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Full Length Research Paper

The inhibitory effect of *Curcuma longa* extract on telomerase activity in A549 lung cancer cell line

Pourhassan Mohammad, Zarghami Nosratollah*, Rahmati Mohammad, Alibakhshi Abbas and Ranjbari Javad

Department of Clinical Biochemistry and Laboratory Medicine, Division of Medical Biotechnology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran and Tuberculosis and Lung Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

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Telomerase is reactivated in lung cancer cells, the most prevalent cancer worldwide, but not normal cells. Therefore, targeting it, preferably with natural compounds derive from medicinal plant such as curcumin, could have important effect on treatment of lung cancer. Curcumin, derived from *Curcuma longa* rhizome, has many anti-cancer activities. Therefore, the main objective of current work was to study inhibitory effect of *C. longa* total extract on telomerase in A549 lung cancer cell line as *in vitro* model of lung cancer. First, total extract of *C. longa* were prepared by n-hexane, methanol and dichloromethane. Then, cytotoxic effect of n-hexane phase was studied on A549 lung cancer cell line with 24, 48 and 72 h MTT assays. Finally, after determination of IC50, cells were treated with n-hexane extract and TRAP (Telomeric Repeat Amplification Protocol) assay was done to measure amount of relative telomerase inhibition by the extract. Data analysis showed that n-hexane extract of *C. longa* has dose-dependent cytotoxic effect on A549 lung cancer cell line with IC50 = 0.23 - 0.28 mg/ml and the extract inhibits telomerase activity with dose-dependent manner. In conclusion, n-hexane extract of *C. longa* has cytotoxic and telomerase-inhibitory effect on cell line A549 and could be exploited as potential source for developing novel drugs against lung cancer.

Key words: Telomerase, lung cancer, *Curcuma longa*, A549 lung cancer cell line.

INTRODUCTION

Telomerase is a ribonucleoprotein reverse transcriptase which maintains telomeric ends of eukaryotic chromosomes during DNA replication. It has been shown that telomerase is active in more than 85% of cancers including lung cancer but not normal cells (Lingner et al., 1995; Kim, 1997). On the other hand, lung cancer is the most prevalent cancer worldwide (Jemal et al., 2007). Therefore, targeting the telomerase in this cancer could be promising step in its treatment. Different agents have been proposed for telo-merase inhibition (Strahl and Blackburn, 1996; Damm et al., 2001; Zou et al., 2006; Massard et al., 2006; Nakamura et al., 2005; Mizushina et al., 2000; Yeo et al., 2005). But, they have undesirable complications (Mittal et al., 2004) and it is necessary to seek alternative telomerase-inhibiting agents, preferably natural compounds such as curcumin.

Curcuma longa is a medicinal plant which its constituents, especially curcumin, has diverse anti-cancer properties including anti-telomerase action (Sanjay et al., 2007; Anand et al., 2008). Anti-telomerase activity of pure curcumin has been studied previously in some cancer cells other than lung cancer cells (Cui et al., 2006; Chakraborty et al., 2006; Ramachandran et al., 2002; Singh and Singh, 2009; Mukherjee et al., 2007). However, to date any study for investigating the effect of curcuma total extract on telomerase in the lung cancer cells remains unperformed. Therefore, the main aim of current work was to study the effect of *C. longa* total extract on telomerase activity in A549 lung cancer cell line as *in vitro* model of lung cancer for its possible therapeutic application in the future.

*Corresponding author. E-mail: zarghami@tbzmed.ac.ir. Tel.: +98-4113363234. Ext 241. Fax: +98-4113363231.

MATERIALS AND METHODS

Extract preparation

100 g powder of *C. longa* rhizome was dissolved in 200 ml n-hexane and solution was shaken for 4 h at 45°C. Then, supernatant was transferred to a tube. This step was performed three times. In the next step, residue of n-hexane extraction was dissolved in 200 ml dichloromethane instead of n-hexane and same steps repeated using dichloromethane. In the next step, debris of previous extraction step with dichloromethane was dissolved in 200 ml methanol and at the same way supernatant was collected. Finally, solvents of all three phases were dried by rotatory-evaporator and remained powders were stored at - 20°C until used.

Selection of appropriate phase

Based on the fact that Curcuminoids have maximum absorbance at λ_m = 420 - 430 nm (Jayaprakasha et al., 2005), the curcuminoids content of n-hexane, dichloromethane and methanol phases of extract was determined spectrophotometrically. Briefly, ODs of 0.038, 0.0304, 0.019, 0.0152, 0.0076 and 0.0019 mg/ml solutions of pure curcumin (sigma) dissolved in 10% DMSO and ODs of 0.0038 mg/ml solutions of extract phases in 10% DMSO were determined in λ_m = 420 nm and λ_m = 430 nm with three times reading in the spectrophotometer. Then, standard curve was plotted using mean of pure curcumin OD×100. Finally, relative curcumin content of phases was determined according to standard curve.

Cell culture and cytotoxicity

A549 lung cancer cell line (kindly dedicated from pharmaceutical nanotechnology research center, Tabriz University of Medical Sciences, Tabriz, Iran) were cultured in RPMI1640 (Gibco, Invitrogen, UK) supplemented with 10% heat-inactivated fetal bovine serum (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) and incubated in 37°C with humidified air containing 5% CO_2. After culturing sufficient amount of cells, cytotoxic effect of n-hexane extract was studied by 24, 48 and 72 h MTT assays (Carmichael et al., 1987). Briefly, 1000 cell/well were cultivated in a 96 well plate. After 24 h incubation in 37°C with humidified atmosphere containing 5% CO_2, cells were treated with serial concentrations of n-hexane extract of *C. longa* (0 mg/ml to 0.57 mg/ml) for 24, 48 and 72 h in the quadruplicate manner as cells which received 0 mg/ml extract + 200 µl culture medium containing 10% DMSO served as control. After incubation, the medium of all wells of plate were exchanged with fresh medium and 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 well and plate was covered with aluminum foil and incubated for 4.5 h. After removing of wells' content, 200 µl pure DMSO was added to wells. Then, 25 µl Sorensen's glycine buffer was added and immediately absorbance of each well was read in 570 nm using ELx800 Microplate Absorbance Reader (Bio-Tek Instruments) with reference wavelength of 630 nm.

Cell treatment

After determination of IC50, 1×10^6 cells were treated with serial concentrations of n-hexane extract (0.028, 0.057, 0.114, 0.142, 0.171 and 0.199 mg/ml). For control cells, the same volume of 10% DMSO without n-hexane extract was added to flask of control cells. Then, culture flasks were incubated in 37°C containing 5% CO_2 with humidified atmosphere incubator for 24 h exposure duration.

Telomeric repeat amplification protocol (TRAP) assay

After 24 h, cells were harvested, their total protein were 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 determined for each sample by Quick Start™ Bradford Protein Assay (Cat. No. 500-0206, Bio-Rad Laboratories, Inc., USA). Finally, for determining relative telomerase activity in the samples, we used Holt PCR-ELISA based TRAP assay method (Holt et al., 1996) according to instructions of TeloTAGGG Telomerase PCR ELISA^{PLUS} kit and our previous work (Zarghami and Asadi, 2007). First, negative control for each sample was constructed by heating at 95°C for 20 min. Then, 50 µl reaction mixture (5 µg extracted total protein, 5 µl dNTPs, 60 ng from each primer, 5 µl Internal Standard and 16 µl double-distilled nuclease free water) 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 20 min at 25°C (primer elongation); 1 cycle for 20 min at 95°C (telomerase inactivation); 30 cycles for 30 s at 94°C, 30 s at 50°C and 90 s at 72°C and 1 cycle for 10 min at 72°C (PCR)]. Then, PCR amplicons were detected by ELISA-based hybridization. For this purpose, 3 µl PCR products were immobilized on streptavidin coated modules and incubated for 2 h at room temperature. Then, module wells were washed three times and horseradish 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, wells incubated in room temperature for 20 min for color development and more color development was stopped. Finally, absorbance of developed blue color was measured at 450 nm by STAT-FAX 2100 ELISA reader (with a reference wavelength of 630 nm).

Data analysis

For data analysis of MTT assays, mean OD of each well was calculated. Then, OD of tests and controls was corrected by subtracting mean OD of tests and controls from mean OD of blanks (medium alone). Then, percent of viability of control was calculated according this formula: percent of viability of control = corrected mean OD of test/corrected control×100. Finally, graph of results was plotted using SPSS 16.0 and IC50 (half maximal inhibitory concentration) of n-hexane extract on A549 was calculated.

Relative telomerase activity percent (%RTA) of each sample was calculated according to instructions of TeloTAGGG Telomerase PCR ELISA^{PLUS} kit. Then, %RTA of control cell was calculated for each sample and related graph was plotted using SPSS 16.0.

RESULTS

Data analysis of spectrophotometric measurement of extracted phases curcuminoids content showed that n-hexane phase has more curcumin than methanol and dichloromethane phases and dichloromethane phase has the lowest content of curcuminoids (Figure 1 and Table 1). Therefore, we investigated the effect of n-hexane extract

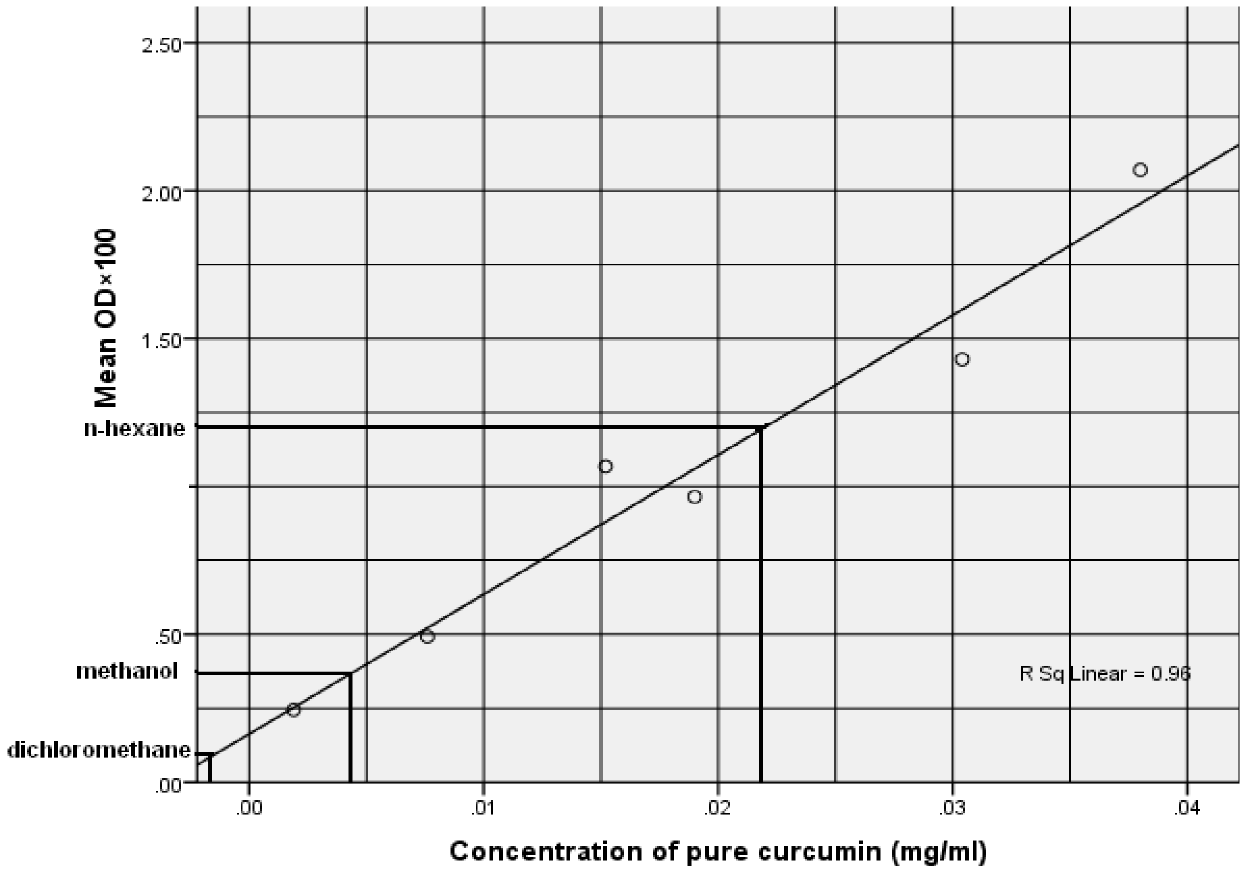

Figure 1. OD_{420} and OD_{430} of *Curcuma longa* extracts in comparison with pure curcumin (as standard).

Table 1. Mean absorbances of pure curcumin, n-hexane extract, methanol extract and dichloromethane extract solutions in λmax = 420 nm and λmax = 430 nm.

Parameter	Pure curcumin (as standard)						n-hexane extract	Methanol extract	Dichloro-methane extract
Conc. (mg/ml)	0.0019	0.0076	0.0152	0.019	0.0304	0.038	0.0038	0.0038	0.0038
λ_{max} = 420 nm	0.0025	0.0049	0.0108	0.0097	0.0143	0.0207	0.0120	0.0038	0.0006
λ_{max} = 430 nm	0.0024	0.0050	0.01055	0.0096	0.0143	0.0207	0.0123	0.0037	0.0008
Mean OD×100	0.245	0.492	1.0675	0.965	1.43	2.07	1.215	0.37	0.07

of *C. longa* on telomerase activity in the A549 cell line. Data analysis of cytotoxicity assay showed that IC50 of n-hexane extract on A549 lung cancer cell line is 0.28, 0.27 and 0.23 mg/ml for 24, 48 and 72 h MTT assays, respectively (Figure 2). Figure 2 shows that IC50 of n-hexane extract on A549 lung cancer cell line is dose-dependent but not time dependent. Therefore, we investigated telomerase-inhibitory effect of n-hexane extract on A549 for 24 h exposure time (Figure 3). Morphological analysis showed no significant difference between control and treated cells.

Data analysis obtained from TRAP assay showed that n-hexane extract of *C. longa* inhibits telomerase activity dose-dependently and doses higher than 0.114 mg/ml completely inhibit telomerase relative activity (Figure 4, Tables 2 and 3). Table 2 shows that concentration of 0.142 mg/ml is the least effective dose for complete inhibition of telomerase activity in A549 lung cancer cell line. In addition, in this dose telomerase activity was inhibited 46.30 folds in comparison with untreated cells. Therefore, dose of 0.142 mg/ml may be a cut-off point for telomerase inhibition by n-hexane extract in the A549 cell

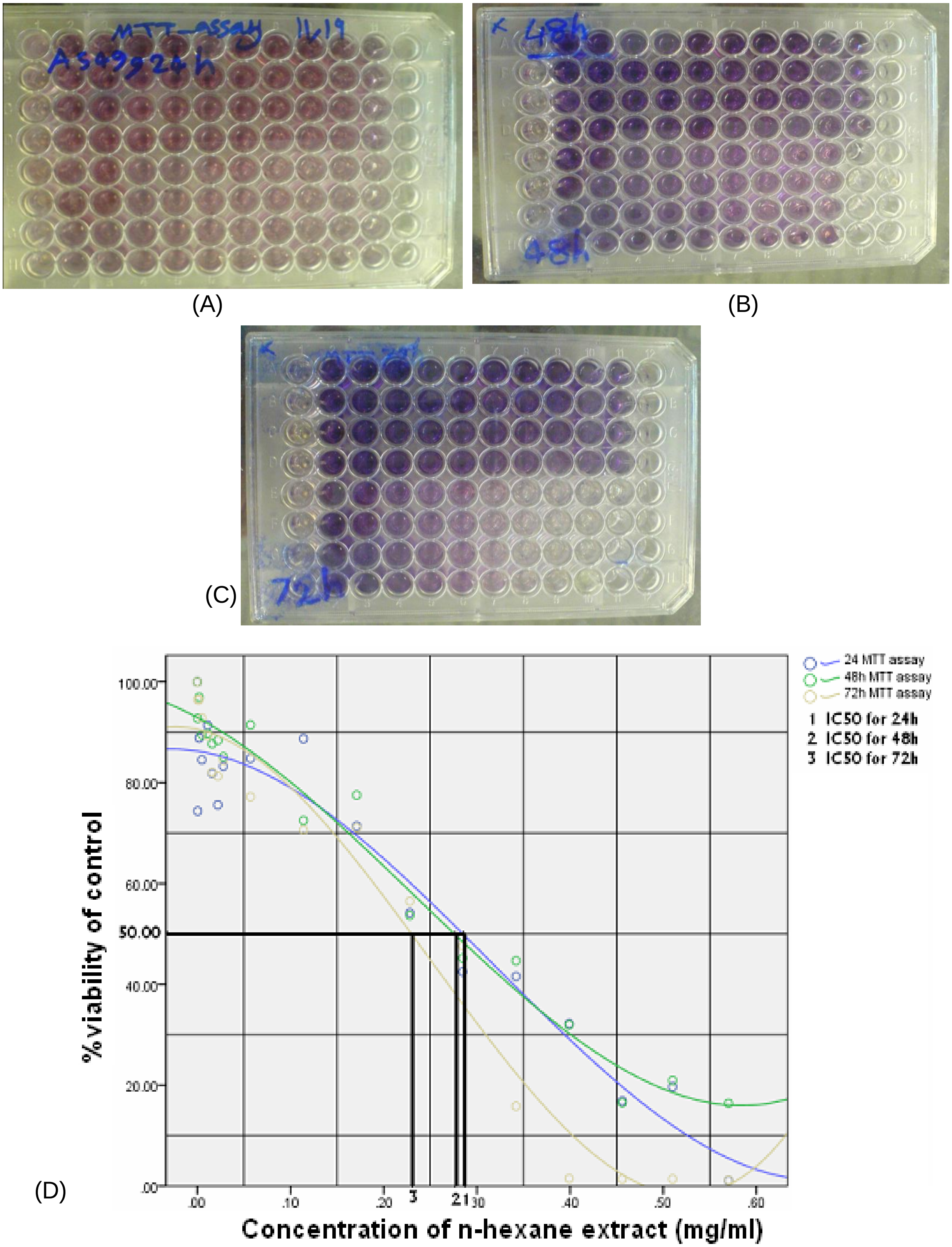

Figure 2. Cytotoxic effect of n-hexane extract on A549 lung cancer cell line after 24 h (A), 48 h (B) and 72 h (C) exposure. (D). IC50 of n-hexane extract of *Curcuma longa* on A549 tumor cell line after 24, 48 and 72 h treatment.

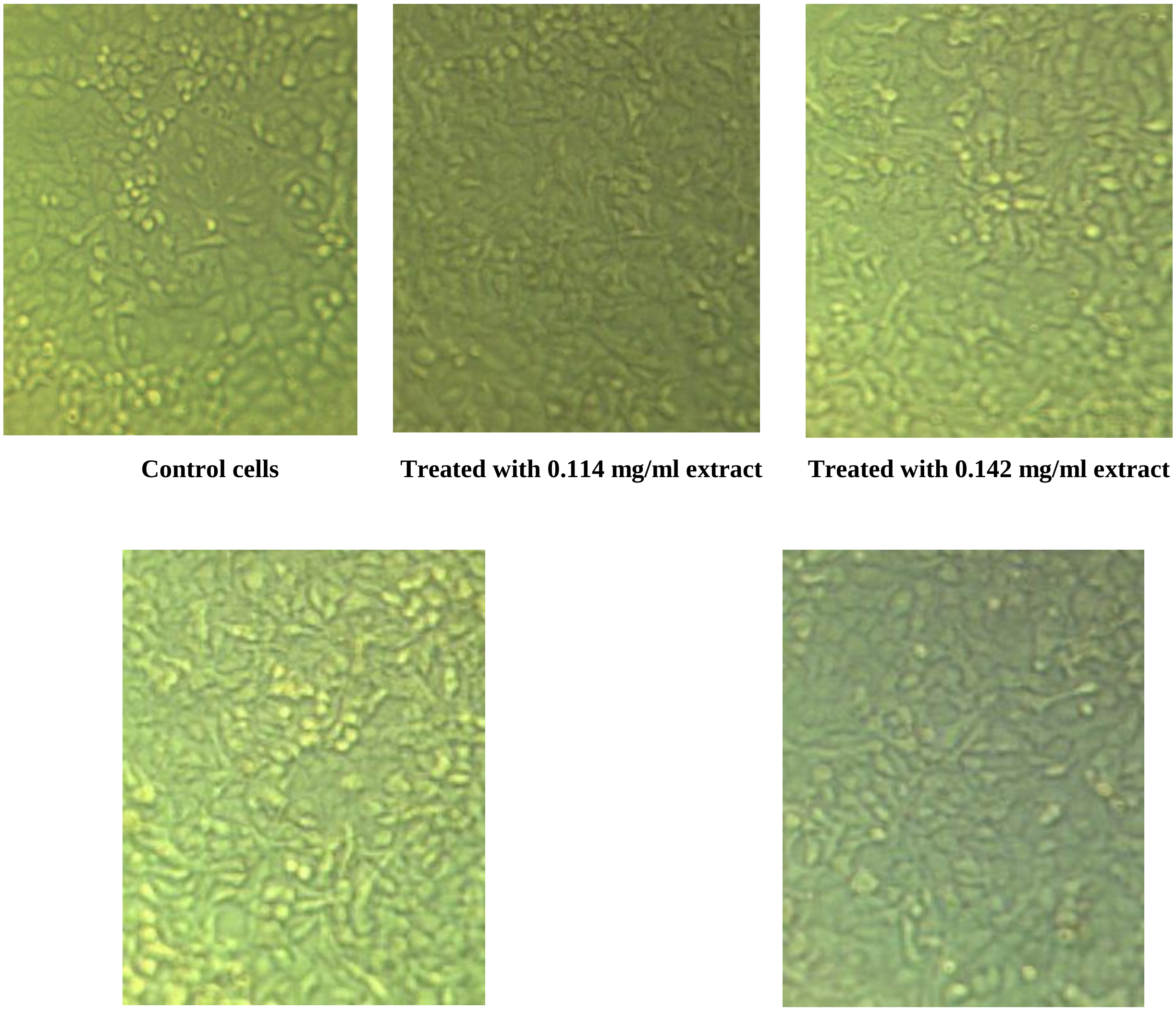

Figure 3. Morphological effect of n-hexane extract of *Curcuma longa* on A549 lung cancer cell line after 24 h treatment.

line.

DISCUSSION

In the present study, total extract used instead of pure curcumin. Because, beside synergistic effects of curcuminoids on each other in a solution (Deshpande and Maru, 1995) and based on Jayaprakasha et al. (2005) report, extraction of pure curcumin from rhizome of *C. longa* is time consumable and needs for using HPLC methods.

In the current work, MTT assay showed that n-hexane extract of *C. longa* has dose-dependent but not time-dependent cytotoxicity on the A549 lung cancer cell line (IC50 = 0.23 to 0.30 mg/ml). This finding is in accordance with result of a study (Radhakrishna et al., 2004) in which pure curcumin had dose-dependent but not time-dependent cytotoxicity on A549, but IC50 of pure curcumin was 0.01841 mg/ml (50 µM) for 24 h exposure in that study. In addition, results of same study showed IC50 = 0.01474 mg/ml (40 µM) of pure curcumin on H1299 cells (Radhakrishna et al., 2004). This difference in the IC50 may be due to purity of curcumin and existence of other substances other than curcumin which may interfere with action of curcumin. Therefore, there is need for separation and further study of n-hexane extraction constituents in the future. Shi et al. (2006) showed that 40 µM curcumin inhibits growth of the human epithelium ovarian cancer cell line Ho-8910 in vitro by 52% for 48 h exposure ($P < 0.05$) (Shi et al., 2006). However, results of current work demonstrated that IC50 of *C. longa* extract

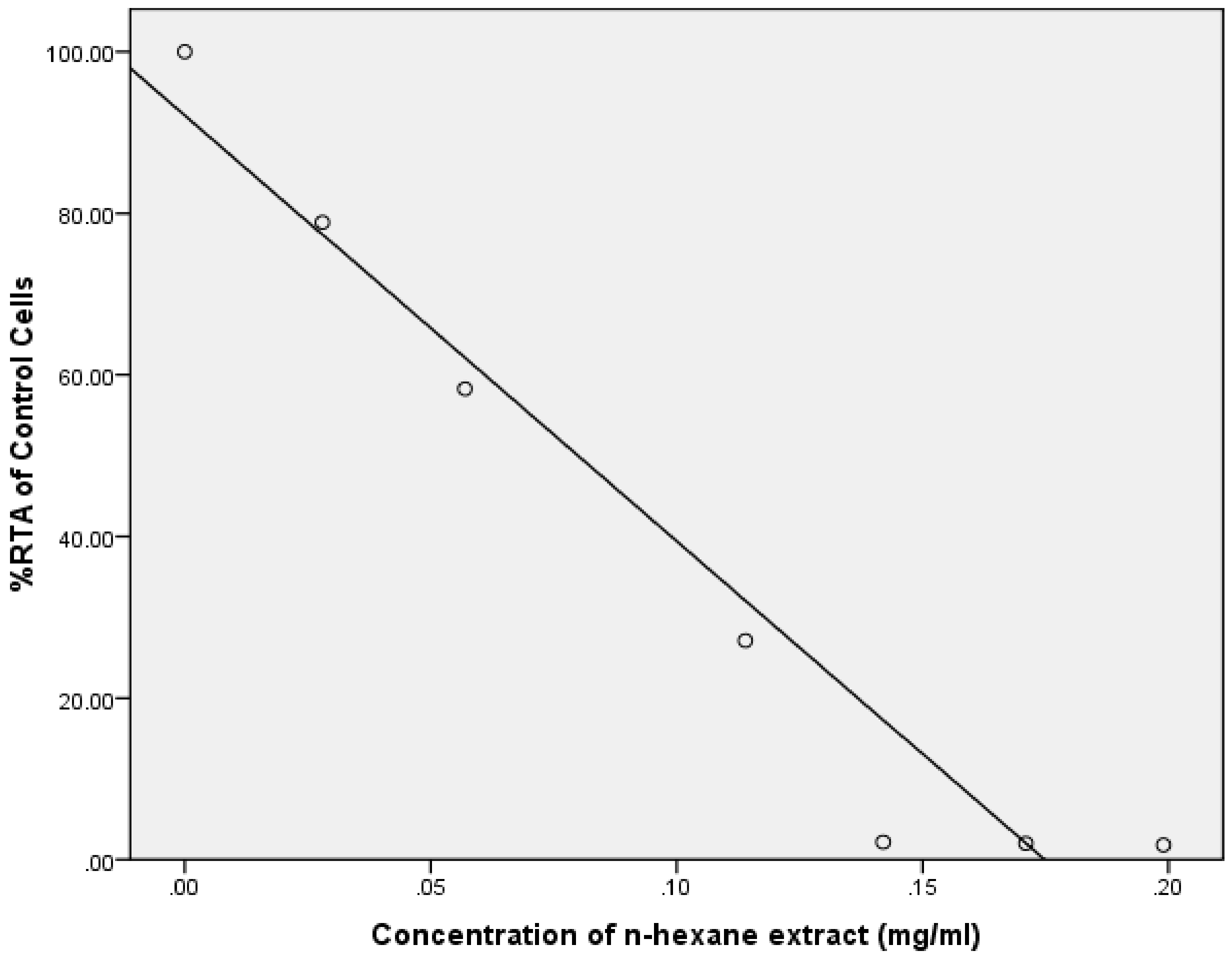

Figure 4. Correlation between different concentrations of n-hexane extract of *Curcuma longa* and telomerase activity in A549 tumor cell line after 24 h treatment.

Table 2. Determination of telomerase activity in A549 tumor cells treated with different concentrations of n-hexane extract of *Curcuma longa*.

Dose (mg/ml)	Telomerase activity*
0.0	+
0.028	+
0.057	+
0.114	+
0.142	_
0.171	_
0.199	_

*The cut off point of being positive (+) and negative (-) was calculated based on following formula:
OD of sample - OD of negative control = A
If A > 2× (OD of negative control), then, telomerase activity considered positive and if not so, activity considered negative.

is about 0.26 mg/ml in A549 lung cancer cell line. This different finding may be is due to different behavior of two cells in response to curcumin and shows that these cancers may differently response to exposure with curcumin *in vivo*.

Currently there are different types of standard treatment methods for lung cancer patients such as surgery, radiation, chemotherapy, laser therapy, photodynamic therapy (PDT), biologic therapy and gene targeted therapy (Thatcher, 2008). However, there are harsh side effects about all (Geamanu, 2008). Therefore, with regarding to these undesirable effects and result of current study, it seems that *C. longa* may be an appropriate source for developing novel drugs against lung cancer and can be a safer alternative for current treatment regimens.

In conclusion, results of current study showed that n-hexane extract of *C. longa* has inhibitory effect on telomerase in the A549 lung cancer cell line. Inhibition of telomerase by n-hexane extract of *C. longa* in these cells is reported for the first time and even any study, in which

Table 3. Comparison Between fold increases of telomerase inhibition by different concentrations of n-hexane extract in A549 lung cancer cell line.

Concentration of n-hexane extract (mg/ml)	RTA of control cells (%)	Fold increase in inhibition of telomerase activity
0.0	100.0	1
0.028	78.9	1.27
0.057	58.26	1.72
0.114	27.13	3.69
0.142	2.16	46.30
0.171	2.0	50
0.199	1.8	55.56

the effect of pure curcumin on the telomerase in the A549 has been investigated, has not been reported to date. However, similar works on other cell lines have been performed with pure curcumin on the other cell lines (Cui et al., 2006; Chakraborty et al., 2006; Ramachandran et al., 2002). Results of these studies have showed that pure curcumin has inhibitory effect on telomerase. Inhibition of telomerase by pure curcumin based on these studies, approve results of current research. Because based on our work n-hexane extract of *C. longa* which contains curcumin, inhibited telomerase dose-dependently in A549 tumor cell lines. Therefore, it can have potentially telomerase-targeting compounds, especially for exploiting in treatment of lung cancer.

Current work demonstrated that n-hexane crude extract of *C. longa* has potent anti-growth effect on A549 and dose-dependently inhibits telomerase in this cell line as in vitro model of NSCLC. Therefore, *C. longa* can be natural potent chemopreventive and chemotherapeutic plant for NSCLC patients and constituents of its n-hexane extract can be appropriate candidate for drug development.

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