

## RESEARCH ARTICLE

# Curcumin and Silibinin Inhibit Telomerase Expression in T47D Human Breast Cancer Cells

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### Abstract

**Background:** Telomerase has been considered as an attractive molecular target for breast cancer therapy. The main objective of this work is to assess the inhibitory effects of silibinin and curcumin, two herbal substances, on telomerase gene expression in breast cancer cells. **Materials and Methods:** For determination of cell viability tetrazolium-based assays were conducted after 24, 48, and 72 h exposure times and expression of human telomerase reverse transcriptase gene was measured with real-time PCR. **Results:** Each compound exerted cytotoxic effects on T47D cells and inhibited telomerase gene expression, both in a time- and dose-dependent manner. The mixture of curcumin and silibinin showed relatively more inhibitory effect on growth of T47D cells and hTERT gene expression as compared with either agent alone. **Conclusions:** These findings suggest that cell viability along with hTERT gene expression in breast cancer cells could be reduced by curcumin and silibinin.

**Keywords:** Curcumin - silibinin - breast cancer - telomerase

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### Introduction

Breast cancer is one of the most common cancers diagnosed in women. It is the second leading cause of cancer death after lung cancer (Jemal et al., 2010). It has been shown in more than 85% of human tumors and 90% of breast carcinomas telomerase is active whereas in normal tissues it is not active or detectable. Telomerase is a ribonucleoprotein reverse transcriptase which maintains telomeric ends of eukaryotic chromosomes during DNA replication (Wang et al., 2002; Ju and Rudolph, 2006). Telomerase consists of two essential components: the functional RNA component (in humans called hTR or hTERC), which serves as a template for telomeric DNA synthesis and the other is a catalytic protein (hTERT) with reverse transcriptase activity. hTERT is highly expressed in all tissues regardless of telomerase activity, but in cancer cells generally have fivefold-higher expression. Therefore, targeting the telomerase in cancers could be promising step in its treatment (Wang et al., 2002). Different agents have been proposed for telomerase inhibition (Nakamura et al., 2005; Yeo et al., 2005; Massard et al., 2006; Zou et al., 2006). But, some of these treatments have no significant improvement on metastasized cancer and survival rate and they may have even some harsh side effects such

as chemotherapy, which causes neutropenia, anemia, thrombocytopenia, nausea, and hair loss (Bundred, 2001). In recent years, the focus of cancer control has been on the search for anticancer agents, which are safer and have higher acceptability for patients. In this regards, various natural agents such as turmeric and polyphenolic, which are generally a part of human diet or traditional herbal medications, have been taken attention (Kaur et al., 2009).

Silibinin, a naturally polyphenolic flavonoid widely consumed as a dietary supplement, constitutes a major biologically active portion of the plant extracted from *Silybum marianum* (known as milk thistle). Many studies have shown that silibinin has anti-inflammatory, anti-tumor, anti-proliferative and anti-oxidant properties and also the protective effects on doxorubicin-induced toxicities (Tyagi et al., 2004; Kim et al., 2009; Lin et al., 2009; Wang et al., 2010). Anti-telomerase activity of silibinin has been studied previously especially in prostate cancer (Thelen et al., 2004).

The other natural agent is curcumin, a yellow dye the rhizome of *Curcuma longa* (Turmeric), has shown anticancer effects against a broad range of cancers, especially in breast cancer (Choudhuri et al., 2002; Ramachandran et al., 2002). Curcumin has also impressive antioxidant, anti-inflammatory, anti-tumor, anti-

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proliferative, and anti-allergic, anti-telomerase activities (Ramachandran and You, 1999; Mukherjee et al., 2007).

Developing the previous studies about the effects of silibinin and curcumin on cancer cell lines, the aim of the current work is to determine the upregulation of these two natural plant components in both separately and mixed on expression of hTERT.

## Materials and Methods

### Cell culture and cytotoxicity

T47D breast cancer cell line (Pasteur Institute of Iran, Tehran, Iran) was grown in RPMI 1640 (Gibco, Invitrogen, UK) supplemented with 10% FBS (Gibco, Invitrogen, UK), 2 mg/ml sodium bicarbonate, 0.05 mg/ml penicillin G (Serva co, Germany), 0.08 mg/ml streptomycin (Merck Co, Germany). Culture was maintained on plastic flask and incubated at 37°C in 5% CO<sub>2</sub>. After growing sufficient amount of cells, cytotoxic effect of silibinin and curcumin was studied by 24, 48 and 72 h MTT assays in which 1000 cell/well were cultivated in a 96 well plate. After 24 h incubation in 37°C with humidified atmosphere containing 5% CO<sub>2</sub>, the cells were treated with serial concentrations of curcumin (5, 10, 20, 30, 40, 50, 60, 80, 100 μM), silibinin (20, 40, 60, 80, 100, 120, 140, 180, 200 μM), and curcumin-silibinin mixture (each of them 5, 10, 20, 30, 40, 50, 60, 80, 100 μM) for 24, 48 and 72 h in the quadruplicate manner, in addition to cells with 200 μl culture medium containing 10% DMSO for control. After incubation, the medium of all wells of the plate were exchanged with fresh medium and the cells were leaved for 24 h in incubator. Then, medium of all wells were removed carefully and 50 μl of 2 mg/ml MTT (Sigma Co., Germany) dissolved in PBS was added to each wells and the plate was covered with aluminum foil and incubated for 4.5 h again. After removing content of the wells, 200 μl pure DMSO was added to the wells. Then, 25 μl Sorensen's glycine buffer was added and immediately absorbance of each wells was read in 570 nm using ELx800 Microplate Absorbance Reader (Bio-Tek Instruments) with reference wavelength of 630 nm.

### Cell treatment

After determination of IC<sub>50</sub>, 1×10<sup>6</sup> cells were treated with serial concentrations of curcumin, silibinin and mixture of them that are below the total IC<sub>50</sub> (5μM, 10μM, 15μM and 17.5μM). For control cells, the same volume of 10% DMSO without drugs was added to flask of control cells. Then, culture flasks were incubated at 37°C in 5% CO<sub>2</sub> for 24 h.

### RNA extraction

The cells were mixed with 1 ml of RNX plus reagent in a clean RNase-free tube and then incubated for 5 min at room temperature (RT). After pipetting, 200μl chloroform was added, shaken rigorously for 15 seconds, and incubated for another 5 min. The mixture was centrifuged at 12000×g for 15 min. The aqueous phase was transferred to a clean RNase-free tube and total RNA was precipitated by adding 0.5 ml isopropyl alcohol and incubated for 15 min at RT. The pellet including total RNA

was washed using 75% ethanol and centrifuged at 7500×g for 8 min. After drying the ethanol, the RNA pellet was dissolved in TE buffer. At the end the concentration and quantity of total RNA was calculated based on OD260/280 ratio measurements and its quality was assessed by electrophoresis on 1.5% agarose.

### cDNA synthesis

Complementary DNA, cDNA, was synthesized using First Strand cDNA synthesis Kit (Fermentas). Briefly, 1μg total RNA, 1μl Oligo (dT) primers and 11μl DEPC treated water was mixed on dry ice and incubated at 65°C for 5 min and then stored on dry ice for 1 min. Reaction mixture of the components (4μl 5X reaction Buffer, 1μl RiboLock™ RNase Inhibitor (20 u/μl), 2μl 10 mM dNTP Mix), was added and incubated at 25°C for 5 min. After that, RNA was reverse-transcribed by 2μl M-MuLV Reverse Transcriptase (20 u/μl) and incubated at 25°C for 5 min and again incubated at 42°C for 60 min. Reaction was terminated the using heating at 70°C for 5 min. The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For longer storage, -70°C is recommended.

### Real-time PCR and measurement of hTERT mRNA

The synthesized cDNA was diluted 1:5, 1:10 and 1:20. The concentration of 1:20 of cDNA was used as template for real time PCR. The cDNA was amplified using specific primers (Takapou Zist Co., Iran) (Table 1) for telomerase gene and also β-actin as endogenous control (corbet 6000). RNA Expression levels were analyzed by quantitative real-time RT-PCR using the SYBR Green-I dye (Roche, Germany) by the Rotor-Gene™ 6000 system (Corbett research, Australia). Alternative spliced variants of hTERT mRNA were not measured because they do not reconstitute telomerase activity. The quality of real-time PCR reactions was controlled by running standard samples as duplicated. 5-times serial dilutions of cDNA obtained from the T47D breast cell line served as samples with strong expression of hTERT gene. The program for real-time PCR reaction was as follows; Initial denaturation at 95°C for 10 min, followed by cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. Finally, amplicons were assessed by melting curve analysis of 70°C to 95°C.

### Statistical analysis

SPSS 16 was used for statistical analysis. The difference in mRNA levels of hTERT between control and treated cells was assessed by ANOVA and Tukey's test. A p value<0.05 was considered as significant difference.

**Table 1. Forward (F) and Reverse (R) Primer Sequences of β-actin and hTERT Used in Real-Time PCR**

Genes	Sequence	PCR product size (pb)
hTERT	Forward primer	5'CCGCCTGAGCTGTACTTTGT3' 198
	Reverse primer	5'CAGGTGAGCCACGAAGTGT3'
β-actin	Forward primer	5'TCCCTGGAGAAGAGCTACG3' 131
	Reverse primer	5'GTAGTTTCGTGGATGCCACA3'

## Results

### Effect of curcumin on inhibition of T47D cells growth and hTERT expression

Growth of cell lines was inhibited by curcumin in a dose-and time-dependent manner. Data analysis of the cytotoxicity assay showed that IC<sub>50</sub>s of effect of curcumin on T47D breast cancer cell line are 25, 19 and 17.5  $\mu$ M for 24, 48 and 72 h MTT assays respectively (Figure 1A). hTERT expression in cancer cell lines after exposure to curcumin for 24 incubation times was assessed by real time PCR. Results of real time PCR showed that expression of hTERT in T47D cell lines was decreased by the incubation in 5 to 17  $\mu$ M curcumin in a dose-and time-dependent manner (Figure 2A). Curcumin treatment (24h) reduced hTERT mRNA by 80% at 17.5  $\mu$ M concentration.

### Effect of silibinin on inhibition of T47D cells growth and hTERT expression

Data analysis of cytotoxicity assay showed that IC<sub>50</sub>s of silibinin on T47D breast cancer cell line are 109, 75, 31  $\mu$ M for 24, 48, 72 h MTT assays, respectively (Figure 1B). Figure shows that IC<sub>50</sub> of silibinin on T47D breast cancer cell line is time-and dose-dependent. Real-time PCR results showed a significant decrease in hTERT

expression in the treated cells compared to the control cells (p-value<0.05) (Figure 2B). When T47D cells were treated with silibinin in concentrations between 5 and 17  $\mu$ M for 24 hours, expression of hTERT was markedly decreased.

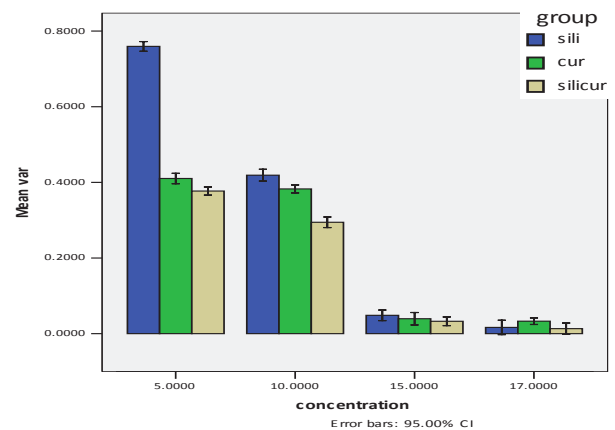
### Effect of curcumin-silibinin mixture on T47D cell growth and hTERT expression

As seen in Figure 1C IC<sub>50</sub> of curcumin and silibinin mixture are 17.5, 15, and 12  $\mu$ M for 24, 48, and 72 h exposure times, respectively (Figure 1C). These IC<sub>50</sub>s were time-and dose-dependent. Mixture of curcumin and silibinin inhibited hTERT gene expression in a dose-dependent manner. Real-time PCR results showed a significant decrease in hTERT expression in the treated cells compared to the control cells (Figure 2C).

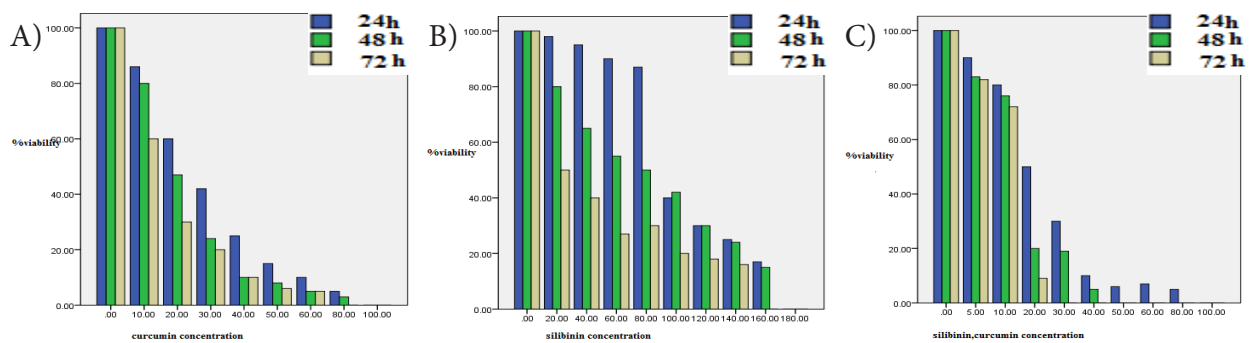
Table 2 and Figure 3 show anti-growth and hTERT-

**Table 2. Effects of Curcumin-Silibinin Mixture on Inhibition of T47D Breast Cancer Cells Growth**

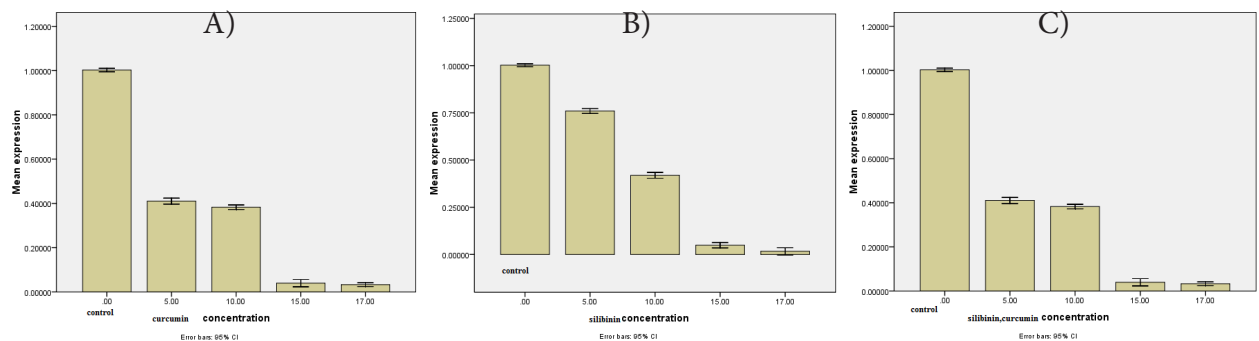
Compound	Mean IC <sub>50</sub> " $\mu$ M"	Statistical difference	
		Curcumin with Curcumin-Silibinin	Silibinin with Curcumin-Silibinin
Curcumin	19.67		
Silibinin	71.67	p-value<0.05	p-value<0.05
Curcumin-Silibinin	14.84		



**Figure 3. Effects of Curcumin-Silibinin Mixture on Inhibition of hTERT Expression in Comparison with Curcumin or Silibinin Alone**



**Figure 1. Cytotoxic Effects.** A) Curcumin on T47D Breast Cancer Cells, B) Silibinin on T47D Breast Cancer Cells and C) Curcumin-Silibinin Mixture on T47D Breast Cancer Cells



**Figure 2. Inhibitory Effects.** A) Curcumin on Expression of hTERT in T47D Breast Cancer Cells, B) Silibinin on Expression of hTERT in T47D Breast Cancer Cells and C) Curcumin-Silibinin Mixture on Expression of hTERT in T47D Breast Cancer Cells

expression inhibitory effects of curcumin-silibinin mixture in compared with those of curcumin and silibinin alone (p value<0.05).

## Discussion

In this study curcumin (diferuloylmethane), one of the major components of turmeric and silibinin (milk thistle) show an antiproliferative effect with a telomerase inhibitory potency on the T47D breast cancer cell line.

This funding is important because toxicity and drug resistance problem of cancer chemotherapeutic agents, has led to challenge in the field of cancer research (Bundred, 2001). As seen in Figure 1A curcumin causes to inhibit the growth of the cells. This effect is increased with increasing the concentration of curcumin, so that the cells were dead completely in a concentration nearly 70  $\mu\text{M}$ . Moreover, its effect on T47D cell line is variable by examining in the three exposure times 24, 48 and 72h. For an evidence, IC50s are different in the mentioned exposure times and decreases with time (IC50s for three exposure times are 22.5, 19 and 17.5  $\mu\text{M}$  respectively). In other words, it seems that the effect of curcumin on T47D breast cancer cell line is time-and dose-dependent. Other studies shows that curcumin has inhibitory effect in the cells growth on the various types of cancer cell lines usually with a time-dependent manner (Syng-Ai et al., 2004; Chakravarti et al., 2010; Watson et al., 2010; Subramaniam et al., 2012). Curcumin exerts its anticancer effect on different cellular factors (Sa and Das, 2008; Wilken, 2011). We have investigated the influence of this substance on expression and activity of the telomerase reverse transcriptase, one of the effective factors which cause the cells to be cancerous. Telomerase inhibitors are being actively considered as potential anti-tumor agents, with the hope that the lack of telomerase expression in normal somatic cells would result in a highly specific treatment with fewer side effects than conventional chemotherapy (Shay, 1997). As observed from Figure 2A, curcumin considerably inhibits expression levels of telomerase mRNA in T47D cell line, especially when its concentration is increased. It should be noted that exposure time also plays a key role in the inhibition of expression levels (a time-and dose-dependent manner similar to that of the cell growth inhibition). Ramachandran et al. also observed an effect inhibitory for curcumin on telomerase activity in breast cancer (MCF-7) cell line (Ramachandran et al., 2002).

In order to augment the inhibition of telomerase expression, we have investigated the effect of another plant-extracted substance on the T47D cell line. Silibinin, extracted from *Silybum marianum*, is a nontoxic agent and consumed widely as a dietary supplement and have strong anticancer activity against different epithelial cancers including breast cancer cells, refer to (Kim et al., 2009; Lin et al., 2009; Wang et al., 2010). Figure 1B illustrates the effect of silibinin on the cell growth in three different exposure times. Similar to curcumin, corresponding IC50s confirm that silibinin effect on the cell line is time-and dose-dependent (IC50s for three exposure times are 109, 75 and 31  $\mu\text{M}$ , respectively). It seems that higher concentrations of silibinin are needed for inhibiting the

cell growth as compared with curcumin. Silibinin exhibits an inhibitory behavior on telomerase expression, which its larger concentrations lead to a more decline in the expression levels of telomerase mRNA (Figure 2B). Thelen et al. reported similar inhibitory influence of silibinin on telomerase activity and expression in prostate cancer cells (Thelen et al., 2004). After that the effect of curcumin and silibinin was examined on the cell growth and also telomerase expression and activity. We made a mixture of curcumin and silibinin in a ration of 1:1. Table 2 and Figure 3 show analysis of Real-time PCR for hTERT expression after subjecting the two substances employed here separately and mixed. Accordingly, it seems that the mixture has a more relatively effect on both the inhibition of T47D cell line growth (IC50s for three exposure times are 17.5, 15 and 12  $\mu\text{M}$ , respectively) (Figure 1C) and expression telomerase inhibition in this cell line (Figure 2C).

In conclusion, the current study shows that curcumin-silibinin mixture is more effective than their use alone for inhibition of T47D breast cancer cell growth and that of hTERT gene expression. Further studies could be performed for further confirmation of this funding.

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