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ORIGINAL ARTICLE

Co-delivery with nano-quercetin enhances doxorubicin-mediated cytotoxicity against MCF-7 cells

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Abstract Quercetin, the plant-derived phenolic compounds, plays a pivotal role in controlling hemostasis, by having potent antioxidant and free-radical scavenging properties. This flavonoid in combination with chemotherapeutic drugs improves the efficacy of these agents in induction of apoptosis in cancer cells. This study investigated the role of nano-quercetin (phytosome) in doxorubicin-induced apoptosis. Nanoparticles were characterized for particle size, zeta potential, scanning electron microscopy (SEM) and differential scanning calorimetric assessments. Anti-proliferative effect of formulations was evaluated by MTT assay. mRNA expression levels of target genes were measured by real time RT-PCR. The mean size of nanoparticles was 85 ± 2 nm with nearly narrow size distribution which was confirmed by SEM analysis. Our results showed that co-treatment of MCF-7 breast cancer cells with nano-quercetin and doxorubicin increased the percentage of apoptosis from 40.11 ± 7.72–58 ± 7.13 ($p < 0.05$). Furthermore, mRNA expression levels for downstream genes including NQO1 and MRP1 showed a marked decrease ($p < 0.05$). Taken together, our results suggest that phytosome technology can elevate the efficacy of chemotherapeutics by increasing the permeability of tumor cells to chemical agents. Our findings introduce a novel phytosome-dependent strategy to improve delivery of doxorubicin to the breast cancerous tissues.

Keywords Quercetin · Chemoresistance · Phytosome · MCF-7

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Introduction

Breast cancer is one of the most common cancers among women worldwide [1]. Despite improvements in efficacy of chemotherapeutic agents, fully understanding of mechanisms, for chemoresistance and finding novel strategies to overcome tumor cell survival still remains poorly understood [2–5]. Finding key target molecules and also safe and stable delivery systems with superior tactics for overcome resistance and diminish the side effects of chemotherapeutic agents are the main goals of any ideal cancer treatment protocol [6–8]. Nuclear factor-E2-related factor 2 (Nrf2) is a member of the cap n collar (CNC) subfamily transcription factor which serves as a cellular sensor to maintain redox homeostasis [9, 10]. Under basal conditions, Nrf2 is taken in the cytosol by binding to Kelch-like ECH-associated protein1 (Keap1). In response to oxidative stress, Nrf2 separates from Keap1, translocates into the nucleus, and binds to the ARE sequence to activate transcription of a number of cytoprotective genes including endogenous antioxidants, phase II detoxifying enzymes, and several ATP-dependent drug efflux pumps such as multidrug resistance-associated protein (MRP1and MRP2) [11]. Overexpression of Nrf2 has been shown in many type of cancers which has a main role in tumor cell growth and survival [12, 13]. Therefore, effective and discerning Nrf2

inhibitors would be beneficial for adjuvant therapy to reduce the development of cancer resistance to chemotherapy and increase the efficacy of anticancer agents [14, 15]. Flavonoids, a diverse family of natural poly phenolic compounds, commonly occurring in plants, showed strong anti-proliferative activity against many types of cancer cells with an ability to sensitize cancer cells to anticancer agents. Several flavonoid compounds, have been reported to act as Nrf2 inhibitors that can reverse drug resistance effectively, such as, luteolin and brusatol [16–18]. In this study we determined whether quercetin, a natural flavonoid that has been reported to possess anti-inflammatory, powerful radical scavenger, and anti-cancer properties, can sensitize tumor cells to anticancer drugs by modulating the Nrf2 signaling pathway. We also examined how phytosome delivery system can improve this effect [19, 20]. Phytosoms, advanced nanoparticles, recently applied for drug delivery due to different advantages including high bioavailability and improved molecular size which can enhance the passage of chemotherapeutic agents through biological membranes [21, 22]. In this study quercetin and lecithin mixture was prepared as nanoparticle complex for reaching to high stability, excellent size for absorption in tumor cells and high solubility. Then, the particles were characterized for molecular size, polydispersity index and drug loading. Quercetin diminished cellular NQO1and MRP1 gene expression levels and sensitized MCF-7 cells to doxorubicin. This study reported potential ability of nano quercetinas an adjuvant to chemotherapy protocols.

Materials and methods

Materials

Quercetin, tert-butylhydroquinone (tBHQ), and doxorubicin were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Lecithin was purchased from Lipoid GmbH, (Germany). Roswell Park Memorial Institute 1640 medium, penicillin–streptomycin and fetal bovine serum (FBS) were provided (Invitrogen Life Technologies, Auckland, New Zealand). Primers were supplied from MWG Biotech (Ebersberg, Germany). RNA isolation kit, (Trizol) was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). RevertAid^{TM} First Strand cDNA Synthesis Kit was purchased from (Fermentas Ontario, Canada).Power SYBER Green PCR Master Mix (5 ml) was obtained from Applied Bio systems (Warrington, UK). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was provided from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of phytosomes (nano-quercetin) formulation

The synthesis routes for preparation of nano-quercetin was based on thin layer method [23]. First, quercetin and phospholipid (lecithin) were dissolved in ethanol and chloroform solvent, respectively. Then the solvents were dried by using of evaporator to form thin film and finally, hydration of thin layer was done to achieve nano-quercetin suspension.

Determination of particle size

Mean particle size distribution of nanoparticles exhibited by laser light scattering Particle Analyzer (Sald 2101, Shimadzu, Japan). The size and size distribution of formulations were showed by the volume mean diameter (VMD) and number mean diameter (NMD).

Determination of quercetin entrapment efficiency

The proportion of encapsulated quercetin was determined by measuring the concentration of the free drug in the lower chamber of Amicon tube (Ultra-30 kDa molecular weight cut-off membrane, Millipore, Germany). To determine the amount of non-encapsulated (free drug), 1000 µl of formulation was placed in the outer chamber and centrifuged at 5000 rpm (Beckman Avanti TM 30, Beckman, Spain) for 20 min. The percentage of Entrapment efficiency (EE) was calculated according to the following equations:

$$EE(\%) = \frac{W\,(initial\,drug) - W\,(free\,drug)}{W\,(initial\,drug)} \times 100$$

Scanning electron microscopy (SEM) analysis

The investigation of surface morphology is often essential in detecting the entrapment behavior. The scanning electron microscopy (SEM) provided photomicrograph of the nanoparticles at appropriate magnification after covering it with a very thin layer of gold (MV2300, Czech Republic).

Determination of zeta potential

The zeta potential of nano-quercetin was measured at 25° C, under an electoral field of 40 V/cm (Malvern Instruments Ltd. Zeta sizer 2000 Malvern UK).

Differential scanning colorimetry (DSC)

Thermal analysis was done using a differential scanning calorimeter (model 200 F 3 Maia, Germany). Thermograms

were obtained at a scanning rate of 30 °C/min. The analyses were done using 2.5 mg of each sample in standard aluminum pans. Lecithin, quercetin and nano-quercetin were scanned between 25 to 380 °C, and −20 to 380 °C, respectively.

Cell culture

The human MCF-7 breast cancer cells were provided from Pasteur Institute cell bank (Tehran, Iran). Cells were cultured in RPMI 1640 medium containing 10 % fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37° C in a humidified 5 % CO_2 atmosphere. The cells with 80 % confluency were collected from culture flask with 0.05 %trypsin/EDTA solution. The cells were seeded into 96- well micro plate (200 µl/well) with concentration of 15,000 cells and 200 µl growth medium. To find the IC50 values for quercetin and doxorubicin, MCF-7cellswere incubated with increasing concentrations of the quercetin (up to 1000 µM) and doxorubicin (up to 100 µM). Then, the cells were pre incubated with quercetin or nano-quercetin for 6 h before adding doxorubicin concentrations. Non-treated cells and the cells incubated with nano blank were considered as controls.

MTT assay

After incubation of the cells with desired concentrations of chemotherapeutic agent or inhibitor, the medium was replaced with 200 µl of fresh media containing 20 µl of MTT solution (2 mg/ml) and incubated for 4 h at 37 °C. Then, media was removed and 200 µl of DMSO plus 25 ml of Sorenson buffer was added to dissolve the formazon crystals. After shaking the plates for 20 min, the absorbance was measured at 570 nm by using a microplate reader (Biotek, ELX 800, USA). Cell viability (IC50) was determined for each agent by calculating the slope and intercept of different concentrations [24].

Real-time quantitative PCR (RT-PCR)

The total RNA was isolated from cultured MCF-7 cells using Trizol reagent according to the manufacturer's protocol. The amount of RNA was determined by optical density (A260/A280 ratio) with Nano Drop 1000 Spectrophotometer (Wilmington, DE, USA). Then, 10× buffer with $MgCl_2$ 2.5 µl, 10 mM dNTP 0.5 µl, Taq polymerase (5 U/µl) 0.25 µl, cDNA 1.25 µl, 1.25 × 2 µl of forward and reverse primers 1 (5 pmol/µl), 1.25 × 2 µl of forward and reverse primers 2 (5 pmol/µl) for a total reaction volume of 25 µl. Amplification of each cDNA was performed for the 25 cycles that allowed detection of basal mRNA levels in the linear range of each mRNA. Real time PCR amplification was carried out for 35 cycles using the following protocol: 95 °C for 1 min, 94 °C for 15 s, 52.5 °C for 20 s, 72 °C for 20 s and 72 °C for 5 min specific primers for Nrf2 (5′-ACACGGTCCACAGCTCATC-3′) and (5′-TGTCAATCAAATCCATGTCCTG-3′:NQO1, (5′-ATGTATGACAAAGGACCCTTCC-3′) and (5′-TCCCTTGCAGAGAGTACATGG-3′) MRP1, (5′-ATGACCAGGTATGCCTATTATTAC-3′) and CACATCAAACCAGCCTATCTC-3′) were recycled for PCR. The PCR products were applied for electrophoresis on agarose gel and standardized with internal control GAPDH [25].

Statistical analysis

Data were conducted and expressed as mean ± SD from three independent experiments. Statistical analysis was done by applying one-way ANOVA. The significance level was considered as $p < 0.05$.

Results

Characterization of nanoparticles

The mean size of nanoparticles was 85 ± 2 nm with nearly narrow size distribution as demonstrated in Fig. 1. Figure 1a exhibited that size distribution was in the range of 50–150 nm based on VMD and 40–130 nm based on NMD. The SEM analysis confirmed our results from particle size analysis (Fig. 1b). Mean zeta potential value of nano-quercetin was −4.14 ± 0.02 mV (Fig. 1c).

Drug EE determination and stability studies

The mean value of EE was 77 ± 5 %. Nano-quercetin displayed a good stability during 2 months. No significant change in size, appearance and phase separation was observed. There was also no evidence for drug leakage from the nano systems.

Bioactive-carrier interaction with DSC

Bioactive-carrier interaction has been substantiated by DSC analysis of quercetin, the empty nanoparticle (lecithin) and nano-quercetin. As shown in Fig. 2 liposomes, containing quercetin interestingly showed the endotherm at 75 °C (lower temperature compered to empty liposome) and the melting endotherm of quercetin disappeared (Fig. 2c). Therefore, changes in thermal properties can be explained by the insertion of quercetin into the bilayer of nano-quercetin and interchange with phospholipid (lecithin).

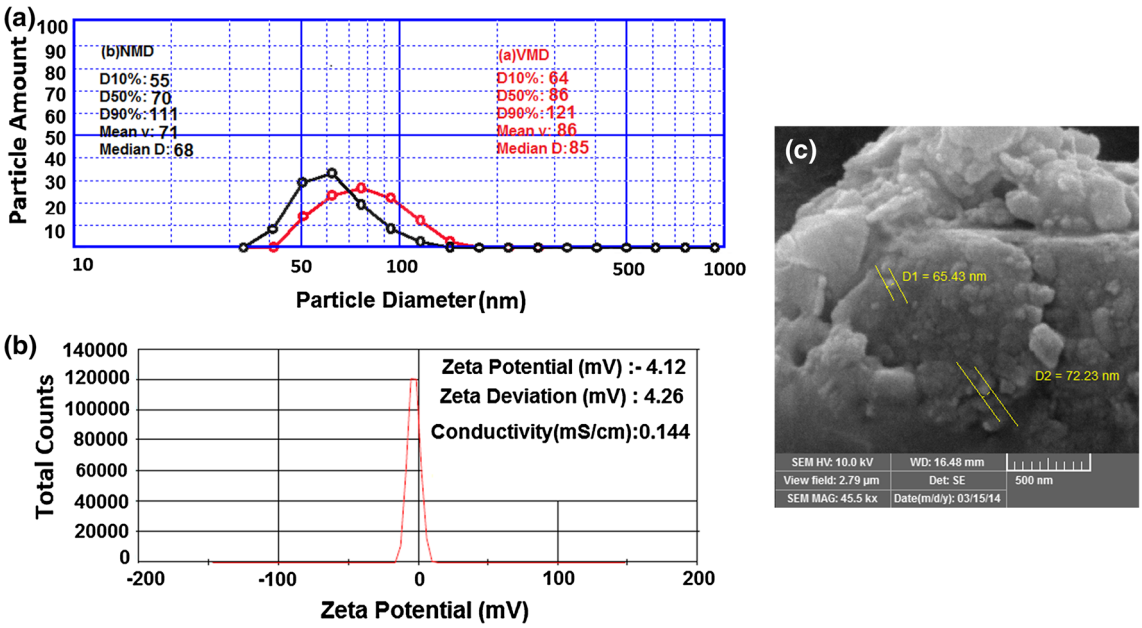

Fig. 1 Characterization of nanoparticle **a** particle size pattern of nano-quercetin based on volume mean diameter (VMD) (*red line*) and number mean diameter (NMD) (*black line*), **b** diagram of zeta potential ZP, **c** scanning electron microscopy(SEM) of nano-quercetin

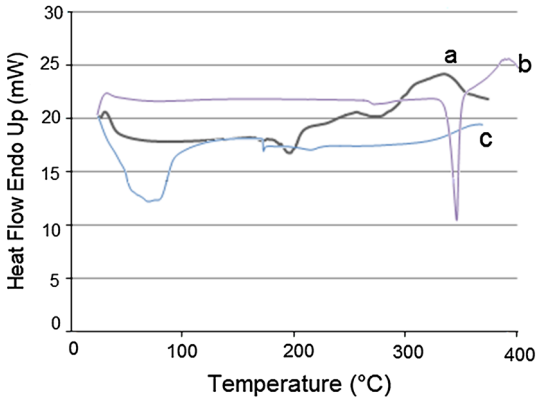

Fig. 2 Differential scanning calorimeter (DSC) pattern of **a** quercetin, **b** lecithin, and **c** nano-quercetin

In vitro cytotoxicity measurement

MTT study was carried out to confirm the impact of nano-quercetin and doxorubicin on cell survival in MCF-7 cells. IC_{50} values for doxorubicin and quercetin were determined as 0.75 µM ± 0.057 and 230 µM ± 4.144, respectively. Our results demonstrated that nano-quercetin along with doxorubicin arrested growth of the cells more effectively ($p < 0.05$). There was no significant differences between the cells treated with nanoparticle alone and non-treated cells ($p > 0.05$) (Fig. 3).

The expression of Nrf2, NQO_1 and MRP_1

Our results from real time RT-PCR showed that applying nano-quercetin caused no significant effect on Nrf2 mRNA expression. However, there was a marked decrease in the expression levels of Nrf2-target genes including NQO_1 (35 %) and MRP_1 (43 %) ($p < 0.05$) (Fig. 4).

Discussion

One of the fundamental challenges to succeed in treatment of cancer is chemoresistance. There are several mechanisms of resistance to anticancer drugs including increased expression of ATP-dependent drug efflux pumps, increased drug metabolism enzymes, and superior DNA repair. Recently, Nrf2 has been introduced as a potent therapeutic target to overcome chemoresistance [26, 27]. Therefore, identification of powerful Nrf2 inhibitors to sensitize cancer cells to chemotherapeutic agents are urgently needed [28, 29]. Flavonoids, have antioxidant properties with low toxicity which candidate them for cancer chemoprevention [30–32]. Several flavonoids have been suggested to be effective as Nrf2 inhibitors which were applied in vivo and vitro. Previous studies have shown overexpression of Nrf2 in the gastric cancer biopsies and lung cancer cell [33, 34]. Quercetin is one of the most common flavonoid that is abundant in celery, honey and chamomile tea. Many

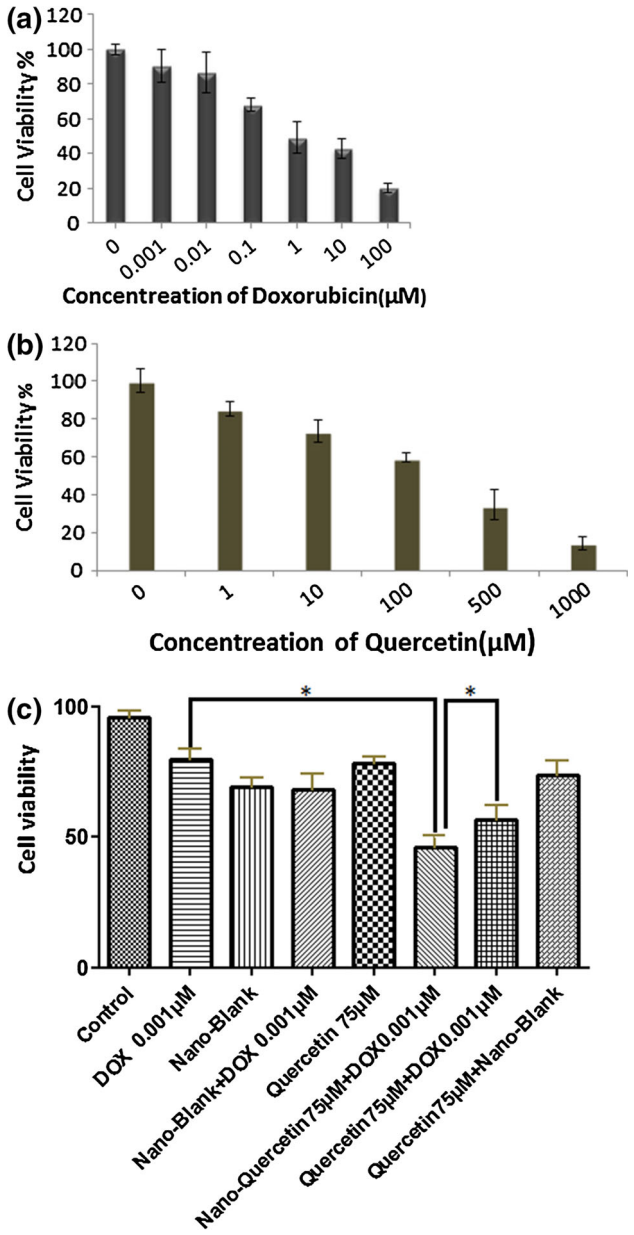

Fig. 3 Nano-quercetin sensitized cancer cells to doxorubicin. **a** MCF-7 cells were treated with various concentration of doxorubicin for 24 h and then cell viability was measured by MTT assay. **b** IC_{50} determined for quercetin against MCF-7 cells, **c** Nano-quercetin had more cytotoxicity effects in comparison with quercetin. *$p < 0.05$, **$p < 0.01$

studies have showed that quercetin play an essential role in reserving of the human body against inflammation, reactive oxygen species and cancer [35, 36]. Flavonoids, can also reverse efflux of doxorubicin out of the cells [37, 38]. However, low solubility and poor permeability across the cells are the major challenges, which restrict the application of quercetin in therapeutic protocols [39]. Using of the novel and effective delivery systems that can ameliorate the side effects of these components is the pivotal purposes in cancer treatment. Phytosome, as an advanced formulation can be an attractive candidate for enhancement of bioavailability of quercetin and also improvement in absorption through membrane of cancer cells [40]. In this study, nanoparticles were effectively prepared by thin layer method. The narrow size distribution which was confirmed by number and volume mean diameters assured reproducible delivery outcomes (Fig. 1a). Due to planar configuration, quercetin has high affinity to phosphatidylcholine to form phytosome [41]. DSC experiments revealed that quercetin was uniformly and molecularly distributed in the matrix of lecithin particles. Thermogram of nano-quercetin also showed disappearance endothermic of melting peak of quercetin and lecithin (Fig. 2c). There was no cytotoxicity in MCF-7 cells when we applied quercetin up to 75 μM. Serrano demonstrated that treatment of HepG2 cells with 50 μM quercetin for 4 and 18 h had no toxic effect with a cell viability of 82 and 60 %, respectively [42]. Staedler showed combination of quercetin with doxorubicin increased toxic effects of doxorubicin in tumor cells [43]. We employed 75 μM nano-quercetin for enhancement of quercetin effect in sensitization of cancer cells to doxorubicin. In vitro cytotoxic assays exhibited that pretreated cells with nano-quercetin was more effective than quercetin alone in induction of apoptosis when we incubated the cells for 24 h (Fig. 3c). Due to anti-oxidant and anticancer properties of quercetin, we examined major down-stream Nrf2 genes, including cytoprotective enzymes, NQO1 and drug transporter, MRP1. However, the efficacy of nano-quercetin in down regulation of NQO1 and MRP1 genes was markedly higher (Fig. 4). No significant change in Nrf2 expression even after applying nano-quercetin, can be explained by increase in Nrf2 ubiquitination followed by decrease in half-life of Nrf2 molecule which was explored by Ren et al. [44]. However, the detailed functional mechanisms of action for Nrf2 signaling and its inhibitors are under investigation [45]. Our findings suggest that phytosomes technology can help to increase the stability of quercetin in microenvironment of tumor cells which results in higher efficacy of chemotherapeutic agents with low concentration of quercetin especially in chemo-resistant tumors.

Conclusion

The goal of drug delivery systems is to modify drug release profile, and improve product efficacy to achieve a desire therapeutic effect. Encapsulation of anti-cancer agent in nanoparticle is recognized as a novel strategy to overcome resistance through several mechanisms including modulation of drug release, increasing drug penetration into cancer cells and high endocytosis phenomenon. By the way

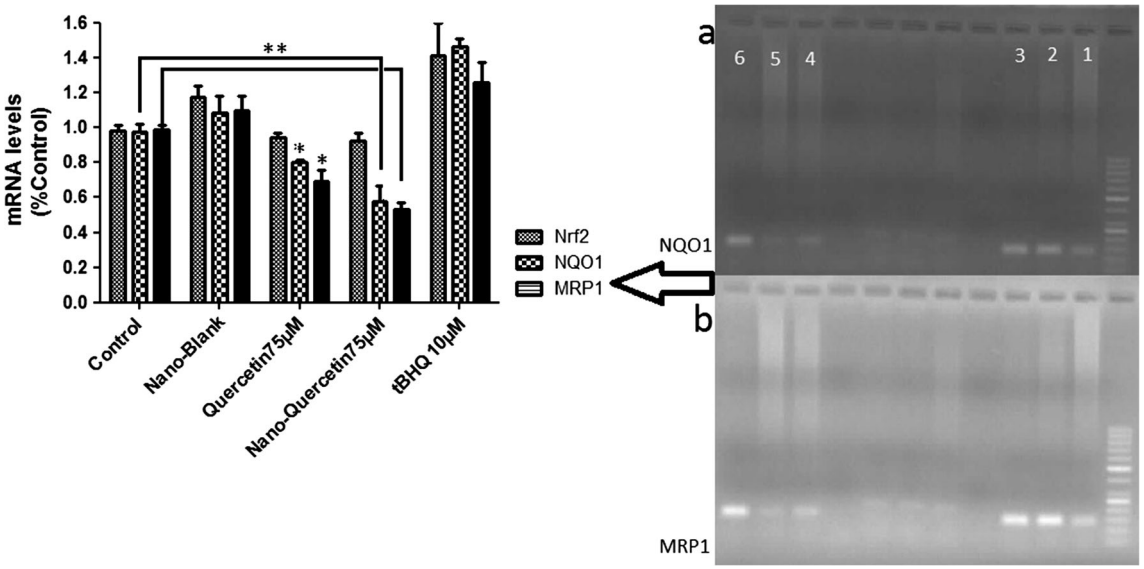

Fig. 4 Nano-quercetin reduced the mRNA level of Nrf2 target genes. MCF-7 cells were exposed to Nano-blank, quercetin 75 μM, Nano-Quercetin 75 μM and tert-butylhydroquinone (tBHQ) 10 μM. The mRNA levels of nuclear factor erythroid 2-related factor 2 (Nrf2), NAD(P)H:quinone oxidoreductase (NQO1) and multidrug resistance associated Protein 1(MRP1) were determined by real time polymerase chain reaction (RT-PCR) analysis. The value for treatment with tBHQ was considered positive control. *$p < 0.05$, **$p < 0.01$

inactivation efflux pumps and other enzymes for accelerate drugs to cancer cells introduced in previous studies [46]. This study addressed low concentrations of nano-quercetin decreased IC50 value of doxorubicin in a dose dependent manner. The higher efficacy of doxorubicin when it was accompanied with nanostructures may be explained by relative increase in quercetin stability in nearby tumor cells. Our data suggest that co-treatment of nano-quercetin and doxorubicin can be considered as a promising strategy for cancer therapy protocols.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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