroscopy at the λ max (277) nm. EE and DL were calculated as per the following formulas(17):

$$EE\% = \frac{\text{mass of tamoxifen citrate in nanoparticles}}{\text{original mass of tamoxifen citrate}} * 100\%$$

$$DL\% = \frac{\text{mass of tamoxifen citrate in nanoparticles}}{\text{mass of nanoparticles}} * 100\%$$

In vitro drug release profile:

The release of tamoxifen citrate from sHDL nanoparticles was evaluated using the dialysis bag method. The sHDL formulation was placed in a dialysis bag with molecular weight cut off 8000-14000 Da), and soaked in 100 mL of PBS (pH 7.4) containing 2.5% (w/v) tween 80 for 48 hours at stirring speed of 120 rpm and 37 ± 0.5ºC [25]. The volume of release medium complies to the sink condition since the concentration of the drug in the medium does not exceed 10 % of the saturated solubility of the drug (the solubility was found to be 0.9 mg/mL) [26]. At specific time points, samples of 5 mL were withdrawn from the release media and replenished with fresh PBS to maintain sink condition. The released tamoxifen was quantified by UV spectroscopy at the λ max (278) nm and the percentage of drug released was calculated. The experiment was performed in triplicate and the data reported represent the mean ± standard deviation [27].

Cell culture

Human breast cancer cells (MCF-7) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture and incubated at 37 ºC in a 5% CO_2 [28,29].

In vitro cytotoxicity by MTT assay

The MTT assay is a reliable indicator of the cellular metabolic activity. The principle of MTT assay is based on the reduction of MTT, a yellow water-soluble tetrazolium dye, by the mitochondrial dehydrogenases of the viable cells, to violet-blue water-insoluble formazan crystals. Briefly, MCF-7 cells at 90-100% confluency were trypsinized (0.25% trypsin in phosphate buffer saline (PBS, pH 7.4), and seeded into 96-well plate at density of 5000 cells per well. Cells were incubated at 37ºC to allow attachment prior initiating treatment for 24 hours. When cells reached 50-60 % growth, they were treated with either free TC (dissolved in DMSO and subsequently diluted with the culture medium) or TC-sHDL (dispersed in D.W.) at concentrations ranging from 0.35 to 25 µg /mL. MTT solution (10 mg /mL) was then added into each well after 48 hours, and plates were further incubated for 4 hours. The supernatants were removed from wells, followed by dissolving formazan crystals with DMSO. Then, optical density (OD) was determined using microplate reader (Bio-Rad, UK) at 570 nm [30,31]. DMSO was used as a negative control for cells treated with free TC, while untreated cells served as the negative control for cells treated with TC-sHDL. The dose response curve and the half maximal inhibitory concentration (IC_{50}) were obtained using Origin Pro 2021(Origin lab Corporation, USA). The cell viability percentage was calculated using the following formula:

$$\text{Cell viability \%} = [OD570(\text{sample}) / OD570(\text{control})] \times 100 \quad (32)$$

Statistical analyses

Statistical analysis was conducted using GraphPad Prism 7. Student's t test was used to compare differences between two groups, and statistically significant differences are indicated by asterisks as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

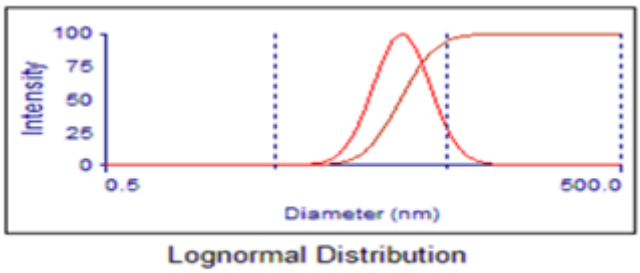
Results and Discussion

Preparation and characterization of TC-sHDL

In this study, sHDL nanoparticles encapsulating TC were prepared by the thin film hydration methodology. The DLS analysis indicated the formation of homogenous dispersion of average diameter of (35.7±12.4) nm and a narrow polydispersity index (PDI) of (0.277±0.02) as shown in Figure (3A). The size of nanoparticles is a key parameter with a significant impact on their biodistribution, biological fate, toxicity, and ability to achieve the enhanced permeability and retention (EPR) effect. Diameter range of

10-100 nm is favorable for cancer therapy; nanoparticles should be optimized so that they are large enough to avoid clearance by the kidney or leakage from normal vasculature into normal tissues. On the other hand, they should be small enough to escape the phagocytosis and clearance of the reticuloendothelial system (RES) [33]. Size distribution, designated by PDI, also exhibits clinical significance since only small variation can elicit change the in vivo outcomes and the toxicity profile of nanoparticles [34];Values of 0.3 and less are considered acceptable for phospholipid vesicles [35].

The formulation exhibited zeta potential (ζ) equals to (-48.4± 0.5) mV, indicating a satisfactory stability attribute, since dispersion is considered stable when the absolute ζ-potential is 30mV or higher [36]. Images obtained by TEM and FESEM, showed spherical particles of nanosized range that agrees with DLS results (Figure3 B&C). In addition, the prepared nanoparticles demonstrated high EE % (96.5±0.7%) and drug loading (9.65±0.1%), which indicates successful encapsulation of the lipophilic drug within the hydrophobic core of the nanoparticles. The TC-sHDL demonstrated a slow release pattern of the encapsulated drug (64.7% after 48 hours), which is a favorable attribute since rapid release behavior is correlated with systemic exposure to TC at *in vivo* setting [37]. The promising results suggest the feasibility of the prepared sHDL nanoparticles as a drug delivery system for TC and encourage further analysis to investigate the cytotoxic potential of TC-sHDL on MCF-7 cells *in vitro*.



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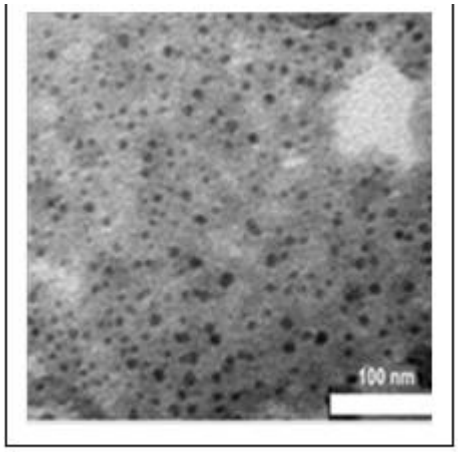
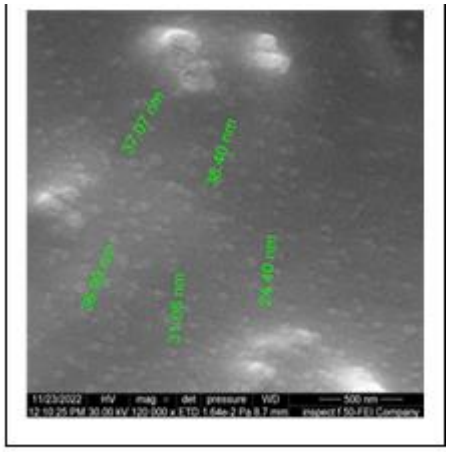


(B)



(C)

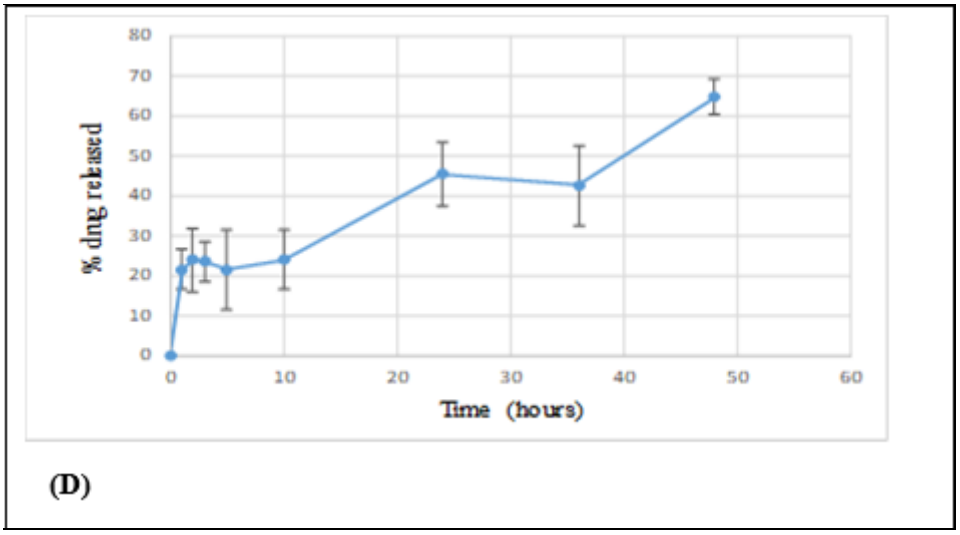


Figure (3): (A) Lognormal distribution of TC-sHDL by DLS. (B) TEM image of TC-sHDL. (C) FESEM image of TC-sHDL (D) The *in vitro* release profile of TC from sHDL in PBS pH 7.4 at 37ºC

***In vitro* cytotoxicity**

Cell survival following treatment with free TC solution or TC-sHDL are illustrated in figure (4). TC-sHDL exhibited significantly ($P<0.001$) lower IC_{50} (2.03µg /mL) than the that of the free TC (2.99 µg/mL), implying an improved cytotoxicity on MCF-7 cells compared to the free drug. Similar findings were obtained with docetaxel-sHDL against breast cancer cells (MCF-7), and valrubicin- sHDL against prostate (PC3) and ovarian (SKOV-3) cancer cell lines. The improved activity was attributed to SR-BI overexpression in cancer cells, thereby enhanced uptake and improved anticancer activity of TC by encapsulation into sHDL [14,16].

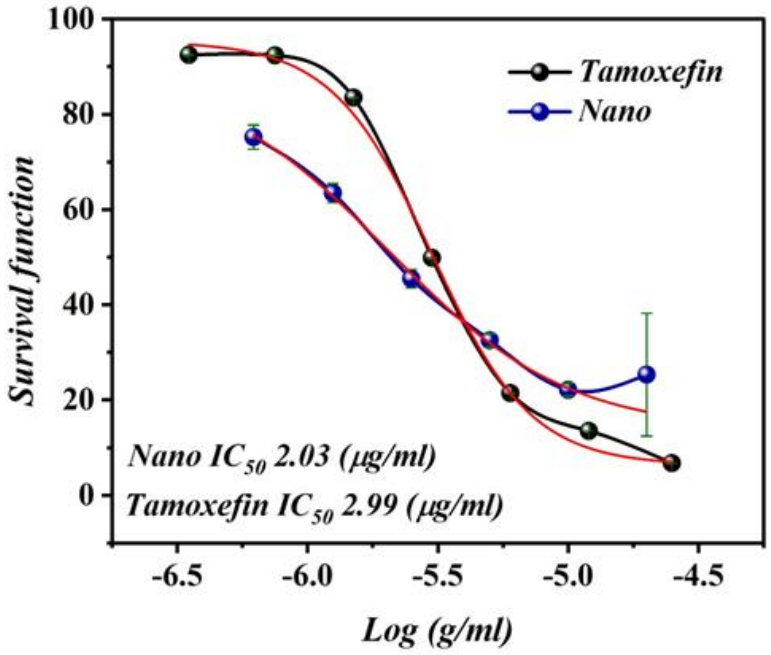


Figure (4:) Cytotoxic effect of tamoxifen and TC-sHDL nanoparticles on MCF7 cell viability

Conclusion

The obtained results suggest that encapsulation of TC in sHDL has improved the cytotoxicity of TC on MCF-7 cells compared to the free drug, implying that the prepared sHDL nanocarrier had significant potential for clinical translation.

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