# Inhibition of hTERT Gene Expression by Silibinin-Loaded PLGA-PEG-Fe$_3$O$_4$ in T47D Breast Cancer Cell Line

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**ABSTRACT**

***Introduction***: Nowadays, using drug delivery is an essential method to improve cancer therapy through decreasing drug toxicity and increasing efficiency of treatment. Silibinin (C$_{25}$H$_{22}$O$_{10}$), a polyphenolic flavonoid which is isolated from the milk thistle plant, has various applications in cancer therapy but it has hydrophobic structure with low water solubility and bioavailability. To increase the effect of silibinin, silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ was prepared to determine the inhibitory effect of this nanodrug on Telomerase gene expression. ***Methods***: The rate of silibinin loaded into PLGA-PEG-Fe$_3$O$_4$ was measured. Then, the cytotoxic effect of silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ was determined by Methyl Thiazol Tetrazolium (MTT) assay. After that, inhibition of Telomerase gene expression was indicated through Real-time PCR. ***Results***: Data analysis from MTT assay showed that silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ had dose dependent cytotoxic effect on T47D cell line. MTT assay showed no cytotoxic effect of free PLGA-PEG-Fe$_3$O$_4$ on T47D breast cancer cell line. Real Time PCR analysis showed that the level of telomerase gene expression more efficiently decreased with silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ than with free silibinin alone. ***Conclusion***: The present study indicates that this nanodrug causes down-regulation of Telomerase gene expression in cancer cells. Therefore, PLGA-PEG-Fe$_3$O$_4$ could be an appropriate carrier for hydrophobic agents such as silibinin to improve their action in cancer therapy.

## Introduction

Cancer is a primary cause of death, and breast cancer is the second-leading cause of cancer death in women.[1,2] Telomerase, a ribonucleoprotein complex, inhibits cellular ageing and retains telomere's length. This enzyme, which attaches telomeric repeats to chromosomal ends, is inactivated in most somatic cells and reactivated in cancer cells.[3] Increased reduction of telomerase activity has been found in approximately 85% of the most common cancers such as breast, prostate, lung, liver, pancreatic and colon cancers. Therefore, its expression could be as an attractive target for cancer therapy.[4,5] Silibinin (C$_{25}$H$_{22}$O$_{10}$), a polyphenolic flavonoid which is separated from the milk thistle plant, is used as a traditional medicine agent. Silibinin has a wide range of pharmacologic effects such as inhibition of cell proliferation, cell cycle progression, and induction of apoptosis in various cell lines including fibroblasts and breast cancer cells.[6]

Also, previous studies show that silibinin has negative effect on the expression of catalytic subunit of Telomerase (hTERT).[7] Due to the low side effects and silibinin-loaded PLGA-PEG (poly (D, L-lactic-*co*-glycolic acid) poly (ethylene glycol))-Fe$_3$O$_4$ action which is targeted on cancerous cells, the process of drug delivery is more effective than using free drugs in this way. Because, using an external magnetic field with magnetic nanoparticles make cancerous cells as better target.

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The most commonly used materials for preparing nanoparticle carriers are polymer, liposome, dandrimer and micelles.[8] PLGA-PEG (poly (DL-lactic-co-glycolic acid)-polyethyleneglycol)-$Fe_3O_4$ is a kind of nanoparticle that can increase the therapeutic effect of silibinin. The silibinin and the pharmaceutical drugs can be physically attached to PLGA-PEG-$Fe_3O_4$ surface and could be released into the target site due to its external localized magnetic-field gradient.[9,10] The high possibility of nonspecific toxicities and more accurate target-based dose of PLGA-PEG-$Fe_3O_4$ make better bioavailability and solubility of silibinin.[11]

In this study, we investigated the inhibitory effect of silibinin-loaded PLGA-PEG-$Fe_3O_4$ on Telomerase expression in T47D human breast cancer cell line.

## Materials and methods
### Cell Culture
Breast cancer epithelial-like cell line (T47D) was provided from Pasteur Institute cell bank of Iran (code: C203). This cell line was cultured in PRMI 1640 cell culture medium (Gibco, Invitrogen, UK). One liter of RPMI 1640 was supplemented with 2 mg sodium bicarbonate ( Merck co, Germany), 0.08 mg penicillin G (Serva co, Germany), 50 mg streptomycin (Merck co, Germany), 0.2 mg amphotricin B and 10% heat–inactivated fetal bovine serum (FBS (Fetal Bovine Serum) ) (Gibco, Invitrogen, UK). To culture T47D cells, flasks with 50ml volume were used and then 5 ml culture medium was added to each of them. Finally, they were incubated in 5% $CO_2$ at 37˚C.

### Materials and experiment for preparation of silibinin-loaded PLGA-PEG-$Fe_3O_4$
Ferric chloride hexahydrate ($FeCl_3.6H_2O$), ferrous chloride tetrahydrate ($FeCl_2. 4H_2O$) and ammonium hydroxide (25wt %) were purchased from Fluka (Buchs, Switzerland). D, L-lactide and glycolide were purchased from Sigma and recrystallized with ethyl acetate. Stannous octoate (Sn (Oct) 2: stannous 2-ethylhexanoate), polyethylene glycol (PEG) (molecular weight, 4000) and DMSO were purchased from Sigma (St Louis, USA). PEGs were dehydrated under vacuum at 70°C for 12 h and were used without further purification. Silibilin was purchased from Sigma (St Louis, USA). XR D, Rigaku D/MAX-2400 X-ray diffractometer with Ni-filtered Cu Kα radiation and scanning electron microscopy (SEM) measurements were conducted using VEGA/TESCAN. The drug loading capacity and release behavior were determined using a UV-Vis 2550 spectrometer (Shimadzu).

IR spectra were recorded at RT with Fourier transform infrared spectroscopy (FTIR) Perkin Elmer Series. The magnetic property was measured on a vibrating sample magnetometer VSM/AGFM (Meghnatis Daghigh Kavir Co, Iran) at room temperature. The 1H NMR spectra was recorded at RT with a Brucker DRX 300 spectrometer operating at 300.13 MHz. The samples were homogenized using a homogenizer (Heidolph Instruments GmbH and Co. KG, SilentCrusher M). The organic phase was evaporated by rotary (Rotary Evaporators, Heidolph Instruments, Hei-VAP Series).

### Synthesis of superparamagnetic magnetite nanoparticles
Superparamagnetic magnetite nanoparticles were prepared via improved chemical co-precipitation method.[12] According to this method, 3.1736 g of $FeCl_2·4H_2O$ (0.016 mol) and 7.5684 g of $FeCl_3·6H_2O$ (0.028 mol) were dissolved in 320 ml of deionized water, such that $Fe^{2+}/Fe^{3+}$ = 1/1.75. The mixed solution was stirred under $N_2$ at 80˚C for 1 h. Then, 40 ml of $NH_3·H_2O$ was injected into the mixture rapidly, stirred under $N_2$ for another 1 h and then cooled to room temperature. The precipitated particles were washed five times with hot water and separated by magnetic decantation. Finally, the magnetic nanoparticles were dried under vacuum at 70˚C.

### Preparation of PLGA–PEG tri-block co-polymer
Poly(lactide–*co*–glycolide)–poly(polyethylene glycol), PLGA–PEG co-polymers with molecular weight of polyethylene glycol ($PEG_{4000}$) as an initiator was prepared by a melt polymerization process under vacuum using stannous octoate ($Sn(Oct)_2$: stannous 2-ethylhexanoate) as catalyst.[13] D, L-lactide (14.4 g), glycolide (3.86 g) and PEG4000 8g (45% w/w) in a bottle-neck flask were heated to 140˚C under nitrogen atmosphere for complete melting. The molar ratio of D, L-lactide and glycolide was 3:1. Then, 0.05% (w/w) stannous octoate was added and the temperature of the reaction mixture was raised to 180˚C. The temperature was maintained for 4 h. The polymerization was carried out under vacuum. The co-polymer was recovered by dissolution in methylene chloride followed by precipitation in ice-cold diethyl ether. The synthesis process of PLGA–PEG co-polymer is shown in Fig. 1(a). A tri-block co-polymer of PLGA–PEG was prepared by ring opening polymerization of D, L-lactide and glycolide in the presence of $PEG_{4000}$ (Fig. 1(b)).[14]

### Preparation of silibinin-loaded PLGA-PEG-$Fe_3O_4$
First, $Fe_3O_4$ and the polymer were mixed through the double emulsion method and then the mixture encapsulated the drug physically.

To prepare silibinin-loaded PLGA-PEG-$Fe_3O_4$, it was physically conjugated to the PLGA-PEG-$Fe_3O_4$ in compliance with Dilnawaz et al. protocol.[15] For conjugation, 240 mg of nanoparticle was dissolved in 20 ml chloroform and then 8 mg of $Fe_3O_4$ was added. In the next step, 20 mg of silibinin was added to mixture, and then these two solutions were homogenized by homogenizer. 20 ml 1% PVA (poly vinyl alcohol) was added as a stabilizer and was mixed for 8 min. It was then centrifuged for 30 min at 11000 rpm to become sediment. After that the mixture was leached, put in room temperature for 5 hours to dry, and pulverized into powder by mortar. Successful loading of silibinin was confirmed by FTIR measurement (Shimadzu FTIR 8400S).
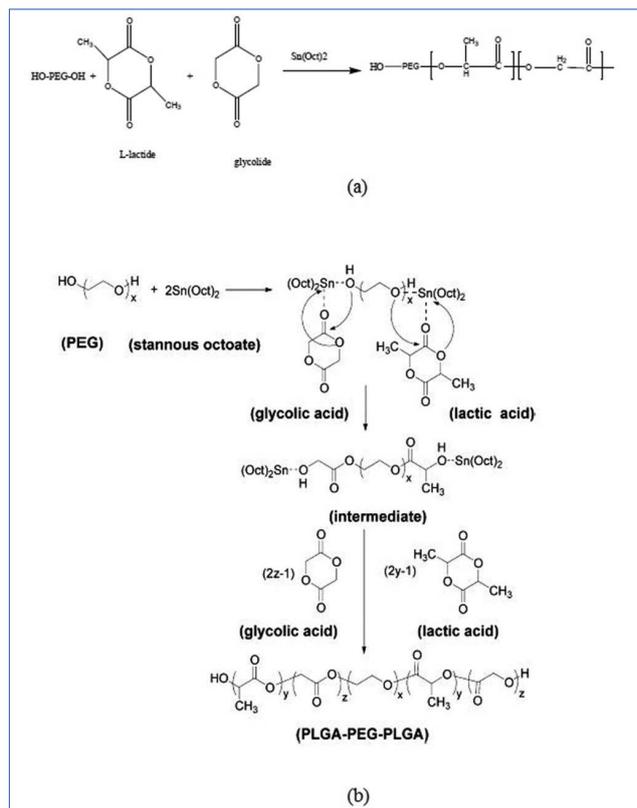
**Fig. 1(a)**. Preparation of a tri-block co-polymer of PLGA-PEG, (b). Mechanism of PLGA-PEG prepared by Sn (Oct)$_2$ as catalyst.

### Drug Release Experiment

3 mg silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ was dissolved in 30 ml PBS (PH=7); it was incubated at 37°C while being shaken. At specific times, with an interval of 3 h, 3 ml was picked up from the solution and 3ml fresh PBS was added. Supernatant was collected for estimations. The amount of released silibinin was measured through UV-vis spectrophotometery at 325 nm.

### Cytotoxicity Assay and Cell Treatment

Cytotoxicity of silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ was measured at 24, 48 and 72 h using the MTT (3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide)(MTT; Sigma-Aldrich ) assay. First, $2 \times 10^4$ cells per well were seeded and kept for 24 h in the incubator to promote cell attachment. Then, cells were treated with different concentrations of free silibilin and silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ (20-120 μM) in replicates of four. T47D cells were exposed to free silibilin and the silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ in the logarithmic phase of growth. Three controls were used; the first was 1% DMSO; the second was PLGA-PEG-Fe$_3$O$_4$ control for assessment of nanoparticle effect; and the third one was cells alone. After 24, 48 and 72 h exposure times, the cell culture medium was replaced with 200 μl fresh medium for 24 h, after that the cells were incubated with MTT solution for 4 h. Then, contents of all wells were removed and 200 μl of pure DMSO were added to the wells followed by adding 25 μl Sorensen's glycine buffer to each well. The absorbance was read at 570 nm ELISA-reader and IC50 was calculated at most within 1 h.

A concentration of 70 μM of free Silibilin and 3 concentrations of 20, 40 and 60 μM of silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ were used for T47D cells treatment. The control and treated cells were incubated at 37°C and 5% CO$_2$ for 24 h.

### Real-time PCR Assay

After 24 h, cells were washed with PBS and their total RNA was extracted from each sample using TRIZOL re-agent (Invitrogen, USA). Through absorbance measurement, RNA concentrations were determined by UV-vis Spectrophotometer (Eppendorf BioPhotometer) and at 260-280 nm purity of RNAs were estimated. The integrity of RNA was confirmed by electrophoresis of the individual samples on a 2% agarose gel. Complementary DNA (cDNA) was reverse-transcribed using the First Strand cDNA Synthesis Kit (Fermentase). To synthesize cDNA, the reaction of mixture was prepared on ice as for each reaction, 2 μl of 5 X PrimeScript Buffer, 0.5 μl of Prime-Script RT Enzyme Mix1, 0.5 μl of Oligo dT Primer and 0.5 μl of Random 6 mers accompanied by 500 ng of total RNA were used that reached to 10 μl by adding RNase Free dH20. The reaction mixture was incubated under the following conditions: 37°C, 15 minutes (Reverse Transcription); 85°C, 5 sec (inactivation of reverse transcriptase with heat treatment); 4°C. Levels of hTERT expression were determined by real–time PCR (RT-PCR). We used hTERT primers (Genbank accession: NM_198255, bp 2165-2362) and beta-actin primers (Genbank accession: NM_ 001101, bp 787-917) for real time PCR.[16] For hTERT, a 198 bp amplicon and for beta actin, a 131 bp amplicon were generated in a mixture that contained 10 picomolar of forward and reverse primers of hTERT (5′CCGCCTGAGCTGTACTTTGT3′, 5′CAGGTGAGC-CACGAACTGT3′) or beta-actin (5′TCCCTGGAGA-AGAGCTACG3′, 5′GTAGTTTCGTGGATGCCACA3′), 2μl template cDNA and 2x PCR master mix of Syber Ex Taq II.

Each RNA sample was divided into equal amounts and then, hTERT and beta-actin in parallel with each other were amplified by real-time PCR in triplicate. 20 μl of PCR reaction mixture contained 10 μl of SYBER Ex Taq II (2x) PCR master mix, 2 μl template cDNA, 6.4 μl dH$_2$O water per reaction. Negative controls were prepared each time with 2 μl ddH$_2$O instead of the cDNA template. Real time PCR amplification was performed using a Corbett (Rortor Gene 6000) system with the following setting as manufacture protocol. The reaction mixture was incubated under the following conditions: 95°C, 2 minutes, 1 cycle (Holding step); 65°C, 20 seconds, 45 cycles (Annealing); 72°C, 20 seconds, 45 cycles (Extension); 75-99 °C, 1 cycle (Melting).

### Statistical analysis

SPSS 16 was used For Statistical analysis. The differences

in mRNA levels of hTERT between control and treated cells were assessed by ANOVA and Tukey's test. A p-value < 0.05 was considered as significant.

## Results

### *1H NMR spectrum of PEG–PLGA co-polymer*

The basic chemical structure of PEG–PLGA co-polymer was confirmed by 1H NMR spectra that were recorded at RT with a Brucker DRX 300 spectrometer operating at 400 MHz. Chemical shift (δ) was measured in ppm using tetramethylsilane (TMS) as an internal reference (Fig. 2). One of the striking features was a large peak at 3.65 ppm, corresponding to the methylene groups of the PEG. Overlapping doublets at 1.55 ppm were attributed to the methyl groups of the D-lactic acid and L-lactic acid repeat units. The multiples at 5.2 and 4.8 ppm corresponded to the lactic acid CH and the glycolic acid CH, respectively, with the high complexity of the 2 peaks resulting from different D-lactic, L-lactic and glycolic acid sequences in the polymer backbone.[17]

### *Size and size distribution (SEM)*

The surface morphology of the nanospheres during the incubation period was observed by SEM. The nanographs of pure $Fe_3O_4$ nanoparticles (and silibinin-loaded $Fe_3O_4$ magnetic nanoparticles modified with PLGAPEG copolymers are shown in Fig. 3(a) and Fig. 3(b), respectively. Observing the photograph, it can be seen that the nanoparticles were well aggregated due to the nanosize of the $Fe_3O_4$ of about 10 nm. After encapsulation and modification of the silibinin-loaded $Fe_3O_4$ magnetic nanoparticles with PLGA-PEG copolymers, the size of particles changed to 25–75 nm and dispersion of the particles was greatly improved (Fig. 3(b)). This can be explained by the electrostatic repulsion force and steric hindrance between the copolymer chains on the encapsulated $Fe_3O_4$ nanoparticles. The samples were coated with gold particles.

### *FTIR spectroscopy*

The FTIR spectrum is consistent with the structure of expected copolymer. FTIR spectroscopy was used to show the structure of $Fe_3O_4$ and PLGA-PEG copolymer nanoparticles. From the infrared spectra shown in Fig. 4, the absorption peaks at 720 cm-1 belonged to the stretching vibration mode of Fe–O bonds in $Fe_3O_4$. In addition, the absorption band at 3509.9 cm-1 is assigned to terminal hydroxyl groups in the copolymer which PEG homopolymer has been removed from. The bands at 3010 cm-1 and 2955 cm-1 are due to C–H stretch of CH, and 2885 cm-1 due to C–H stretch of CH. A strong band at 1630 cm-1 is assigned to C=O stretch. Absorption at 1186–1089.6 cm-1 is due to C–O stretch. FTIR spectroscopy was done by Shimadzu spectrophotometer (Fig. 4).

Standard curve of silibinin concentration was drawn via UV-VIS spectrophotometer at 325 nm in PBS. Correlation coefficient $r^2$ was 98%.

### *In Vitro silibinin-loaded PLGA-PEG-$Fe_3O_4$ Releasing*

UV-vis spectrophotometer of 325 nm wavelength was used for drawing the curve indicating samples 1 to 15 of the nanoparticle release with nearly the same proportion of silibinin during the time (Table 1). 16th sample was a solution which remained from the aqua phase of loading procedure and showed percentage of silibinin in the environment. It is measured about 24%. 1 mg of silibinin-loaded PLGA-PEG-$Fe_3O_4$ included 760 μg silibinin. The curve indicates that this nanodrug has an appropriate amount of magnetic nanoparticle and silibinin in total.

### *MTT Assay*

Cell viability was assessed by MTT assay through exposing T47D cell line to different concentrations of free silibinin and silibinin-loaded PLGA-PEG-$Fe_3O_4$ (20-120
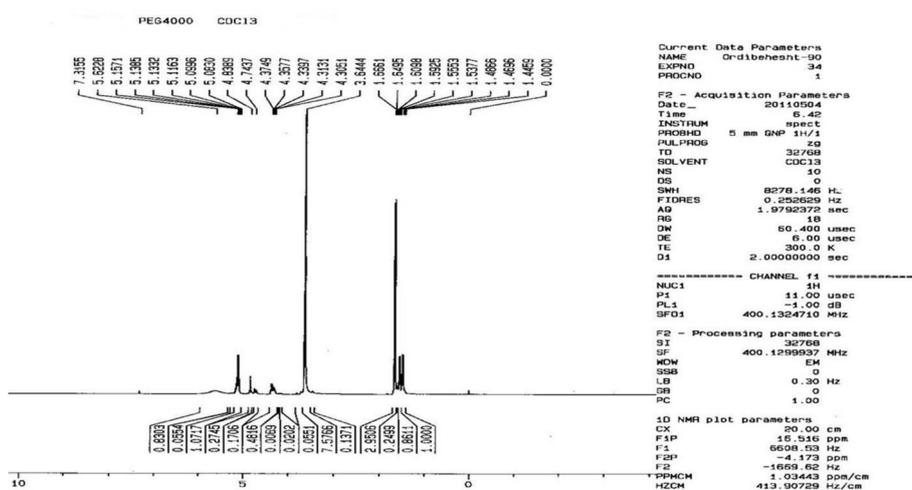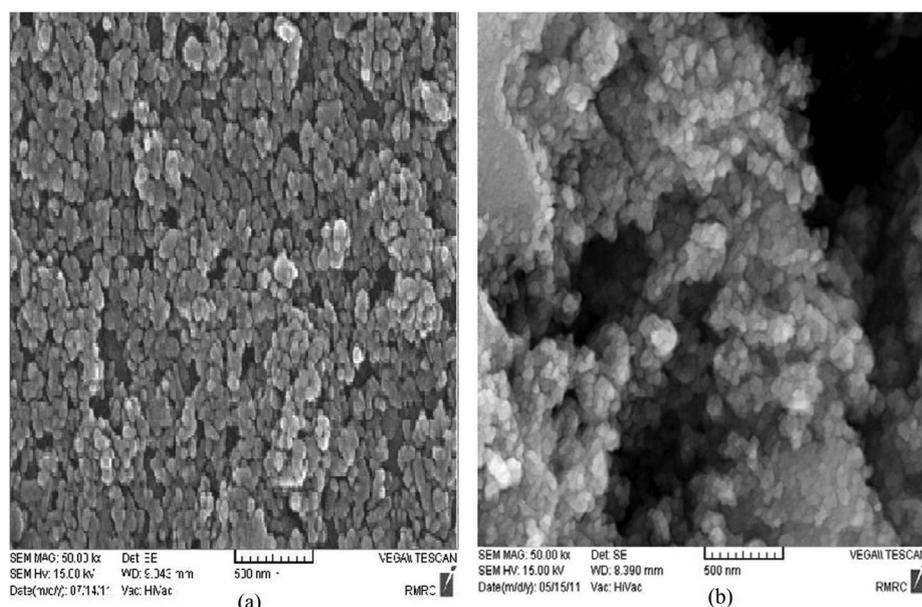


**Fig. 2**. 1H NMR spectrum of PEG-PLGA co-polymer.

**Fig. 3**. Scanning electron microscopy of (a) Fe3O4 magnetic nanoparticles, (b) Silbinin-loaded Fe3O4 magnetic nanoparticles modified with PLGA-PEG copolymers.

Abbreviations: PEG, poly (ethylene glycol); PLGA, poly (D, L-lactic-co-glycolic acid).

µM) during 24, 48, 72 h. The results in all cases show that the toxicity effect increased by increasing the drug dose, leading to the fact that these drugs are dose-dependent. This is quite opposite for the viability factor.

The free silibinin had cytotoxic effect on T47D cell line with inhibitory concentration at 50% (IC50), 107 µM for 24 h, 73 µM for 48h and 47 µM for 72h (Fig. 5).

The nanodrug, on the other hand, had cytotoxic effect of 73 µM for 24 h, 77 µM for 48 h and 74 µM for 72
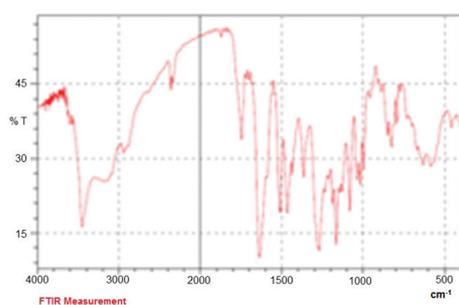


**Fig. 4**. FTIR plot of loading silibinin in nanoparticle PLGA-PEG-$Fe_3O_4$

h at IC50 (Fig. 6). The sameness of results for different periods shows time-independency of this drug. The IC50 of nanodrug was dose-dependent and did not show significant time dependency. The graph was drawn by SPSS 16 (Figs. 5 and 6). Sub-optimums cytotoxic dose of free silibinin (70 µM) and the silibinin-loaded PLGA-PEG-$Fe_3O_4$ (20, 40 and 60 µM) were taken.

*Quantitative mRNA analysis*

The levels of hTERT gene expression were measured by Real-Time PCR. Changes in hTERT expression

**Table 1**. 1-15 Silibinin-loaded PLGA-PEG-$Fe_3O_4$ samples dissolved in PBS. They show drug release during time (every 3 h); sample 16 was aqueous phase of drug releasing which remains after loading drug.

| Sample ID | Conc | WL 325.0 |
|---|---|---|
| 1 | 0.041 | 0.029 |
| 2 | 0.036 | 0.012 |
| 3 | 0.037 | 0.015 |
| 4 | 0.035 | 0.008 |
| 5 | 0.035 | 0.008 |
| 6 | 0.036 | 0.009 |
| 7 | 0.036 | 0.011 |
| 8 | 0.041 | 0.028 |
| 9 | 0.037 | 0.014 |
| 10 | 0.038 | 0.016 |
| 11 | 0.041 | 0.029 |
| 12 | 0.038 | 0.016 |
| 13 | 0.037 | 0.014 |
| 14 | 0.040 | 0.023 |
| 15 | 0.039 | 0.022 |
| 16 | 0.247 | 0.747 |

levels between the Control and treated T47D cells were normalized to beta-actin mRNA levels and then calculated by the 2-ΔΔct method. As amount of nanodrug increased, levels of hTERT gene expression decreased accordingly (Table 2). Real-time PCR data analysis indicated that by increasing amount of silibinin-loaded PLGA-PEG-$Fe_3O_4$, hTERT mRNA level expression would be decreased (Fig. 7). Each experiment was repeated three times.
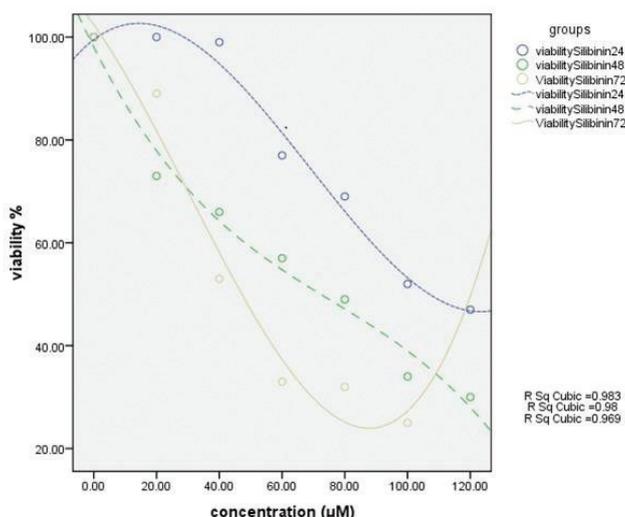
**Fig. 5**. Results of MTT assay. Cytotoxic effect of different concentrations of silibinin on T47D cell line for 24h, 48h and 72h
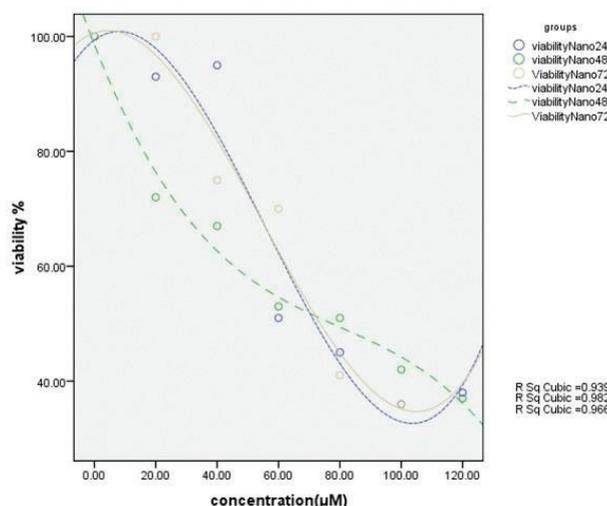


**Fig. 6**. Results of MTT assay. Cytotoxic effect of different concentrations of silibinin-loaded PLGA-PEG-Fe₃O₄ on T47D cell line for 24 h, 48 h and 72 h

Gene expression can be reduced to 70% in dose of 70 μM of this free drug. In addition, at doses of 20, 40 and 60 μM, nanodrug injection can inhibit gene expression up to 71, 90 and 98%, respectively.

**Discussion**

In this work, we tested anti-growth factor of silibinin-loaded PLGA-PEG-Fe₃O₄ on breast cancer cell line T47D and MTT assay showed that silibinin-loaded PLGA-PEG-Fe₃O₄ inhibited Telomerase in a dose–dependent manner. The results show that Silibilin can significantly inhibit hTERT gene expression (70%) and also silibinin-loaded PLGA-PEG-Fe₃O₄ has more inhibitory effect than free drug (up to 98%) in this cell line.

silibinin-loaded PLGA-PEG-Fe₃O₄ was used instead of free silibinin, demonstrating that PLGA-PEG-Fe₃O₄ enhances silibinin delivery through higher uptake by

cells; Telomerase gene expression in comparison with control cell was significantly inhibited via using silibinin-PLGA-PEG-Fe₃O₄ in T47D breast cancer cell line because it has poor bio-availability and solubility in water; thus, PLGA-PEG- Fe₃O₄ was used to enhance drug delivery. Then based on these results, the nanodrug could be an appropriate inhibitor for telomerase expression and could inhibit efficiently hTERT expression in T47D breast cancer cell line.

Silibinin has anti-carcinogenic effect on human prostate, breast and cervical carcinoma cells.[18,19] Evidence shows that anti-cancer properties of silibinins make it a great agent for cancer therapy and these activities of silibinin have been published in the scientific literature.[20-22]

One reason to select PLGA-PEG is that the PEG is located on the surface of the nanoparticles and easily exposed to the water surrounding the nanoparticles, whereas the
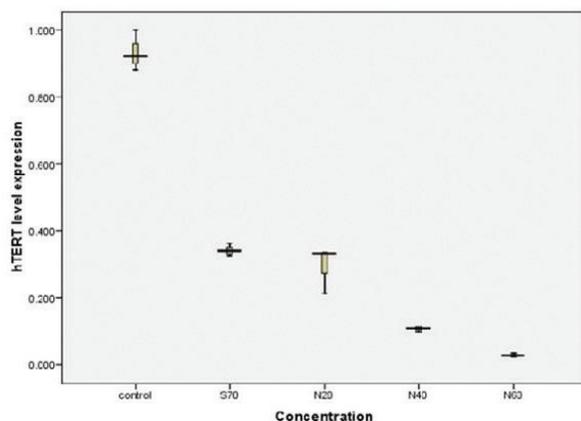
**Fig. 7**. Decreasing trend in the hTERT mRNA levels with increasing silibinin-loaded PLGA-PEG$_{4000}$-Fe$_3$O$_4$ concentration in T47-D breast cancer cell line (concentration: 70 μM is concentration of free silibinin. 20, 40 and 60 μM show silibinin-loaded PLGA-PEG$_{4000}$-Fe$_3$O$_4$ concentrations

PLGA part of the copolymer is mainly located in the relatively hydrophobic core of nanoparticles.[10]
The silibinin is loaded to the surface of the PLGA-PEG with Fe$_3$O$_4$ released at the target site due to its external localized magnetic field gradient. It is a suitable object with nonspecific toxicities producing more accurate effect on targeted tissue even when used in lower amount of silibinin. The surface and size of the PLGA-PEG are important for monitoring systemic tumor response to drug treatment.[9] PLGA-PEG$_{4000}$ which was used in this study captured maximum percentage of polymer compared to 2000 and 3000. As the rate of PEG drug loading will have better outcome, irrespective of PLGA-PEG composition, the average molecular weight and the molecular weight distribution of the PLGA-PEG remain stable over an incubation period of 7 days and can improve bioavailability of the silibinin. PLGA-PEG-Fe$_3$O$_4$ remains for few days and its rapid nanoparticle degradation in vivo would be advantageous because it would result in rapid polymer removal from the body. More interestingly, silibinin and nanoparticle with silibinin are suitable inhibitors of transcription factors like NFKB and AP-1.[10] In this study,

different concentrations of silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ were used for three different times 24,48 and 72 h by MTT assay to demonstrate cytotoxic effect of silibinin-PLGA-PEG-Fe$_3$O$_4$ on T47D cell line. For this aim, preparation of silibinin-PLGA-PEG-Fe$_3$O$_4$ was made.15 The proportion of silibinin loaded into PLGA: PEG4000 was about 76%, which means about 76% of silibinin was loaded into PLGA-PEG- Fe$_3$O$_4$. This proportion is appropriate for silibinin uptake by cells. As can be seen, the result is much better than those modified with PLGA: PEG$_{2000}$, PLGA: PEG$_{3000}$ copolymers which were 69.5% and 73%, respectively.[11]
With this system, the nanoparticle could be used as drug-loading vehicle for hydrophobic drugs with the advantage of being directed into the target tissue by its magnetic section.
Based on our knowledge, the effect of silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ on hTERT gene expression in T47D cell line has never been studied up to now. Although there is no study in the literature to be compared with this study, this nanoparticle was used and confirmed for drug delivery system in other research and articles.

**Conclusion**
The results of this work demonstrate that silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ in T47D had inhibitory effect on T47D breast cancer cell line more than free silibinin. This inhibition was dose-dependent, but it was not time-dependent. Silibinin-loaded PLGA-PEG4000-Fe$_3$O$_4$ cytotoxic effect increased by increasing the concentration of silibinin-loaded PLGA-PEG-Fe$_3$O$_4$. Analysis of data distinguished that decreasing hTERT expression level is directly associated with increasing silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ concentration. In summary, our data showed that silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ inhibited hTERT expression and this complex could be used as an anti-cancer drug in the breast cancer.

**Table 2**. hTER mRNA level in Real-time PCR measurement

| Silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ | Sample Ct | Internal control Ct | Δct | 2-ΔΔct |
|---|---|---|---|---|
| 60μM | 24.38 | 18.86 | 5.42 | 0.029 |
| 40μM | 22.55 | 18.89 | 3.66 | 0.106 |
| 20μM | 21.17 | 18.94 | 2.23 | 0.294 |
| 70 μM | 20.85 | 18.87 | 1.99 | 0.342 |
| DMSO control | 19.56 | 18.93 | 0.63 | 0.876 |
| PLGA-PEG- Fe$_3$O$_4$ control | 19.51 | 18.95 | 0.56 | 0.920 |
| Untreated cell control | 19.41 | 18.97 | 0.44 | 1.000 |

**Ethical issues**
Not applicable in this study.

**Competing interests**
The authors report no competing interests.

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