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Preparation, characterization and antiproliferative activity of thymoquinone-β-cyclodextrin self assembling nanoparticles

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Thymoquinone (TQ) was complexed with β-cyclodextrin (CD) to form nanosized aggregates. Various TQ:CD ratios were tested and it was found that the ratio of (1:0.25) TQ:CD formed distinguishable nanoparticles with minimum toxicity towards normal cells. These nanoparticles had an average size of 445 ± 100 nm with a charge 21.8 mV using Zeta-sizer. Particle size measurement using scanning electron microscopy (SEM) showed an average size of 400 nm and it also revealed the presence of smaller structures, with an average size of 50 nm. The *in vitro* antiproliferative activity on MCF7 cells was determined using MTT assay and an IC_{50} of 4.70 ± 0.60 μM for TQ-CD nanoparticles in comparison to 24.09 ± 2.35 μM of free TQ solution after 72 h of incubation. Simultaneously, TQ-CD nanoparticles showed lesser toxicity than TQ solution using human periodontal fibroblasts as a model for normal cells. It could be concluded from the results that TQ loaded cyclodextrin nanoparticles might serve as a potential nanocarrier to improve TQ solubility as well as its antiproliferative activity with little toxicity to normal tissues.

1. Introduction

The seeds of *Nigella sativa* Linn. (family Ranunculaceae), known as the black seed, are used in folk medicine all over the world for medicinal as well as culinary purposes (Ali and Blunden 2003). The seeds contain both fixed and essential oils, proteins, alkaloids and saponins. Much of the biological activity of the seeds has been proven to be due to thymoquinone (TQ), the major component of the essential oil, which is also present in the fixed oil portion. The seed/oil has anti-inflammatory, analgesic, antipyretic, antimicrobial, hypoglycemic as well as antioxidant activities (Ali and Blunden 2003). It has also been proven to have anti carcinogenic and mutagenic activity on variety of cancers that has been experimented *in vitro* using cell lines, including Caco-2, LoVo, HT-29, DLD-1 and HCT-116 (Gali-Muhtasib et al. 2006). The antiproliferative activity of TQ has been proposed through induction of apoptosis by p53 dependent and p53 independent pathways in cancer cell lines (El-Mahdy et al. 2005; Gali-Muhtasib et al. 2008).

Although TQ is a proven antioxidant and anticancer drug, its administration is limited due to poor water solubility (Ganea et al. 2010). In addition, it has been reported that the administration of high doses to rats resulted in hypoactivity and difficulty in respiration associated with reduced glutathione in the liver and kidney (Badary et al. 1998). To overcome these limitations, different drug delivery systems have been approached as alternatives to TQ's direct administration. These include poly(D, L lactide-co-glycolide) PLGA nanoparticles (Ganea et al. 2010; Ravindran et al. 2010), and PHA-mPEG copolymeric nanocontainers (Shah et al. 2010) and liposomes (Odeh et al. 2012). Other possible delivery systems would include polymers, chitosans, dendrimers, niosomes, cyclodextrins, etc. (Xiong et al. 2011).

Cyclodextrins (CD) are cyclic oligosaccharides usually comprising 6–8 D-glucose units, forming inclusion complexes with different molecules in aqueous solution and in solid state (Ishiguro et al. 2011). Cyclodextrins have been applied pharmaceutically to enhance the solubility, stability, safety and bioavailability of different drugs as well as to improve safety for anticancer drugs (Uekama et al. 1998; Bilensoy et al. 2008; Gao et al. 2012).

Recently, it has been suggested, that drug inclusion/complexation in cyclodextrins would enhance drug permeation through biological barriers and it was specific for lipophilic drugs that show permeation layer dependent drug absorption (Loftsson 2012).

At higher concentrations, lipophilic drug molecules, CDs and CD complexes have been reported to form aggregates in aqueous solutions, with a diameter of around 100 nm (Messner et al. 2010). Formation of such nanoparticles is expected to have important effects on both physicochemical and biological properties of the complexes and has been investigated for several model molecules (Messner et al. 2011).

In addition to their pharmaceutically favorable action, amphiphilic cyclodextrins were reported to be non-hemolytic and non cytotoxic according to studies on human blood samples and L929 mouse fibroblast cells regardless of their different chemical structures (Memisoglu et al. 2003; Memisoglu-Bilensoy el al. 2006).

The objective of this study was to compare the safety and efficacy of novel, non-surfactant, thymoquinone-loaded amphiphilic β-cyclodextrin nanoparticles to thymoquinone solution. The safety is to be estimated through cell viability studies using normal periodontal fibroblasts and antiproliferative activity using adenocarcinoma cell line (MCF7).

Table 1: Particle size and zeta-potential of the various formulations with different TQ:CD ratios

TQ: CD Ratio	Physical Appearance	Particle size (nm)	Size distribution (100%)	Zeta potential (mV)
1: 0.25	Turbid	445	100	−21.76
1:1	Clear	420	90.5	−27.89
		6.97	5.1	
		0.96	4.1	
0.5:1	Clear	352	63.9	−21.76
		172	36.1	
0.25:1	Clear	341	100	−18.24
0.15:1	Clear	210.6	100	−16.42

2. Investigations, results and discussion

For optimal drug efficacy, the ability of the drug to get dissolved, penetrate the biological barrier and reach its target within cells is of prime importance. Nanoparticle technology is able to enhance drug solubility and stability as well as drug biological availability *in vivo*. The aim of this study was to find a novel and safe delivery system for TQ where its solubility is enhanced and biological activity is at least preserved.

2.1. Nanoparticle preparation

After complete dissolution of TQ and cyclodextrin, the two aqueous solutions were mixed and particle size of the resultant conjugates was determined. Various ratios have been prepared and characterized (Table 1). The ratio that gave the most homogenous particles in the submicron range and preserved the antiproliferative activity of TQ was that of TQ:cyclodextrin of 1:0.25. This formula was freshly prepared each time and subjected to physical characterization. The presence of cyclodextrin enabled the conjugation of double the amount of TQ for biological testing.

2.2. Nanoparticle characterization

Various techniques were used for the characterization of TQ-CD nanoparticles. These include spectroscopic, gravimetric, particle sizing, zeta potential and SEM.

Table 1 shows particle sizing of TQ-CD nanoparticles after drying and re-suspension in water. These particle sizing data were in agreement with SEM imaging shown in Fig. 1. As obvious in the SEM images, TQ-CD nanoparticles are composed of smaller ones with sizes in the range of $\sim$ 50 nm which aggregate to form larger particles. However, any variation in the TQ:CD ratio can lead to sheet like aggregates that can be seen under the light microscope and could be detected using the particle sizer under the unweighted mode (Table 1).

In order to better understand the physical properties of these TQ-CD nanoparticles, DSC studies were conducted. These data are shown in Fig. 2. For β-CD alone (Fig. 2.A) the transition temperature was at 120 °C while for TQ alone (Fig. 2.B) this temperature was at $\sim$ 48 °C. However, for the TQ-CD nanoparticles the transition temperature was 110 °C with 10 degrees drop indicating the presence of inclusion complexes of TQ.

The FTIR spectra of β-CD alone, TQ alone and TQ-CD nanoparticles (Fig. 3) show the presence of a shift in TQ's main peak at 1555 cm^{-1} (due to the C = O bond) to 1672 $cm-^{1}$ in addition to the disappearance and/or weakening of TQ peaks in the region 400–1500 cm^{-1}, which can be strongly attributed to the formation of the inclusion complexes and nano-structures formation. Cardoso et al. (2012) described TQ-CD inclusion complexes with regards to physical properties in liquid and solid state. Their experimental results indicated the formation of 1:1 cylodextrin inclusion compounds with TQ in a guest-host complex formation. TQ in Cardoso et al. model was interposed between CD molecules and was a fully solubilized in contrast to what is done here. Nevertheless, in agreement with the reported results, FTIR data indicate the inclusion of TQ in the colloidal carrier system.

2.3. Antiproliferative activity of TQ and TQ-CD nanoparticles

In this study, the MTT assay was used to determine cell viability after the exposure to either free TQ, free CD or TQ-CD nanoparticles. The effect on the proliferation of cells was followed for 24, 48 and 72 hours. Cell proliferation results are shown in Fig. 4 A and B.

Firstly, TQ antiproliferative activity was tested against MCF7 cells and IC_{50} was calculated. TQ showed dose and time dependent toxicity and its antiproliferative activity increased gradually with increasing the incubation time from 24 to 48 and then to 72 h. The maximum reduction was seen after 72 h with an IC_{50} value of 24.09 ± 2.35 μM (a). These findings come into agreement with those done earlier by Woo et al., who reported time and dose dependency and an IC_{50} of 32 μM after 48 h of incubation (Woo et al. 2011). Arafa el al. recorded a 35% reduction in cell viability after the incubation of MCF7/Dox resistant cells with 100 μM TQ (Arafa et al. 2011).

Secondly, when TQ was loaded in CD nanoparticles, the antiproliferative effect showed time dependency with maximum potency after 72 h. IC_{50} value for these nanoparticles after 72 h

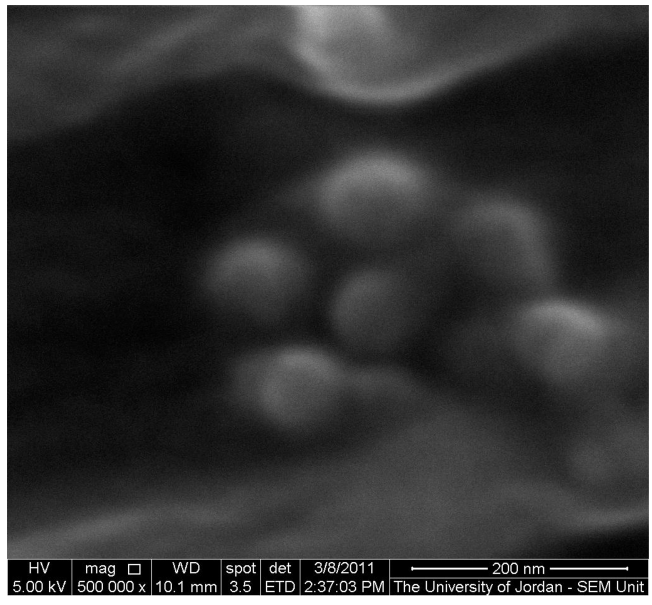

Fig. 1: Scanning Electron Micrograph of cyclodextrin loaded thymoquinone nanoparticles.

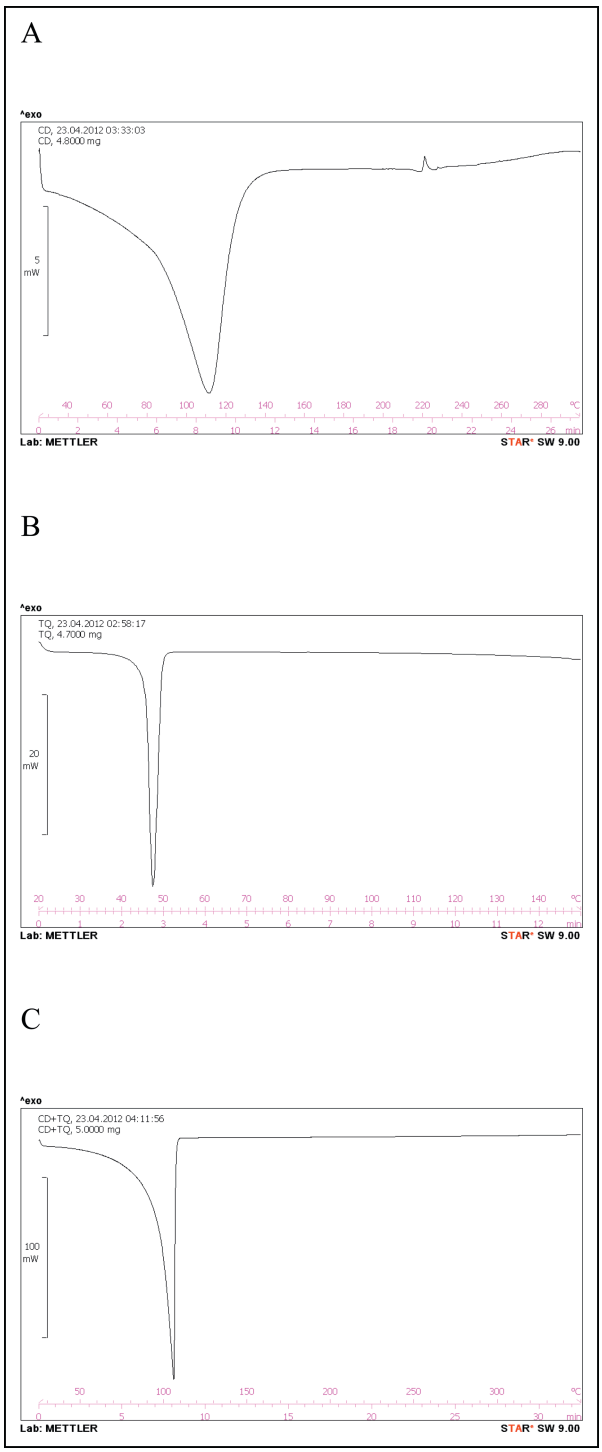

Fig. 2: Differential scanning calorimetry thermograms of (A) pure β-cyclodextrin, (B) pure thymoquinone and (C) thymoquinone:cyclodextrin nanoparticles.

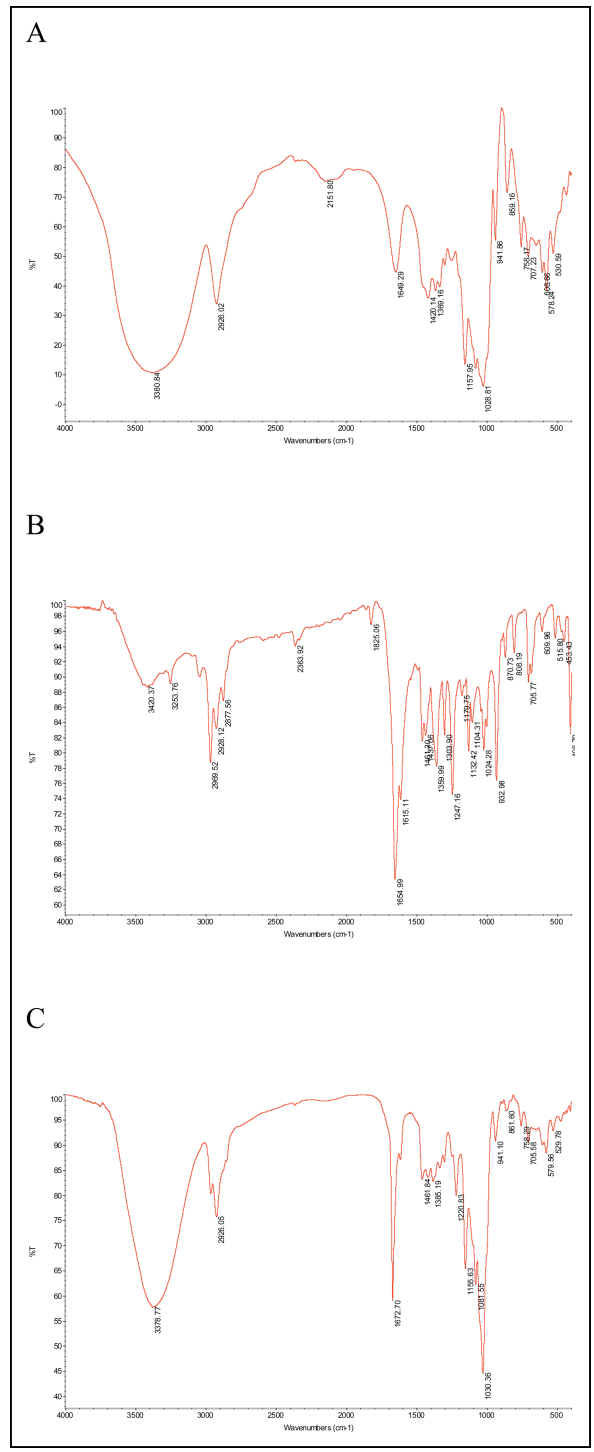

Fig. 3: Fourier transform infrared spectra of (A) pure β-cyclodextrin, (B) pure thymoquinone and (C) thymoquinone:cyclodextrin nanoparticles.

of incubation was 4.70 ± 0.60 μM (b). This enhanced antiproliferative effect is most likely due to improved cellular permeation. Ravindran et al. designed PLGA-PEG nanoparticles of TQ and those were tested for antiproliferative activity against a panel of cell lines including human chronic myeloid leukemia, colon cancer, breast (MCF7) and prostate cancer. The nanoparticles had a size range of 150–200 nm. In all the cell lines tested, TQ nanoparticles had enhanced antiproliferative effect over free TQ after 72 h of incubation. TQ nanoparticles were twice as toxic to cancer cells than free TQ and this was attributed to enhanced cellular uptake (Ravindran at al. 2010). Similar results were obtained with nanoencapsulation of other naturally occurring anticancer agents prepared with the same group such as curcumin (Zhao et al. 2012).

Chitosan TQ nanoparticles were also prepared using the ionic gelation method and the resultant particles showed enhanced permeation using *in vitro* cultured nasal epithelium (Alam et al. 2012). In another recent study, solid lipid nanoparticles (SLN) were prepared with an average diameter of 160 nm and a zeta potential of -11.34 mV. The SLN were designed for oral administration and showed higher bioavailability and a better hepatoprotective activity against paracetamol induced liver cirrhosis (Singh et al. 2013).

Free CD was not toxic in the concentration range under investigation and cell viability was not reduced below 75% after

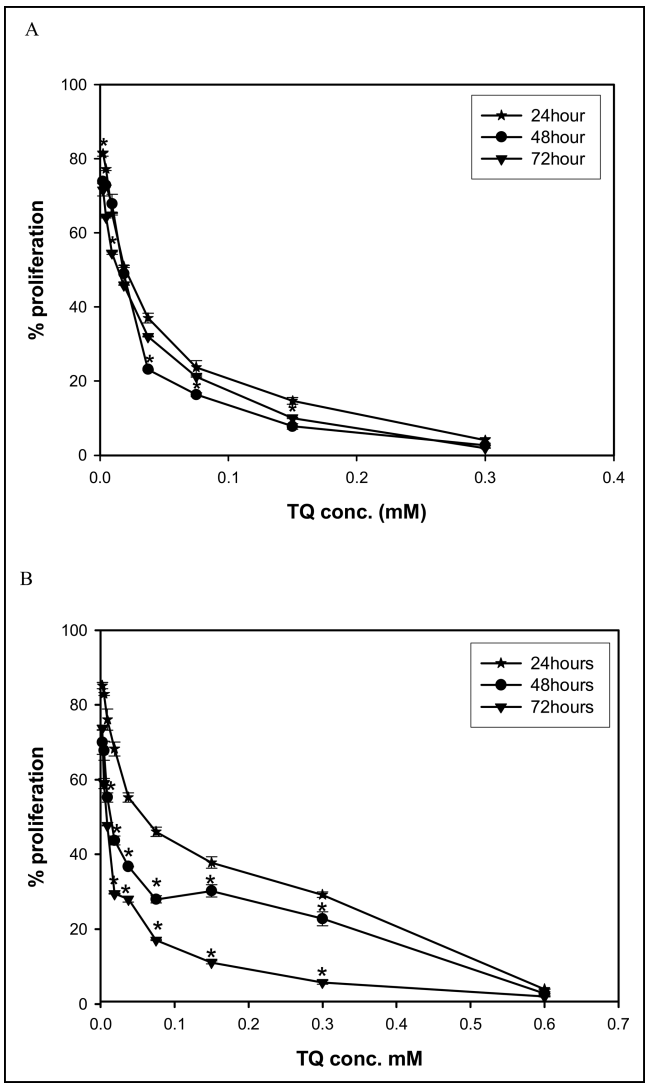

Fig. 4: Antiproliferative activity of thymoquinone (A) and thymoquinone loaded nanoparticles (B) against breast adenocarcinoma cells (MCF7). Effect of incubation time is demonstrated. Cell proliferation was determined by MTT assay. Results are expressed as percentages of un-treated cells. Each value is the average ± standard deviation of at least four replicates and reproduced using two passages. A one-tailed t-test used for each TQ concentration (* $P < 0.01$).

72 h incubation. The safety of free β-CD as been proved earlier and specifically against MCF7 cells as well as other tissues (Memisoglu-Bilensoy et al. 2005).

To investigate the toxicity of the nanoparticle system against normal cells, free TQ, CD and TQ-CD conjugates were incubated with human periodontal fibroblasts at one of the highest loaded TQ concentrations of 0.3 mM and incubation for 24, 48 and 72 h followed. Here, maximum toxicity was shown for free TQ and TQ-CD nanoparticles at 72 h. Nevertheless, toxicity was always significantly lower for the nanoparticles than for free TQ (Fig. 5). Parallel to the known anticancer and antioxidant activities of TQ, it has been reported that TQ has low toxicity after oral and peritoneal administration to animals as well as selective anticancer activity. Alhosin et al. (2012) compared the anti-neoplastic activity of TQ in human astrocytoma and T lymphoblastic leukemia cells. TQ showed time and concentration dependent degradation of a α/β tubulin in both cancer cell lines, whereas its toxicity to normal human fibroblasts was minimal. The selective antitumor activity has also been reported for other cell lines in comparison to normal fibroblasts (Ivankovic et al. 2006). After its conjugation with CD, TQ toxicity went even lesser, which might indicate lower uptake of the nanoparticles

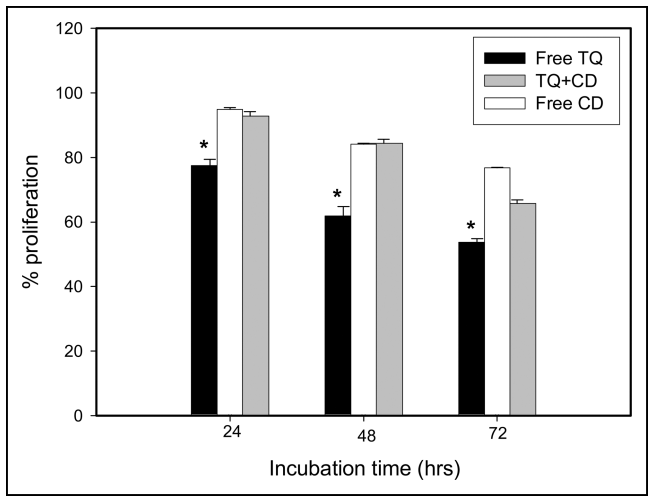

Fig. 5: Comparison of the toxicity of thymoquinone nanoparticles versus thymoquinone solution using freshly excised human periodontal fibroblasts. Cell proliferation was determined by MTT assay. Results are expressed as percentages of un-treated cells. Each value is the average ± standard deviation of at least four replicates and reproduced using two passages. A one-tailed t-test used for each treatment (* $P < 0.01$). Significant difference between free TQ and TQ-CD nanoparticles is shown.

by normal human fibroblasts or a difference in the uptake mechanism. Nevertheless, while the highest concentration of TQ-CD liposomes used reduced the viability of MCF7 cells to 5.75% after 72 h of incubation, it only reduced that of normal human fibroblasts to 65.75%. This demonstrates the potential selectivity of prepared TQ-CD nanoparticles against cancer cells.

2.4. Conclusion

β-Cyclodextrin successfully conjugated thymoquinone to give self assembling nanostructures with an average size of 400 nm and a surface charge of 21.8 mV. These aggregates allowed double the amount of thymoquinone to be solubilized and hence used for biological testing. The *in vitro* assay showed that TQ-CD nanoparticles presented higher antiproliferative and sustained effects on MCF7 cells. At the same time, TQ-CD nanoparticles were less toxic against human periodontal fibroblasts. It could be concluded that CD nanoparticles developed in this study may be considered as a promising delivery system for TQ.

3. Experimental

3.1. Materials

Thymoquinone (TQ) and β-cyclodextrin (CD) ($C_{24}H_{70}O_{35}$) were purchased from Sigma-Aldrich, (USA). All used chemicals and reagents were of analytical quality.

3.2. Preparation and characterization of TQ-CD loaded nanoparticles

TQ (2.0 mg) was added to 10 mL of distilled water and completely dissolved using vortex and water bath sonicator for one hour. β-CD (3.5 mg) was added to 10 mL distilled water and dissolved using a water bath sonicator. TQ solution was mixed with CD solution in a molar ratio of 1:0.25 (TQ:CD). The dispersion was freshly prepared each time and used within 24 hours for physical characterization and biological activity.

3.3. Characterization of TQ loaded nanoparticles

3.3.1. Size measurements and determination of zeta potential

The mean diameter and size distribution of the freshly prepared nanoparticles were measured using a Zetasizer (Nano-ZS; Micotrac Germany). Measurements were performed at 25 °C with an angle detection of 90 °C. The zeta potential of freshly prepared nanoparticles was determined by laser doppler

electrophoresis using a Zetasizer (Microtrac, Germany). All measurements were performed in triplicates.

3.3.2. Scanning electron microscope (SEM)

A drop of the TQ-CD nanoparticles dispersion was dispersed on carbon adhesive tape applied on aluminium stub, then dried completely under fume hood. The dried nanoparticles were coated with platinum nanoparticles using sputter coater Emitech (K550X, England), and then the SEM images were obtained using Inspect ^{TM} F50 Scanning Electron Microscope (FEI, Netherlands) with voltage of 15 kV.

3.3.3. Differential scanning calorimetry (DSC)

The differential scanning calorimetry (DSC) method was used to determine the phase changes in the CD melting temperature due to the inclusion of TQ. The TQ-CD nanoparticles were analyzed on a Mettler Toledo DSC823^{e} and Netzsch DSC 204 F1 instrument (Germany) with heating rate of 5 °C/ min.

3.3.4. FTIR spectroscopy

The TQ-CD nanoparticles were also examined using FTIR spectroscopy (Thermo Nicolet NEXUS 670, USA). FTIR spectra of the free TQ and CD in addition to physical mixtures of TQ and CD and TQ-CD nanoparticles were obtained using KBr discs.

3.4. Antiproliferative activity of TQ-CD nanoparticles

Human breast adenocarcinoma cell line was used (MCF7, ATCC no. HTB-22). Cells were cultured in RPMI media supplemented with 10% heat inactivated fetal bovine serum, 1% of 2 mmol/L L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin.
Cells were seeded with a density of 5000 cell/well and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 h, the cells were treated with TQ, TQ and CD nanoparticles or CD alone. Test compounds were incubated with the cells for 24, 48 or 72 h at 37 °C in humidified conditions containing 5% CO_2. At the end of the exposure time, cells were treated with MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide) 20 μL and left for 1 h. Thereafter the absorbance was measured at 490 nm using a microplate spectrophotometer (Bio Tek uQuant). Cell media was used as controls. Percentage cell viability was calculated as the ratio between the absorbance in the treatment group and the absorbance for media. All experiments were performed in triplicates. As positive controls, cisplatin and doxorubicin at their IC_{50} values of 0.70 and 0.2 μM, respectively, were used (Afifi and Abu-Dahab 2012).
Under sterile conditions in a class II microbiological safety cabinet, sample teeth included 2 fully erupted sound maxillary first molars extracted from an 11-year-old healthy male, was held with a sterile forceps and the middle third of the root surface was mechanically scraped with a number 15 scalpel to obtain samples of periodontal ligament (PDL) tissue. The PDL tissue was diced into small tissue explants of 1 mm^3. Thereafter, these tissue explants were placed in 25 cm^2 tissue culture flasks. The explants were incubated with DMEM culture medium supplemented with 10% FBS. The 10^{th}- 15^{th} passage of periodontal ligament fibroblasts (PLF) cells were used in the experiment.

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