Dichlorodiaportinol A – A new chlorine-containing isocoumarin from an endophytic fungus *Trichoderma* sp. 09 from *Myoporum bontioides* A. Gray and its cytotoxic activity

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ABSTRACT

Background: *Myoporum bontioides* A. Gray, an evergreen shrub from the Myoporaceae family, is a commonly used medicinal plant. Many studies have been conducted on the biologically active constituents of whole parts of *M. bontioides*. However, the endophytes of *M. bontioides* have not been intensively investigated. A new chlorine-containing isocoumarin, named dichlorodiaportinol A (1) was isolated from the endophytic fungus *Trichoderma* sp. 09 isolated from the root of *M. bontioides*. Its cytotoxic activity against human breast cancer (MCF-7) and human liver cancer (HepG2) cell lines was evaluated. **Materials and Methods:** Different open silica gel column chromatographic techniques with different solvent systems were used for the separation of the constituents of the ethyl acetate extract of the culture broth of the endophytic fungus *Trichoderma* sp. 09. The structure of compound one was identified by analysis of spectroscopic data [one-dimensional (1D), two-dimensional (2D)-nuclear magnetic resonance (NMR), ultraviolet (UV), infrared (IR) and Mass spectrometry (MS)]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay method was used for the evaluation of cytotoxic activity of compound one against MCF-7 and HepG2 cell lines. **Results:** Compound one was identified as 3-(3,3-dichloro-2,3-dihydroxy-propyl)-8-hydroxy-6- methoxy-isochromen-1-one. It inhibited MCF-7 and HepG2 cell lines, with half maximal inhibitory concentration (IC_{50}) values of 17.8 and 39.6 μg/mL, respectively. **Conclusions:** Compound one is a new chlorine-containing isocoumarin with moderate cytotoxic activity against MCF-7 and HepG2 cell lines. Thus, endophytes of *M. bontioides* are worthy of consideration for the development and research of antitumor agents.

Key words: Cytotoxic activity, dichlorodiaportinol A, isocoumarin, *Trichoderma* sp.

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INTRODUCTION

Endophytes are microorganisms that reside in the tissues of living plants without causing any damage to the host.[1] They have proven to be a rich source of structurally unique and biologically active secondary metabolites which are of interest for specific medicinal or agrochemical value.[2] *Myoporum bontioides* A. Gray belonging to genus *Myoporum*, family Myoporaceae, is a small evergreen shrub distributed along the coastal regions of southern Kyushu islands through Okinawa, Taiwan, southern China, and Indochina. In China, the decoction of *Myoporum bontioides* A. Gray is usually used as a folk medicine for antidermatosis and as antipyretic and antipsychotic agent.[3,4] The phytochemistry and pharmacological studies conducted on this medicinal plant have successfully isolated a serial of natural products including sesquiterpenoids, iridoids, monoterpenes, phenylethanoids, and flavonoids etc.[5-8] However, the chemical and medicinal values of its endophytes seldom have been investigated.

During our continuing search for structurally new bioactive secondary metabolites from endophytes from *M. bontioides*,[9] we examined the chemical constituents of

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Trichoderma sp. 09, an endophytic fungus isolated from the root of the semi-mangrove plant. In the study, we describe the isolation and characterization of a new chlorine-containing isocoumarin, 3-(3,3-dichloro-2,3-dihydroxy-propyl)-8-hydroxy-6-methoxy-isochromen-1-one, Dichlorodiaportinol A (1) obtained from the culture broth of this fungus. Its *in vitro* cytotoxic activity against human breast cancer (MCF-7) and human liver cancer (HepG2) cell lines were evaluated.

MATERIALS AND METHODS

General

Melting points were detected on X-4 micromelting point apparatus (Cany Precision Instruments Co., Ltd., Shanghai, China), uncorrected. Ultraviolet (UV) absorptions were measured in MeOH on a Shimadzu UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Infrared (IR) spectra were obtained on a Nicolet 5DX-Fourier transform infrared (FTIR) spectrophotometer (Thermo Electron Corporation, Madison, USA). Nuclear Magnetic Resonance (NMR) data were recorded on a Bruker AVIII 600MHz NMR spectrometer (Bruker BioSpin GmbH company, Rheinstetten, Germany), using deuterated acetone (CD_3COCD_3) as solvent and tetramethylsilane (TMS) as internal standard,and coupling constants (*J*) are in Hz. Electrospray ionization mass (ESIMS) and high resolution electrospray ionization mass (HRESIMS) were operated on LCQ-DECA-XP (Thermo,USA), and LCMS-IT-TOF (Shimadzu, Janpan) mass spectrometers, respectively. Chromatography was carried out on silica gel column (200-300 mesh; Qingdao haiyang chemicals Co., Ltd., Qingdao, China). All other reagents used were analytical grade.

Fungus and cell material

The *Trichoderma* sp. 09 strain was isolated from the root of *M. bontioides* A. Gray collected in Leizhou Peninsula, Guangdong Province, China. Stock cultures were maintained on slant solid cornmeal seawater agar. Plugs of agar supporting mycelia growth were cut and transferred aseptically to a 250 mL Erlenmeyer flask containing 100 mL of liquid medium (glucose 10g/L, Peptone 2 g/L, yeast extract 1 g/L, NaCl 30 g/L). The flask was incubated at 30ºC on a rotary shaker for 5–7 days. The mycelium was aseptically transferred to 500 mL Erlenmeyer flasks containing culture liquid (200 mL). The flasks were then incubated at 30ºC for 25 days.

Breast MCF-7 and Liver HepG2 cell lines were purchased from the American Type Culture Collection (ATCC). They were cultured in Dulbecco's modification Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin (Invitrogen). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Microplate reader (TECAN, Inc.). Flat-bottom microtiter plates, 96 well were from Falcon, NJ, USA. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for viability assay was purchased from Sigma (St. Louis, Mo., USA).

Extraction and isolation

The cultures (30 L) were filtered through cheesecloth. The filtrate was concentrated to 5 L *in vacuo* below 50ºC and extracted five times by shaking with an equal volume of ethyl acetate. The combined extract (18.6 g) was subjected to silica gel column chromatography and eluted with petroleum ether–ethyl acetate (90:10, 80:20, 70:30, 50:50, and 0:100) to afford fractions 1-5 (0.9, 1.6, 2.2, 3.7, and 5.8 g, respectively). Fraction four (3.7 g) was subjected to silica gel column chromatography and eluted with petroleum ether–ethyl acetate in a gradient of ethyl acetate (petroleum ether-ethyl acetate, 100:0-50:50). Fifty-two fractions of 30 ml each were collected, combined on the basis of their thin layer chromatography profiles, and concentrated to dryness to give nine major fractions A–I. Repeated slow recrystallization of Fraction H (85 mg, petroleum ether-ethyl acetate 60:40, v/v) at room temperature from petroleum ether-ethyl acetate (65:35, v/v) yielded 3.2 mg white crystalline needle shape solid of Dichlorodiaportinol A (1).

Assessment of Antitumor Activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) Assay

Cells were harvested during logarithmic growth phase and seeded in 96-well plates at a density of 1×10^4 cells/mL, and cultured at 37ºC in a humidified incubator (5% CO_2) for 24 h, followed by exposure to various concentrations of compound one tested for 48 h. Negative control cells were incubated without test samples. Cells treated with epirubicin served as a positive control. Subsequently, 20 μL of MTT (Genview, Houston, TX, USA) solution (5 mg/mL) was added to each well and mixed, the cells were then incubated for an additional 4 h. Culture supernatant was removed and 150 μL of Dimethyl sulfoxide (DMSO) (Sangon Biotech, Shanghai, China) was added to each well to fully dissolve the MTT-formazan crystals. Cell growth inhibition was determined by measuring the absorbance (Abs) at λ = 570 nm using a microplate reader and calculated according to the following equation: Growth inhibition = (1 − OD of treated cells/OD of control cells) ×100%. The half maximal inhibitory concentrations (IC_{50}) were obtained from liner regression analysis of the concentration-response curves plotted for each tested compound.

RESULTS

Dichlorodiaportinol A (1)

White needles, m.p. ($CHCl_3$) 137-139°C [α] $^{25}_{D}$ + 7.5° (*c* 0.04, CH_3COCH_3). UV (MeOH) λ_{max} (nm): 243, 280, 326. IR (KBr) υ_{max} (cm^{-1}): 3,421, 2,925, 1,685, 1,637, 1,570, 1,508, 1,241, 1,207, 1,166, 1,047 cm^{-1}. ^{1}H-nuclear magnetic resonance (NMR) (600 MHz, CD_3COCD_3), ^{13}C-NMR (150MHz, CD_3COCD_3), Heteronuclear Multiple Bond Correlation (HMBC) and ^{1}H-^{1}H COSY spectral data are listed in Table 1. ESIMS *m/z* 335[M+H]$^+$, 356 [M+Na]$^+$. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) *m/z* 335.0082 ([M+H]$^+$, calcd. 335.0084), 356.9902 ([M+Na]$^+$, calcd. 356.9903).

DISSCUSSION

Dichlorodiaportinol A (**1**) m.p. 137–139°C, has a molecular formula of $C_{13}H_{12}Cl_2O_6$ as determined by HR-ESI-MS *m/z* 335.0082 ([M+H]$^+$, calcd. 335.0084) and *m/z* 356.9902 ([M+Na]$^+$, calcd. 356.9903). Its UV spectrum with absorption maxima at 243, 280 and 326 nm was indicative of a conjugated chromophore. Its IR spectrum with absorption bands at 3,421, 1,685, 1,637, 1,570, and 1,508 cm^{-1} suggested the presence of hydroxyl, α,β-unsaturated δ-lactone carbonyl, and olefinic functionalities, respectively. These spectra revealed the presence of typical isocoumarin moiety in comparison with known isocoumarins. [10,11] In the ^{1}H-NMR spectrum, a lowfield, one-proton singlet signal at δ 10.47 (s, 1H, exchangeable) indicated the presence of a hydrogen-bonded phenolic proton at C-8.[12] Therefore, a pair of meta-coupled doublets (*J* = 2.4 Hz) at δ 6.53 and 6.64, were assigned to H-5 and H- 7, respectively. Another aromatic singlet at δ 6.76 (s, 1H), connected to the carbon at δ 108.6 (C-4) in the HSQC spectrum, was ascribed to H-4 of the isocoumarin moiety. A sharp three-proton singlet at δ 3.92 belonging to a methoxy group was connected to C-6 that was determined by the HMBC correlations from 6-OCH_3, H-5, and H-7 to the carbon at δ 169.0 (C-6). The remaining substituent of the isocoumarin was revealed to contain a -CH-CH-CH- fragment by the ^{1}H-^{1}HCOSY correlations from H-9 to H-10 and H-10 to H-9 and H-11. Two hydroxyls at δ 5.60 (s, 1H, exchangeable) and 5.70 (s, 1H, exchangeable) were, respectively, assigned to be at the positions of C-9 and C-10 by the ^{1}H-^{1}HCOSY correlations from 9-OH to H-9 and 10-OH to H-10. Therefore, the two remaining chlorine atoms were both connected to C-11. The HMBC correlations from H-9 to C-4 and H-4 to C-9 further demonstrated the connectivity of C-3 and C-9. Hence, the structure of compound 1 was established as 3-(3,3-dichloro-2,3-dihydroxy-propyl)-8-hydroxy-6-methoxy-isochromen- 1-one [Figure 1]. We named it Dichlorodiaportinol A, according to its similar structure with the known compound, Dichlorodiaportin.[13]

The relative configuration of compound one was assigned based on the method of J-based configurational analysis and Nuclear Overhauser effect (NOE) data.[14,15] For compound one, H-9 and H-10 were anti due to their large vicinal coupling constant (9.0 Hz). In this case, C-11 and C-3 might be gauche in the threo diastereomer or anti in the erythro diastereomer. Since the NOE interactions between 9-OH and H-11 were also observed, it suggested that 9-OH and H-11 were gauche, and C-11 and C-3 were anti. Accordingly,

Table 1: NMR data for compound 1 (CD_3COCD_3, TMS)

	^{1}HNMR	^{13}CNMR (DEPT)	^{1}H-^{1}HCOSY	HMBC
1		167.6(C)		
3		157.4(C)		
4	6.76(s, 1H)	108.6(CH)		C-3,5,8a, 9
4a		141.0(C)		
5	6.64(d, 2.4Hz, 1H)	103.9(CH)	H-7	C-4,6,7,8a
6		169.0(C)		
6-OCH_3	3.92(s, 3H)	57.4(CH3)		C-6
7	6.53(d, 2.4Hz, 1H)	102.6(CH)	H-5	C-5,6,8,8a
8		165.2(C)		
8a		102.0(C)		
9	4.40(d, 9.0Hz, 1H)	73.9(CH)	H-10	C-4,10,11
10	4.28(dd, 1.8, 9.0Hz, 1H)	77.7(CH)	H-9,11	C-9,11
11	6.42(d, 1.8Hz, 1H)	77.0(CH)	H-10	C-9,10
9-OH	5.70(s, 1H)		H-9	
10-OH	5.60(s, 1H)		H-10	
8-OH	11.09(s, 1H)			

NMR: Nuclear magnetic resonance, HMBC: Heteronuclear multiple bond correlation, TMS: Tetramethylsilane, HNMR: Proton Nuclear Magnetic Resonance, CNMR: Carbon Nuclear Magnetic Resonance

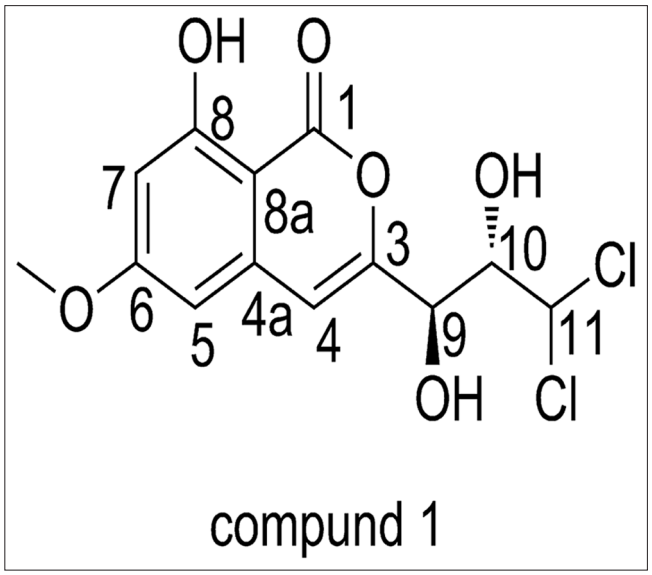

Figure 1: The structure of compound 1

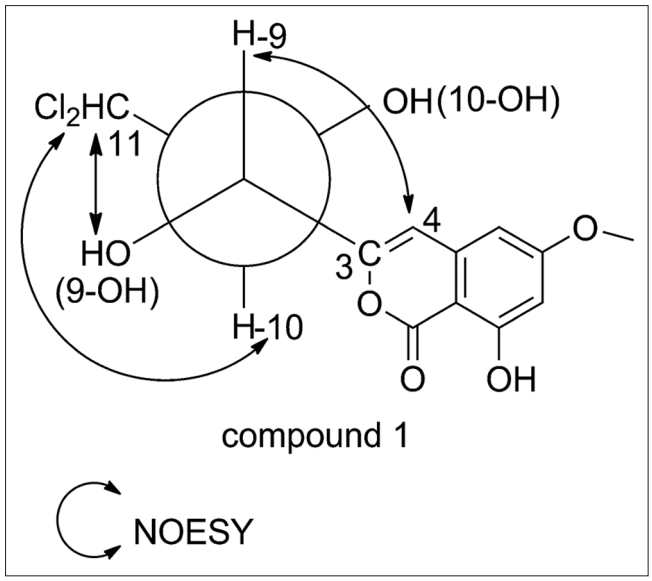

Figure 2: The key NOESY correlations of compound 1

Table 2: Cytotoxicity of compound 1 against MCF-7 and HepG2 cells (IC_{50}, μg/mL)

Sample	Cells	
	MCF-7	HepG2
Compound 1	17.8	39.6
Epirubicin[a]	5.3	5.2

a: Used as a positive control

compound one was confirmed as the erythro diastereomer. The key Nuclear Overhauser effect spectroscopy (NOESY) correlations were shown in Figure 2.

Compound one was evaluated for *in vitro* cytotoxic activity against two human cancer cell lines including human breast MCF-7 and human Liver HepG2 by MTT assay[16,17] using epirubicin (an anticancer drug used widely in the clinic[18,19]) as positive control. The results are summarized in Table 2. Compound one was moderately active against MCF-7 and HepG2 cells as compared with the standard. Thus, this study suggests that endophytes of *M. bontioides* are worthy of consideration for the development and research of antitumor agents.

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In vitro anti-telomerase activity of novel lycopene-loaded nanospheres in the human leukemia cell line K562

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ABSTRACT

Background: Lycopene, a plant carotenoid, has potent effects against the various types of cancer cells. To date, the effect of lycopene in the free and encapsulated forms on the telomerase activity in human leukemia cell line K562 have not been investigated. The aim of the present study was to prepare a novel lycopene-loaded nanosphere and compare its anti-telomearse activity in K562 cell line with those of free lycopene. **Materials and Methods:** The lycopene-loaded nanospheres were prepared by nanoprecipitation method. The lycopene entrapment efficacy was measured by high-performance liquid chromatography (HPLC) method. The anti-proliferation effect of the lycopene in the free and encapsulated forms in the different times (0-72 h) and the different doses (0-100 μg/ml) on K562 cell line was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The changes of telomerase activity, following treatment with the lycopene in the free and encapsulated forms, were detected using the telomeric repeat amplification protocol-enzyme-linked immunosorbent assay. **Results:** The entrapment efficacy of lycopene was 78.5% ± 2. Treatment of the K562 cell line with lycopene, in particular in encapsulated form, resulted in a significant inhibition of the cell growth and increasing of percentage of apoptotic cells. It has also been observed that the telomerase activity in the lycopene-loaded nanospheres-treated cells was significantly inhibited in a dose and time-dependent manner. **Conclusion:** Our data suggest a novel mechanism in the anti-cancer activity of the lycopene, in particular in encapsulated form, and could be provided a basis for the future development of anti-telomerase therapies.

Key words: Apoptosis, K562, lycopene, nanosphere, telomerase

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INTRODUCTION

Telomerase is a cellular reverse transcriptase enzyme that can add nucleotide repeats to telomeres by using ribonucleic acid template.[1,2] Telomerase activity is closely dependent on the expression of subunit human telomerase reverse transcriptase (hTERT) that is widely expressed in many different cancers.[3-8] Many studies have indicated that the activation of telomerase and telomere stabilization is a crucial step in tumorigenesis.[3] Recent studies showed that the telomerase activity is present in the almost all tumor-derived human cell lines analyzed and in at least 85% of human tumor samples.[4,5] Additionally, many studies have demonstrated that the inhibition of telomerase triggers apoptotic death in various cell types.[6,7] In this case, many different synthetic chemical agents have been proposed for telomerase inhibition,[8,9] but most of these compounds have also influenced to the normal cell system with severe toxic side effects.[10] Extensive studies have been carried out in search of substances that are capable of inhibiting telomerase activity for retarding the growth of cancer cells without affecting normal cells.[11-13] In this field, certain plant-derived compounds have been found to be able to inhibit telomerase activity through transcriptional repression of hTERT leading to induction of cell death in several cancerous cell lines.[5,7-13]

Lycopene [Figure 1], a water insoluble carotenoids are found in some red fruits and vegetables, such as red carrots, papayas, and tomatoes.[14] Lycopene has an inhibitory effect on different kinds of cancer cells including colon, breast, endometrial, and prostate cancer cells.[15,16] However, the insolubility of the lycopene in the water has restricted its use in the biomedical research.[17]

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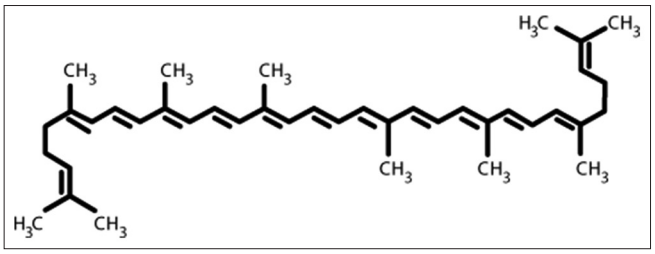

Figure 1: Lycopene chemical structure

Some studies showed that the encapsulation of plant-derived materials in the nanoparticles markedly alter their pharmacokinetics and compensated their water insolubility.[18,19] Nanoparticles as biodegradable and biocompatible polymeric submicron carriers have a general name to describe nanocapsules, nanospheres, and mixed micelles.[20,21] The nanospheres have a polymeric matrix and have been developed as drug targeting delivery system, using poly lactide, poly lactic acid-co-glycolid (PLGA) and other polymers.[22] PLGA is synthesized from two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid and used in drug delivery, owing to its biodegradability and biocompatibility.

To date, little is known about the molecular mechanism involved in the anti-cancer effect of lycopene on K562 cells,[23] and there is no report regarding the inhibitory effect of lycopene in the free and loaded forms on telomerase activity in this cell line. The aim of present study was therefore, to investigate the molecular mechanism involved in induction of apoptosis by lycopene in the free form and loaded in PLGA nanospheres in human leukemic cell line K562 with special emphasis on their role in inhibiting telomerase activity.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), Roswell Park Memorial Institute medium 1640 (RPMI)-1640, penicillin, streptomycin, and trypan blue were purchased from Gibco BRL (Gaithersburg, MD, USA). Lycopene (purity ≥ 90%), Hoechst 33342, PLGA, mineral oil, sorbitan monostearate, and tetrahydrofuran were obtained from Sigma (St. Louis, MO, USA). Annexin V-propidium iodide (PI) apoptosis Detection Kit and TeloTAGGG telomerase polymerase chain reaction (PCR)-enzyme-linked immunosorbent assay (ELISA) Plus kit were purchased from Roche (Applied Science, Germany). Methanol, methyl-*tert*-butyl ether and ammonium acetate were purchased from Merck (Darmstadt, Germany).

Preparation of lycopene-loaded nanospheres

The lycopene-loaded nanospheres were prepared by nanoprecipitation method, as described previously.[22] Briefly, the nanospheres suspension was prepared by dissolving the PLGA (l g), lycopene (1 g), and the sorbitan monostearate (0.766 g) in acetone (270 ml). This organic phase was added with moderate magnetic stirring into an aqueous solution (530 ml) containing the polysorbate 80 (0.766 g). Empty nanoparticles were prepared in a similar manner omitting the lycopene.

Characterization of nanospheres

The content of lycoene in nanospheres was determined by high-performance liquid chromatography (HPLC) as previously described.[24] Briefly, 20 mg of prepared nanospheres were dissolved in 0.5 ml of dimethyl sulfoxide (DMSO) and mixed with 0.98 ml of methanol: Water (50:50 vol./vol.) solution. Then, the 50 μl of mixture was injected into the HPLC column. In the HPLC analysis, a C30 column (3 μm, 150 × 4.6 mm, YMC, Wilmington, NC, USA) was used. The mobile phase for the determination of the lycopene was methanol/methyl-tert-butyl ether/water (83:15:2, v/v/v, with 1.5% ammonium acetate in the water; solvent A and methanol/methyl-tert-butyl ether/water (8:90:2, v/v/v, with 1% ammonium acetate in the water; solvent B at a flow rate of 1.2 ml/min and then the percentage of lycoene loading was then calculated as:

Amount of lycoene in nanospheres × total volume tested × 100/total sample volume × initial amount of nanospheres.

The particle size, zeta-potential, and polydispersity index of the lycopene-loaded nanospheres were evaluated using Malvern Zetasizer apparatus (Malvern Instrument, Worcestershire, UK), as reported previously.[22] Briefly, the particle size and zeta-potential of nanospheres were measured at angles of 90° and 120° at 25°C, respectively. These experiments were done in triplicate.

Cell culture

Human chronic myelogenic leukemia cell line K562 was purchased from Pasteur Institute of Iran (Tehran, Iran). The cells were maintained in RPMI-1640 medium supplemented with 10% heat inactivated FBS along with penicillin (100 units/ml) and streptomycin (100 μg/ml) and were grown at 37°C in a humidified atmosphere of 5% CO_2. Lycopene was dissolved in the tetrahydrofuran to obtain a 100 mg/ml stock solution.[25] All subsequent dilutions were made in the RPMI medium.

Cell proliferation assay

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously.[26] Briefly, 5×10^3 cells were incubated in 96 well plates in the presence of different concentration of lycopene in the free and