

Effects of *Curcuma longa* Extract on Telomerase Activity in Lung and Breast Cancer CellsJavad Ranjbari,¹ Abbas Alibakhshi,¹ Roghayeh Arezumand,¹ Mohammad Pourhassan-Moghaddam,¹ Mohammad Rahmati,¹ Nosratollah Zarghami,*² Mohammad Mehdi Namvaran³

1. Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
2. Tuberculosis and Lung Research Center, Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
3. Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Article information	Abstract
<p>Article history: Received: 1 June 2013 Accepted: 2 Aug 2013 Available online: 3 Sep 2013 ZJRMS 2014 Oct; 16 (10): 1-6</p> <p>Keywords: Telomerase Breast cancer Lung cancer <i>Curcuma longa</i></p> <p>*Corresponding author at: Tuberculosis and Lung Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. E-mail: zarghamin@yahoo.com</p>	<p>Background: The purpose of this study is to evaluate the effect of <i>Curcuma longa</i> extract on the telomerase gene expression in QU-DB lung cancer and T47D breast cancer cell lines.</p> <p>Materials and Methods: The present study is an experimental research. Using 3 different phases n-hexane, dichloromethane and methanol, total extract of <i>Curcuma longa</i> in a serial dilution was prepared and three phases was analyzed for determining which phase has more curcuminoids. Then the extract cytotoxicity effect was tested on breast cancer cell line (T47D), and lung cancer cell line (QU-DB) by 24, 48 and 72 h MTT (Dimethyl thiazolyl diphenyl tetrazolium) assay. Then, the cells were treated with serial concentrations of the extract. Finally, total protein was extracted from the control and test groups, its quantity was determined and telomeric repeat amplification protocol (TRAP) assay was performed for measurement of possible inhibition of the telomerase activity.</p> <p>Results: Cell viability and MTT-based cytotoxicity assay show that the total extract of <i>Curcuma longa</i> has cytotoxic effect with different IC₅₀s in breast and lung cancer cell lines. Analysis of TRAP assay also shows a significant reduction in telomerase activity on both cancer cells with different levels.</p> <p>Conclusion: <i>Curcuma longa</i> extract has anti-proliferation and telomerase inhibitory effects on QU-DB lung cancer and T47D breast cancer cells with differences in levels of telomerase inhibition.</p>

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Introduction

The most prominent difference between tumor and normal cells is their proliferative capacity. While normal cells have a limited life span and show replicative senescence, tumor cells are immortal [1]. The biochemical mechanism responsible for tumor cell immortalization, and conversely for normal cell senescence, involves an enzyme termed telomerase. This enzyme permits cancer cells to overcome one of the fundamental limitations to mammalian cell immortality, the progressive loss of telomeric DNA from the ends of the cell's chromosomes that occurs during each round of cell division. Telomerase has therefore emerged as a very attractive target for diagnostic and therapeutic intervention of cancer [1, 2].

Human telomerase has two key components, an RNA (hTR) which serves as template for the simple sequence and the catalytic subunit that acts as reverse transcriptase (hTERT). The inhibitors for telomerase generally target the template region of the RNA component of telomerase (hTR), the telomerase active site, the catalytic component of telomerase (hTERT), or telomeric G-quartet structures [3, 4]. For targeted therapy of telomerase, one of the main

categories is small molecules. Furthermore, using plant anticancer therapies for treatment of cancers have more advantages compared to synthetic agents, because many plant-based products, accomplish multi-targeting naturally and, in addition, are inexpensive and safe [5, 6].

Curcuma longa L., which belongs to the Zingiberaceae family, is a perennial herb, distributed throughout tropical and subtropical regions of the world. As a powder, called turmeric, it has been in continuous use for its flavoring, as a spice in both vegetarian and non-vegetarian food preparations and it also has digestive properties. *C. longa* L. has a great variety of pharmacological activities which exhibit anti-inflammatory, anti-human immunodeficiency virus, anti-bacteria, antioxidant effects and nematocidal activities and also inhibits carcinogenesis and cancer growth. Curcumin (1E, 6E)-1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5 dione) is a major component in *C. longa* L., being responsible for its biological actions. It also has shown that other extracts of the plant has been showing potency [7-9]. Numerous research teams have provided evidences that curcumin contributes in inhibition of tumor formation and

promotion as cancer initiation [10]. The results of experiments have revealed that curcumin can modulate several different factors such as factors are involved in apoptosis in many cancers [11-14], those are involved in cell cycle [15], arylamines N- acetyltransferase in lung cancer cells [16], NF- κ B and AP-1 in human promyelocytic leukemia cells [17], mitotic spindle structure in breast cancer cells [18], proteasome in human colon cancer cells [12]. Also studies show that telomerase activity decreases with increasing concentrations of curcumin [4]. Moreover some studies show that curcumin can have protective effects. In experimental rats, curcumin can protect liver tissue from arsenic induced deterioration of antioxidant levels and oxidative stress [19]. However the effect of *C. longa* total extract on telomerase in cancer cells and comparing this effect on telomerase activity in different cancer cells remains unperformed. Therefore the main aim of the current study was the analysis of *C. longa* extract effect on the telomerase activity and gene expression in QU-DB lung cancer and T47D breast cancer cell lines.

Materials and Methods

Cell culture: For this experimental study, QU-DB lung cancer cell line and T47D breast cancer cell line (Pasteur institute, Iran) were cultured in RPMI1640 medium (Gibco, Invitrogen, UK) supplemented with 10-20% heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen, UK), 2 mM L-glutamine, penicillin G (80 mg/mL) (Serva Co, Germany), streptomycin (50 mg/mL) (Merck Co, Germany) and NaHCO_3 (2 g/mL), at 37°C and in 5% CO_2 .

Extraction of *C. longa* total extract: After rubbing some dried rhizome of *C. longa* mechanically, 100 g powder was dissolved in 200 mL n-hexane and the solution was shaken for 4 h at 45°C. Residue of n-hexane extraction was dissolved in 200 mL dichloromethane and the solution was shaken for 4 h at 45°C. Residue of previous extraction step was dissolved in 200 mL methanol and then same steps were carried out. Finally, solvents of three phases were dried by rotatory evaporator and remained powders were stored at -20°C until used.

In the next steps 0.019 g pure curcumin (Sigma Co., Germany) was dissolved in 5 mL DMSO 10% to a final concentration of 10 mM (3.8 mg/mL) and then a serial dilutions (38, 30.4, 19, 15.2, 7.6 and 1.9 $\mu\text{g/mL}$) from with DMSO 10% was prepared. Also 0.019 g n-hexane, methanol and dichloromethane extracts was dissolved in 5 mL DMSO 10% respectively, to a final concentration of 3.8 mg/mL.

Then 5 μL from each extract solution was added to 4995 μL DMSO 10% to final concentration (38 $\mu\text{g/mL}$). DMSO 10% was used as blank. Then, absorbance of 10 μM , 5 μM and 2.5 μM solutions of curcumin and prepared extracts (38 $\mu\text{g/mL}$) were measured at medium wavelengths (λm) 420 and 430 nm by spectrophotometer (Curcuminoids have maximum absorbance at λm =420-430 nm [20]). The standard curve was plotted using mean of pure curcumin with optical density (OD) $\times 100$ and

relative curcumin content of phases was determined according to standard curve.

Cell viability and MTT-Based cytotoxicity Assay: Cells in the exponential phase of growth were exposed to n-hexane *C. longa* extract. Cytotoxic effect of n-hexane extract was studied by 24, 48 and 72 h MTT assay [(3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] assay which is based on the reduction of MTT (Sigma co., Germany), yellow water-soluble tetrazolium dye, by the mitochondrial dehydrogenase of intact cells to a purple formazan product [11, 21]. 2×10^3 cells/well were plated in a 96-well plate (Coastar from Corning, NY) and after 24 h incubation were treated with different concentrations (0-396 $\mu\text{g/mL}$) of *C. longa* extract for 24, 48 and 72 h in the quadruplicate manner.

After these different exposure durations, medium was removed and then the cells were fed with 200 μL of fresh medium. Cells were leaved for 24 h, then 50 μL of 2 mg/mL MTT (Sigma Co., Germany) was added to each well and plate was covered with aluminum foil and was incubated for 4 h. In the next step content of wells was removed and 200 μL pure DMSO and 25 μL Sorensen's glycine buffer was added to each well. Finally, the absorbance measurement was determined at 570 nm using an ELISA plate reader (with a reference wavelength of 630 nm) [22].

Cell treatment: 2.5×10^5 cells were treated with serial concentrations of the n-hexane extract of *C. longa* for 24 h exposure.

Telomeric repeat amplification protocol (TRAP) assay: Relative telomerase activity was determined according to instructions of TeloTAGGG telomerase PCR ELISA PLUS kit. Total protein was extracted according to instructions of TeloTAGGG Telomerase PCR ELISA PLUS package (Cat. No. 12 013 789 001, Roche Applied Science, Germany) and quantity of total protein was measured by Quick Start™ Bradford protein assay (Cat. No. 500- 0206, Bio-Rad Laboratories, Inc, USA). Briefly, 50 μL reaction mixture (10 μg ex tracted cytosol, 5 μL dNTPs, 60 ng from each primer, 5 μL internal standard and 16 μL double-distilled water (nuclease-free) for each sample was added in a new 0.5 mL microtube. Then, microtubes were transferred to Eppendorf PCR thermal cycler and a combinatory elongation/PCR reaction was performed (1 cycle for 30 minutes at 30°C, 1 cycle for 15 min at 94°C, 1 cycle for 1 minute at 94°C, 27 cycles for 30 seconds at 94°C, 27 cycles for 30 seconds at 50°C, 27 cycles for 90 seconds at 72°C and 1 cycle for 10 min at 72°C). Then, PCR amplicons were detected by ELISA-based hybridization, refer to [23]. For this purpose, 3 μL PCR products were immobilized on streptavidin coated modules and were incubated for 2 h at room temperature (RT). Then, module wells were washed three times and horse radish peroxidase-conjugated anti-digoxigenin antibody was added to wells. After that, substrate solution containing 3, 3', 5, 5', tetra methyl benzene (TMB) was added to wells and wells incubated in RT for 20 min for color development.

Finally, OD of developed blue color was measured at 450 nm by ELISA reader (with a reference wave length of

630 nm), and relative telomerase activity of each sample was calculated according to the instructions of kit manufacturers.

Statistical analysis: Relative telomerase activity percent (RTA%) was calculated according to instructions of TeloTAGGG telomerase PCR ELISAPLUS kit and related graph was plotted using SPSS-16.

Results

According to standard curve n-hexane extract has more curcuminoids compared with methanolic and dichloromethane extracts (Fig. 1). Therefore, n-hexane extract was used for treatment of cells.

Cytotoxic effect of *C. longa* extract on the cell lines: Analysis of results showed that n-hexane extract inhibits growth of QU-DB and T47D cells dose-dependently so that with increasing in concentration of n-hexane extract, the number of dead cells was increased (Fig. 2). Data analysis of cytotoxicity assay showed that IC₅₀s of the n-

hexane extract on QU-DB cells were 79, 74 and 68 µg/mL, and that on T47D cells were 342, 57 and 57 µg/mL, for 24, 48 and 72 h MTT assay, respectively. As a result n-hexane extract of *C. longa* has a dose-dependent effect on growth of both cell lines. Also, the extract has a time-dependent effect on T47D cells for 48 h exposure (Fig. 3 and Fig. 4).

Inhibition of telomerase: Data analysis of TRAP assay showed that RTA (Relative Telomerase Activity) was decreased in treated QU-DB cells in comparison with the control cells. Although, 26 and 47 µg/mL n-hexane concentrations had less inhibitory effects on RTA, but 68 µg/mL concentration showed a decreased RTA to 76.4% (Table 1 and Fig. 5). In T47D cells, it seems that n-hexane *C. longa* extract has more inhibitory effect on telomerase activity in comparison with QU-DB cells. As seen in table 2 and figure 5 with increasing of the extract concentration, RTA in T47D cells was more decreased than in QU-DB cells.

Table 1. Comparison between fold increases of telomerase inhibition by different concentrations of n-hexane extract in QU-DB cells for 24 h exposure

Concentration of n- hexane extract (µg/mL)	Telomerase activity for 24 h exposure (Mean OD at 450 nm)	RTA compared with the control (%)	Fold increase in inhibition of telomerase activity
control	67.11±1.01	100	1
26	65.55±0.90	97.7	1.02
47	65.05±0.88	96.9	1.03
68	51.27±0.94	76.4	1.30

Table 2. Comparison between fold increases of telomerase inhibition by different concentrations of n-hexane extract in T47D cells for 24 h exposure

Concentration of n- hexane extract (µg/mL)	Telomerase activity for 24h exposure (Mean OD at 450 nm)	RTA compared with control group (%)	Fold increase in inhibition of telomerase activity
control	162.33±1.97	100	1
57	132.5±1.011	81.63	1.226
114	92.4±1.32	56.93	1.76
171	85.3±1.1	52.55	1.90
197	79.55±1.54	49	2.04
228	21.5±1.04	13.25	7.55
255	9.1±1.79	5.6	17.84

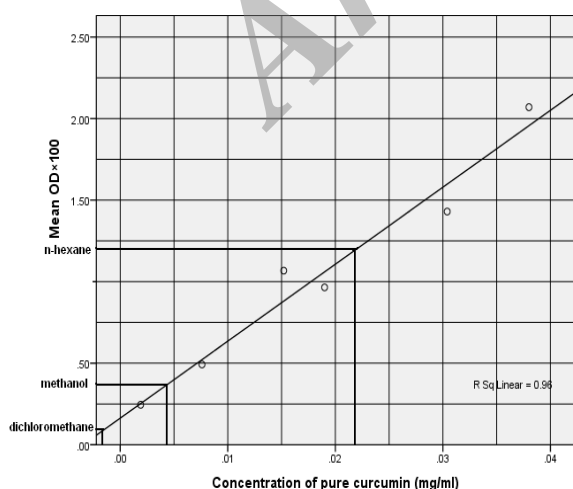
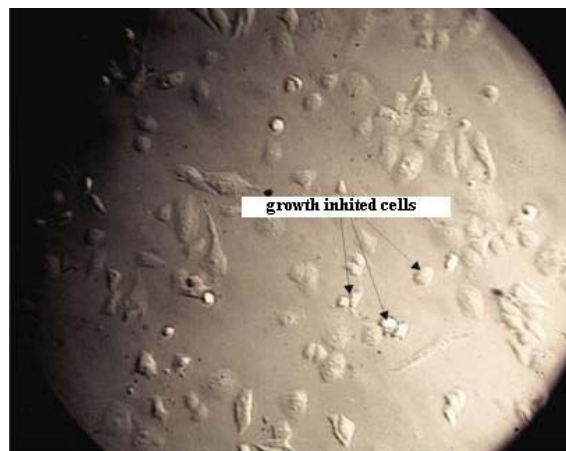


Figure 1. Amount of curcuminoids in n-hexane, methanol and dichloromethane extracts



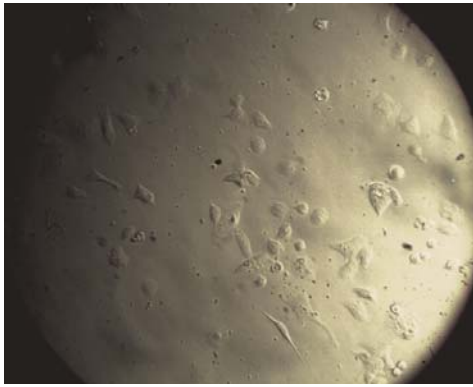


Figure 2. Morphology of growth-inhibited QU-DB and T47D cells after treatment with *C. longa* extract. (Up: low concentration extract. Down: high concentration extract)

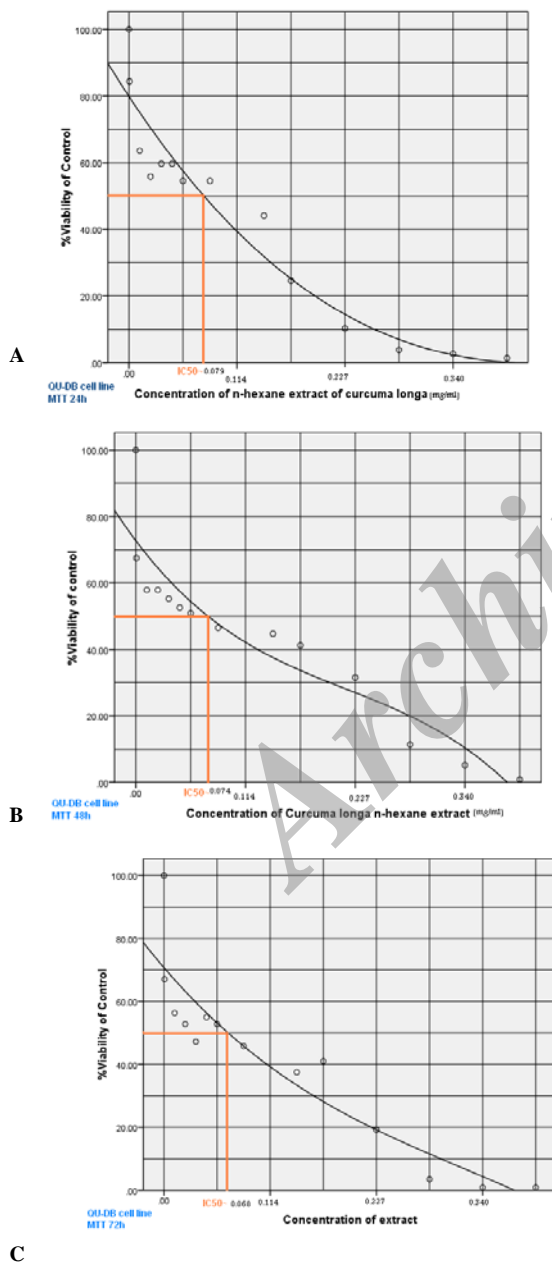


Figure 3. Cytotoxic (growth-inhibitory) effects of n-hexane extract on QU-DB lung cancer cell line after 24 h (A), 48 h (B) and 72 h (C) treatments

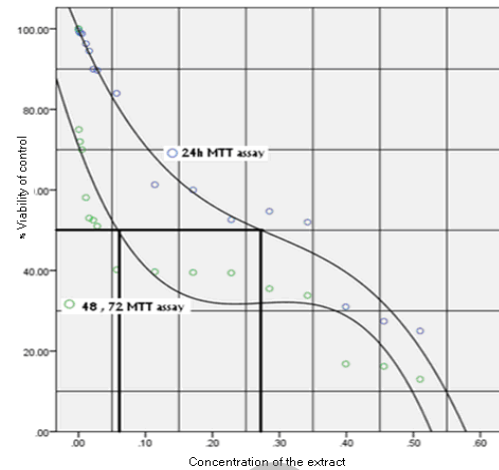


Figure 4. Cytotoxic effect of n-hexane extract on T47D breast cancer cell line after 24 h, 48 h and 72 h treatments

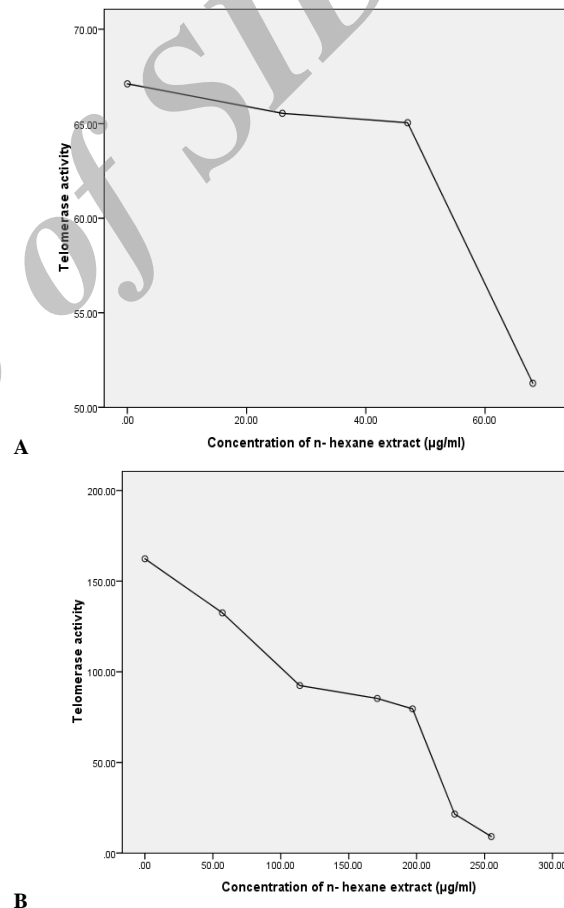


Figure 5. Inhibitory effect of *C. longa* total extract on telomerase activity in QU-DB cells (A) and T47D cells (B)

Discussion

The results of this study show that *C. longa* total extract has anti-proliferative effect on QU-DB and T47D cell lines in a dose-dependent manner. Also the extract has inhibitory effect on telomerase activity in both QU-DB and T47D cancer cell lines with different levels of inhibition.

Because telomerase is detected in majority of malignant tumors, but not in most normal somatic cells, this suggests an important role of telomerase in neoplastic transformation [24]. In the present study telomerase activity decreased with increasing concentrations of *C. longa* extract. Based on the results of TRAP assay, our study shows that the extract has stronger inhibitory effect on T47D cells in comparison with QU-DB cells. The RTA in QU-DB cell line at a concentration of 68 µg/ml of the extract was decreased to 76.4% compared with control cells. Although this activity reduction is significant, but still telomerase has considerable activity. In previous our work, the n-hexane extract of *C. longa* had inhibitory effect on both growth and telomerase in the A549 lung cancer cell line [25].

Also, it seems that effect of the extract on A549 is stronger than in QU-DB cell line for inhibition of telomerase. Radhakrishna Pillai et al. also reported similar growth inhibitory effect of curcumin in both the A549 and H1299 lung cancer cell lines dose-dependently. H1299 cells also showed time dependency toward curcumin cytotoxicity and were more sensitive to curcumin than A549 cells [14]. Ramachandran et al. also reported similar inhibitory influence of curcumin in MCF-7 breast cancer cells. At 100 mM concentration, telomerase activity was decreased to 93.4% [4].

Also telomerase activity in human cancer cell lines Bel7402, HL60 and SGC7901 was decreased by the incubation with 1.0 µM curcumin in a time-dependent manner [26]. Since QU-DB cells and T47D cells are derived from different cancers, basic expression levels of their counterpart genes may be also different from each other and this difference could be attributed to basal levels of telomerase expression in these cell lines. The

difference in basal levels of telomerase expression between QU-DB cell line and T47D cell line may be due to probably different mechanism(s) involved in regulation of telomerase expression in QU-DB and T47D cells. The differences in levels of telomerase inhibition may be mainly due to different response of various cancer cell lines to *C. longa* extract. Because of different levels of telomerase inhibition, it is suggested that the involved molecular mechanisms in control of telomerase activity may be different in various cancer cell lines.

In conclusion, *C. longa* extract has anti-proliferation and telomerase inhibitory effects on QU-DB lung cancer and T47D breast cancer cell lines. It seems that the extract has more inhibitory effect on telomerase in T47D cells than in QU-DB cells. So the extract inhibits telomerase activity on both cells differently, although more investigations are required for deciphering underlying mechanisms.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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