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Full Length Research Paper

Apoptotic and necrotic effects of carboxylated quercetin/polyethylenimine complex on HeLa cells

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The effects of quercetin (Q), carboxylated quercetin (CQ) and carboxylated quercetin/polyethylenimine (CQ/PEI) complex on HeLa cell cultures were investigated. Firstly, carboxylated quercetin was acquired through hydroxyl groups of quercetin, using chloroacetic acid. The complex of CQ/PEI was acquired by electron cooperation path over polietilenimine amine groups and quercetin carboxyl groups. CQ and CQ/PEI obtained were characterised by FTIR and ^{1}H–NMR methods. Cytotoxicity was determined by MTT assay. Apoptotic and necrotic indexes were obtained by immunocytochemical staining with the M30 antibodies and double staining and double staining, respectively. It was determined that quercetin caused lower rates of necrosis and apoptosis on HeLa cells by itself, but CQ/PEI complex resulted in high levels. As a result, it was observed that transition of quercetin to HeLa via binding it to polyethylenimine increased its anticarcinogenic effects.

Key words: Quercetin, HeLa, cancer, apoptosis, necrosis, cytotoxicity.

INTRODUCTION

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a type of plant-based flavonoid commonly found in nature (Sasaki et al., 2007). There are two different forms of quercetin that contain a sugar group at the 3- and 4-positions. Quercetin 3-*O*-β-glucoside form containing a sugar group at the 3-position is more commonly found in nature compared to quercetin 4′-*O*- β-diglucoside form that contains a sugar group at the 4-position (Murota and Terao, 2003). Quercetin is especially abundant in apple, strawberry, cherry, onion and red wine. As a member of the flavonol group, quercetin is not toxic and has several important functions in terms of cell physiology. Quercetin has antiproliferation, antioxidant, antiulcer, apoptosis induction, protein kinase C inhibition, lipooxygenase inhibition, cell cycle progression, angiogenesis inhibition, anti-allergic and anti-cancer characteristics (Shin et al., 2006). It is reported that quercetin inhibits the members of the MDR family (P-gp, MRP1 and BCRP) (Scambia et al., 1994). It interacts with the ATPase regions of MDR proteins (Van Zanden et al., 2005). It is also reported that quercetin can scavenge intracellular free radicals and show *in-vivo* and *in-vitro* antioxidant activity by inhibiting the oxidation of various molecules (Lamson and Brignall, 2000). The absorption rate of orally taken quercetin is about 20%. It is reported that quercetin should have a serum concentration of approximately 10 µM for anti-cancer activity. As 100 mg single dose quercetin has a serum concentration of 0.8 µM in humans, the recommended daily dose for anti-cancer activity is 1500 mg (Lamson and Brignall, 2000). The intake of quercetin up to 4 g has no side effects in humans (Hannum et al., 2004). Quercetin is the most mutagenic flavonoid, but this mutagenic effect has no carcinogenetic effects (Hertog et al., 1995). It has been found that quercetin reduces the mutant p53 expression in cancer cell series and causes G2/M and G1 cell-cycle arrest (Kalra et al., 2007). Quercetin, which inhibits tyrosine kinase activation, is the fist flavonoid subjected to Phase I study in humans (Birt et al., 2001). As it is reported, quercetin increases the binding site expression of the estrogen receptor II (ER II) in Type I estrogen receptor positive and negative breast cancer cell series and inhibits the growth by binding to ER II (Cantero et al., 2006). Besides, quercetin was found

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to bind to ER II in human melanoma cells with the same affinity as tamoxifen and diethylstilbestrol (Shin et al., 2006).

Several *in-vitro* studies have demonstrated the inhibition of heat-shock proteins (Tsuda et al., 2004) and mutant ras oncogene by quercetin (Zhang et al., 2005). As demonstrated by *in-vitro* studies carried out with quercetin, the dosage interval varies between 7 and 100 µM (Hertog et al., 1995). The transfer of biological molecules via polycationic compounds is one of the preferred approaches. Polycationic compounds are preferred more, as they are easy to prepare and purify and they are open to chemical modifications (Thomas et al., 2006). The interaction of polycationic compounds with DNA and other molecules is based on electrostatic balance. Since this complex is positively more charged than the cell membrane, it penetrates into the cell membrane by endocytosis. The complex must escape the endosomal activity (Lungwitz et al., 2005). For this, it is recommended to use cationic compounds with low pK value under physiologic conditions. Such cationic compounds use the mechanism called “proton sponge” (Piskin et al., 2004). According to this mechanism, cationic compound binds the protons to amine groups and then release them into the organelle. Proton binding to amine groups causes a swelling of the polymer. Besides, Cl^- ions are carried to the endosome so that no charge difference occurs within the endosome. Proton and Cl^- ions increase the osmolarity of the endosome and thereby cause water inflow into the endosome (Tiera et al., 2006). The swelling of the polymer and endosome leads to endosome decomposition and the release of polycationic+DNA or drugs complex (Turk et al., 2004). In the present study, carboxylated quercetin were acquired by using chloroacetic asid.

The objective was to make quercetin soluble in water by electrostatic interaction between poliethylenimine (PEI2000) amine groups and quercetin carboxyl groups, to transfer quercetin into cancer cells, and thus, to increase its anti-carcinogenic effect.

MATERIALS AND METHODS

Human cervix epithelioid carcinoma cell line (HeLa) was obtained from the tissue culture collection of the SAP Institute (Turkey). Cell culture flasks and other plastic materials were purchased from Corning (USA). The growth medium, which is Dulbecco modified medium (DMEM) without L-glutamine supplemented fetal calf serum (FCS) and Trypsin-EDTA were purchased from Biological Industries (USA). Quercetin, M30 CytoDEATH antibody, 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). Hoeshct 33342 and propodium iodide were purchased from Serva (Israel).

Synthesis of carboxylated quercetin/ polyethylenimine (CQ/PEI) complex

40 mg/ml quercetin (Q) was dissolved in 3M sodium hydroxide (NaOH) and chloroacetic acid (CAA) was added in the amount equivalent to 1:1 mol/mol quercetin. The mixture was left to react in magnetic mixer in room temperature for 70 min. The reaction was discontinued at the end of 70 min by adding 1M NaH_2PO_4 buffer to the medium. Later, 6N HCl was added to the medium and pH value was adjusted to 7. Impurities were removed from the medium by using dialysis membrane. 1 mM polyethylenimine (PEI, 2000 Da branched) was dissolved in water. Then, 4 mol of carboxylated quercetin (CQ) was added for 1 mol of polyethylenimine. The mixture was mixed in magnetic mixer in room temperature for 1 h and electron cooperation path binding to polyethylenimine was realized over PEI amine groups and quercetin carboxyl groups. The unreacted parts were removed from the medium by using a dialysis unit with a pore diameter of 1000 Da (Spectrum lab, USA). Thereby, we obtained carboxylated quercetin/polyethylenimine (CQ/PEI) complex with carboxyl group which is electrostatically bound to polyethylenimine and soluble in water. The obtained quercetin with carboxyl group and quercetin/polyethylenimine complex with carboxyl group were characterized by F – TIR and ^1H – NMR methods.

The transfection of quercetin into the cells

Certain concentrations of Q and CQ/PEI complex solutions in water were prepared. The cells were seeded at a density of 20×10^3 cells/well in 48-well microassay plates, and incubated in CO_2 incubator for over night. The Q and CQ/PEI complex were diluted (0 to 500 µg/ml) with the cell culture medium and inoculated to the cells and incubated for 24 h. Then cell medium were washed with PBS and examined by a Leica DMI 6000 fluorescence microscopy with FITC fitler at 200X magnification.

Cytotoxicity

Evaluation of Q, CQ, CQ/PEI and polyethylenimine (PEI 2000da) cytotoxicity was performed by the 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) assay (Aldrich, USA). MTT assay is a simple colorimetric assay to measure cell cytotoxicity, proliferation and viability. Cytotoxicity effects of quercetin (Q), carboxylated quercetin (CQ), carboxylated quercetin/polyethylenimine (CQ/PEI) were investigated on cultured HeLa cells. The cells were seeded at a density of 5×10^3 cells/well in 96-well microassay plates, and incubated over night. The Q, CQ, CQ/PEI and PEI were diluted (0 to 500 µg/ml) with the cell culture medium and inoculated to the cells and incubated for 72 h. Following the incubation, mediums were removed and 100 µl of fresh medium and 13 µl of MTT solution (5 µg/ml, diluted with RPMI 1640 without phenol red) were added to each well. Incubation was allowed for another 4 h in dark at 37°C. Since living cells metabolize the MTT in their mitochondria and form blue formazan crystals, 100 µl/well isopropanol- HCl (absolute ispropanol containing 0.04M HCl) solution added to dissolve the formazan crystals. The wells were read at 570 nm on an ELSA plate reader and percentage of cell viablity was determined. For each MTT assay, the control HeLa cell viability was defined as 100%.

Analysis of apoptotic and necrotic cells

Double staining

Double staining were performed to quantify the number of apoptotic cells in culture on basis of scoring of apoptotic cell nuclei. HeLa cells (25×10^3 cells per well) were placed in DMEM by using 24-well plates. After treating with different concentrations of quercetin, carboxylated quercetin, polyethylenimine and carboxylated

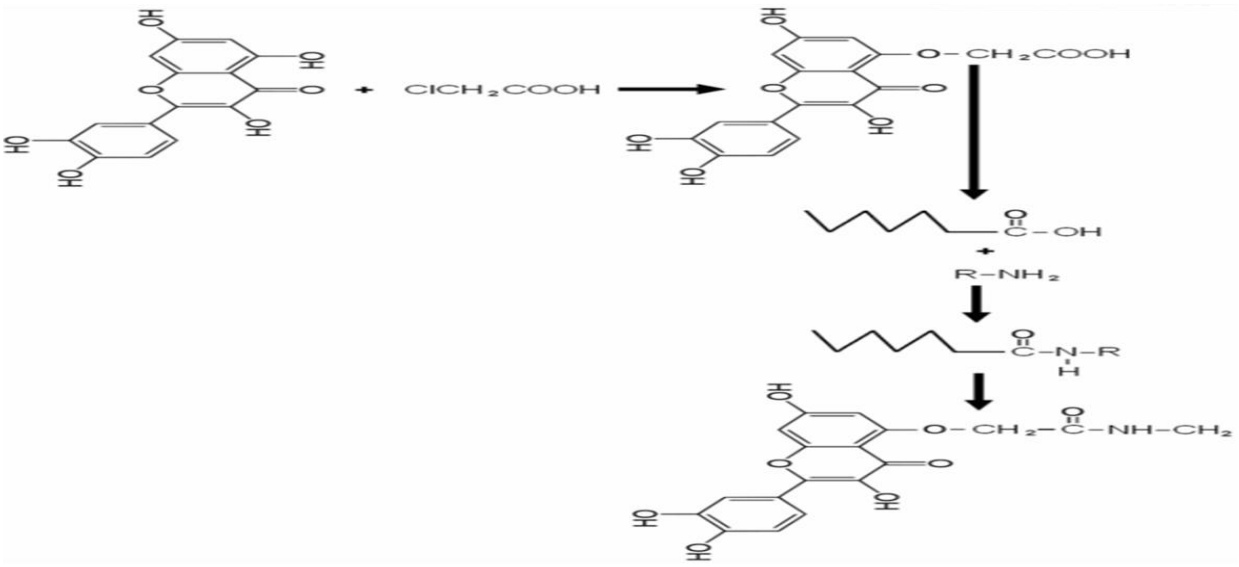

Figure 1. Synthesis of carboxylated quercetin/ polyethylenimine (CQ/PEI) complex.

quercetin/polyethylenimine (about 0 to 500 μg/ml in aqueous solutions) for 24 h period. Both attached and detached cells were collected, then washed with PBS and stained with Hoechst dye 3342 (2 $\mu g.ml^{-1}$), propodium iodide (PI) (1 $\mu g.ml^{-1}$) and DNAse free-RNAse (100 $\mu g.ml^{-1}$) for 15 min at room temperature. After that 10 to 50 μl of cell supension was smeared on slide for examination by fluorescence microscopy (Choi et al., 2009). The nuclei of normal cells were stained light blue but apoptotic cells were stained dark blue by the hoechst dye. The apoptotic cells were identified by their nuclear morphology as a nuclear fragmentation or chromatin condensation (Ulukaya et al., 2001). Necrotic cells were stained red by PI. In the necrotic cells lacking plasma membrane integrity, PI dye diffuses into the cell, does not stain non necrotic cell. The number of apoptotic and necrotic cells in 10 randomly chosen microscopic fields were counted and the result was expressed as a ratio of apoptotic and necrotic to normal cells.

M30 immunostaining for detection of apoptotic cells

The percentage of apoptotic cells was determined by M30 CytoDEATH antibody (Mcpartland et al., 2005). This is a monoclonal mouse immunoglobulin (Ig) G2b antibody (clone M30; Roche, Mannheim, Germany) that binds to a caspase-cleaved, formalin-resistant epitope of cytokeratin 18 cytoskeletal protein. The immunoreactivity of the M30 antibody is confined to the cytoplasm of apoptotic cells. HeLa cells (25 x 10^3 cells per well), treated with quercetin, carboxylated quercetin and carboxylated quercetin/polyethylenimine (about 0 to 500 μg/ml in aqueous solutions) for about 24 h, were fixed in 10% neutral-buffered formalin for 15 min, treated with 0.3% hydrogen peroxide in methanol for 10 min to block the endogenous peroxidase activity, washed in the standard phosphate buffer solution, and then incubated with M30 antibody at room temperature for 1 h. In negative controls, preimmune mouse serum was used instead of primary antibody. Immunoreactions were revealed by the avidin-biotin complex technique using diaminobenzidine (DAB) as substrate. We counted the number of M30-positive cytoplasmic staining cells in all fields found at x400 final magnification. For each image, three randomly selected microscopic fields were observed, and at least 100 cells/field were evaluated.

Statistical analysis

Cytotoxicity, apoptosis and necrosis studies were performed in triplicate. The data are represented as mean ± standard deviation (SD). Data were analyzed using the dependent samples t-test (for two groups). Statistical significance was set at $P<0.05$.

RESULTS

Extraction of carboxylated quercetin/polyethylene-imine (CQ/PEI) complex

While HCl was obtained by interaction between chloroacetic acid and quercetin, carboxyl group from chloroacetic acid was transferred to quercetin through hidroxyl groups of quercetin. The reaction steps are schematized in Figure 1.

FTIR spectras

Comparative analysis of FTIR spectra of CQ (Figure 2b) and CQ/PEI (Figure 2a) indicates that the bands of C = O group in CQ at 1664 cm^{-1} disappeare in the spectra of CO/PEI. The formation of amide bound in CQ/PEI complex is confirmed by the appearance of new bands such as 1600 (amide I. band) and 1550 (amide II. band) in the FTIR spectra of CQ/PEI. These are shown that the polymerization between CQ and PEI is continued through carboxylic acid group. Simultaneously a very broad band at 3420 cm^{-1} appearances in spectra of CQ/PEI complex due to N-H stretching band in amine group of PEI and C-H strecthing band in aliphatic carbon (CH_2) and thus, the synthesis of CQ/PEI complex was verified (Table 1 and Figure 2).

Table 1. Characteristic peaks in FTIR spectra of CQ having a carboxylic group and CQ/PEI Complexes.

Characteristic peak	CQ wavenumbers (cm^{-1})	Characteristic peak	CQ/PEI wavenumbers (v/cm^{-1})
– C – OH (stretcthing)	3400	N – H (imi) (– C – OH)	3420
C = O (symmetrical stretcthing)	1664	C – H (stretcthing, CH_2)	2822
C = O (stretcthing)	1450	C – H (bending, CH_2)	1450
C – O – C (antisymmetrical stretcthing	1262 - 1168	C = O (stretcthing, amide I. band)	1650 - 1660
C – O – C (symmetrical stretcthing)	1130 – 1014	N – H (bending, amide II. band)	1608 – 1561

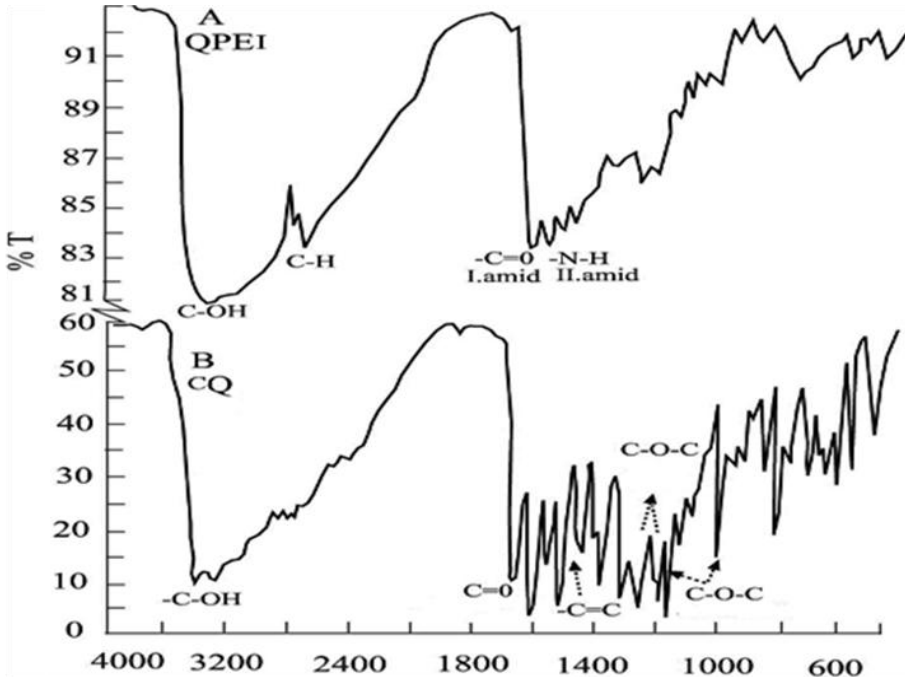

Figure 2. FTIR spectra of synthesized complexes A) CQ/PEI complex (quercetin having a carboxylic group/polyethyleneimine) and B) quercetin having a carboxylic group.

^{1}H NMR spectra of carboxylated quercetin and CQ/PEI complex

The results of ^{1}H NMR spectra of CQ and CQ/PEI complex are illustrated in Figure 3. As seen from ^{1}H NMR spectras of CQ/PEI (Figure 3a), the peaks at 2.50 to 2.90, 4.60 to 4.80 and 6.60 to 6.80 ppm are shown in CH_2 related to amine, – OH in anile and C = O-NH, respectively unlike the NMR spectra of CQ (Figure 3b). As shown in the NMR figures, the peak of carboxyl group at 9.3 to 9.4 ppm disappear after the quercetin having a carboxylic group formed a complex with polyethyleneimine. All of these indicate the formation of complex (Table 2 and Figure 3).

The transfection of quercetin into the cells

Certain concentrations of Q, CQ/PEI complex solutions in water were prepared. While quercetin alone formed a yellow suspension solution in water (falcon tube where on the left of Figure 4), CQ/PEI complex dissolved in water and revealed a brown color (falcon tube where on the right of Figure 4). Besides, quercetin 0.2 µm could not be

Table 2. Characteristic peaks of H-NMR spectra of Carboxylated Quercetin and CQ/PEI complex.

Functional group	CQ Chemical shift (ppm)	CQ/PEI Chemical shift (ppm)
O ‖ Ar – CH_2 – C(b)	3.30 – 3.40	3.30 – 3.50
Aril – H (d)	6.20 – 7.80	7.20 – 7.30
O ‖ C – H	9.30 – 9.40	–
NH – CH_2 – CH_2(a)	–	2.50 – 2.90
Ar – OH (c)	–	4.60 – 4.80
O ‖ C – NH (amide band)(e)	–	6.60 – 6.80

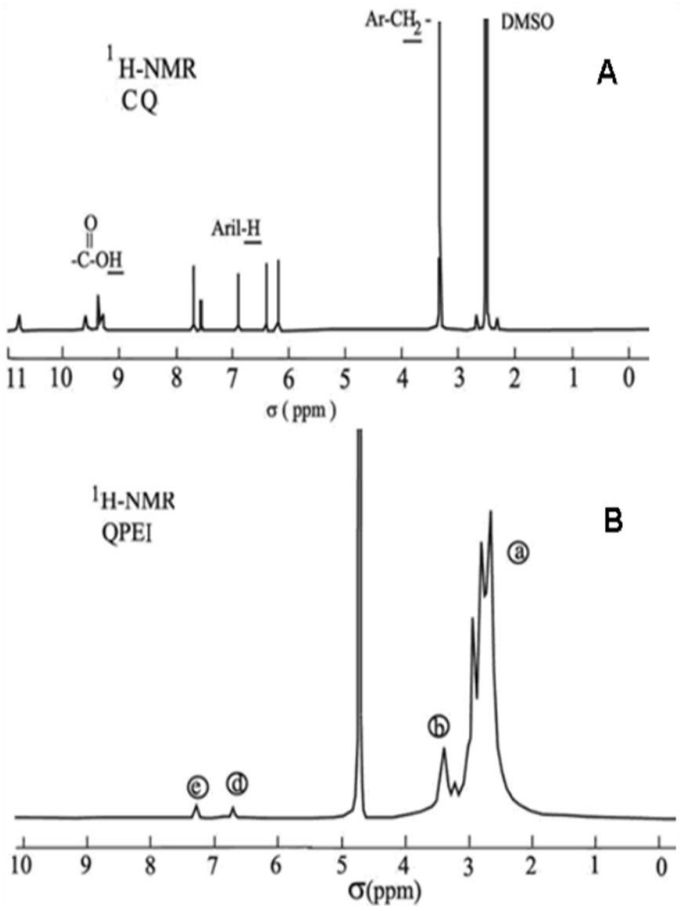

Figure 3. ^{1}H NMR spectra of (A) CQ and (B) CQ/PEI in DMSO-d_6.

filtered, whereas CQ/PEI complex could easily be filtered. The picture showing the dissolution of carboxylated quercetin in water with PEI is given in Figure 4. While the solution of quercetin in water did not penetrate into the cells (Figure 4a), the complex formed with polyethyleneimine was observed to have penetrated into the cells (Figure 4b). Figure 4 shows the photographs of CQ/PEI complex penetrating into the cell, which were taken under the fluorescent inverted microscope.

The toxic effects of quercetin (Q), carboxylated quercetin/polyethylenimine (CQ/PEI) complex on HeLa (cancer) cells

Inhibition concentration (IC_{50}) values killing 50% of the cells were determined by MTT method. The results are given in Table 3. According to the obtained results, the toxic effect of quercetin (Q) on cancer cells was low at low concentrations. The toxic effect at 50 to 300 µg/ml concentrations was close to those observed in the control groups. When the concentration was increased to 400 to 500 µg/ml, a 15% increase in toxicity was observed in HeLa cells. IC_{50} value was found above 500 µg/ml. The same results were found also with the quercetin with carboxyl group (CQ) obtained by quercetin modification and polyethylenimine (PEI 2000 Da) used in the transfection of quercetin into the cell. Regarding the effect of incubation period on toxicity; no toxicity was observed in short-term incubations (2 to 6 h), whereas an increased toxicity was observed in long-term incubations (12 to 24 h). Based on the obtained results, the low toxic effect of quercetin was associated with the fact that quercetin was poorly soluble in water and thus could not penetrate enough into the cell. Different toxicity results were found when carboxylated quercetin/polyethylenimine (CQ/PEI) complex was interacted with cancer cells. The obtained results are given in Table 3. While toxicity was not observed at 50 µg/ml concentration of CQ/PEI complex, toxicity increased with the increase in concentration. When concentration was increased to 100 µg/ml, toxicity was observed to increase by 10 to 15%. Viability of the cells decreased by 50% at 235.345 µg/ml concentration (Table 3). While the toxic effect did not increase much in the incubation period of 2 to 4 h in CQ/PEI complex, high levels of toxicity was observed in 6 to 12 and especially 24 to 72 h of interaction. This finding indicates that the toxic effect of CQ/PEI increases depending on the incubation period and concentration.

According to MTT results, IC_{50} dose of CQ/PEI complex which killed half of the cells was obtained at about 235.345 µg/ml. High toxicity of CQ/PEI was an expected result; because, Q alone could hardly penetrate into the cell and it possibly passed through the cell membrane at higher concentrations with the help of PEI and thus showed toxic effect.

Apoptotic effect

Two different methods were used to determine the

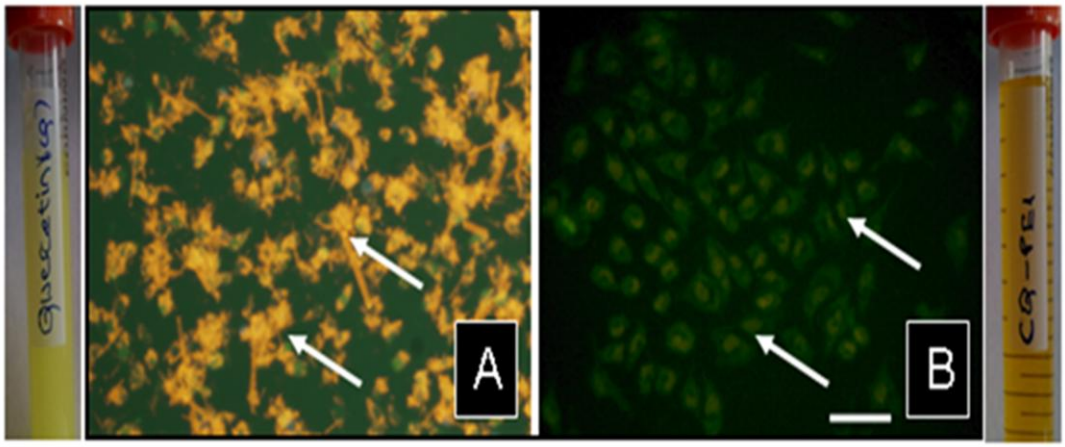

Figure 4. Fluorescent inverted microscopy photos of transfected HeLa cells. (A) shows the photograph of quercetin treated HeLa cells, arrows shows that quercetin did not penetrate in to cells), insoluble form of quercetin (brigth yellow) can be seen on the left of the figure; (B) shows the photograph of quercetin/PEI complex penetrating into the cell (arrows shows quercetin in the cells), soluble form of quercetin/PEI complex (the color brown) can be seen on the right of the figure. All images were recorded with x200 magnification. Scale bar is 40 µm.

Table 3. Cytotoxicity of Q, CQ, PEI polymers, and CQ/PEI complex and with varying concentrations, on HeLa tumor cells (toxic effect of active substances are given as IC_{50} value).

Substance	IC_{50} value (µg/ml)
Quercetin	546.2±18.7
Carboxylated Quercetin	521.6±24.3
Polyethylenimine	537.8±14.6
CarboxylatedQuercetin/polyethylenimine	216.5 ±17.8

apoptotic effect of quercetin and CQ/PEI complex. Apoptotic cells were shown by using double staining method and application of M30 immunocytochemical method in HeLa cells. The apoptotic index results are illustrated in Table 4 and the photographs of apoptotic cells are presented in Figure 5. The hoechst (33342) fluorescent dye in the double staining solution binds to DNA and gives the cell nuclei a blue color (Figure 5A) under the blue fluorescent light. Apoptotic cell nuclei are distinguished from other blue nuclei by their distorted borders and brighter appearance (arrow shows apoptotic bodies in Figure 5B). The cytoplasm of apoptotic cells at the end of M30 staining is brown (Figure 5F), while the cytoplasm of non-apoptotic cells is blue in color (Figure 5E). Generally, the results obtained from immuno-cytochemical and double-staining methods are parallel. The use of quercetin alone revealed a low apoptotic index in HeLa cells at 50 to 200 µg/ml concentrations. It was observed that an increae at concentration level of quercetin caused an increae in apoptosis rate. The comparison of the obtained findings with those of PEI revealed similar results. In the use of carboxylated quercetin, the apoptosis increased to a rate of 12% at these concentrations. The short and long term interaction of cells with the active substances did not cause significant changes in the apoptotic index at both low and high concentrations. The highest apoptosis was observed in the incubation period of 24 h. Results different from ones belonging to other active substances were found when HeLa cancer cells were interacted with CQ/PEI complex. The results are given in Table 4. Apoptotic index was not very high in HeLa cells compared to others at low concentration and shorter incubation periods. Yet, the increase at concentration and incubation period (24 h) led to an increase in the apoptosis of HeLa cells. It was determined that CQ/PEI complex caused a 19±2% apoptosis in cancer cells at 400 µg/ml concentration, and 31±2% apoptosis at 500 µg/ml concentration (Table 4).

Accordingly, the increase in the apoptotic effect was associated with the high penetration of quercetin through PEI into the cells. Statistical difference ($P<0.05$) was found for opoptotic activity between Q, CQ and CQ/PEI complex in different ratios and P values were 0.0108 and 0.0192 respectively.

Necrotic effect

When HeLa cells were stained with propodium iodide fluorescent dye in double-staining solution, the nuclei of necrosed cells appeared in red color under red and green fluorescent light, indicating that cells were necrosed. The necrotic index results are illustrated in Table 4 and photographs are given in Figures 5D. Quercetin, carboxylated quercetin and polyethyleneimine alone had low necrotic effects (approximately 10%) at 50 to 200

Table 4. Apoptotic and necrotic index in HeLa cancer cells formed by (**I**) quercetin(Q), (**II**) carboxylated quercetin (CQ), (**III**) polyethylenimine (PEI) and (**IV)** carboxylated quercetin/ polyethylenimine (CQ/PEI) complex and PEIpolymers at varying concentrations (0-500 µg/ml) (Apoptotic index was determined by taking the average of the values obtained by M30 methods. Data was represented with ±SD. Statistical difference($P<0.05$) was found for opoptotic and necrotic activity between Quercetin(Q), carboxylated quercetin(CQ) and carboxylated quercetin/ polyethyleneimine (CQ/PEI) in different ratio and P values were 0.0108 and 0.0157 respectively.

Substance	Apoctotic indexes (%)				Necrotic indexes (%)			
Amount	I	II	III	IV	I	II	III	IV
0	1±1	1±1	1±1	1±1	1±1	1±1	1±1	1±1
50	2±1	3±1	1±1	4±1	2±1	3±1	3±1	5±1
100	3±1	3±1	2±1	6±1	5±1	5±1	4±1	10±1
200	5±1	9±1	5±1	14±2	12±1	10±1	8±1	17±2
300	7±1	12±2	5±1	17±2	13±2	14±2	10±1	32±3
400	9±1	15±2	8±2	19±2	14±2	16±2	14±2	38±3
500	16±1	17±2	12±2	31±2	20±1	21±2	18±2	51±2

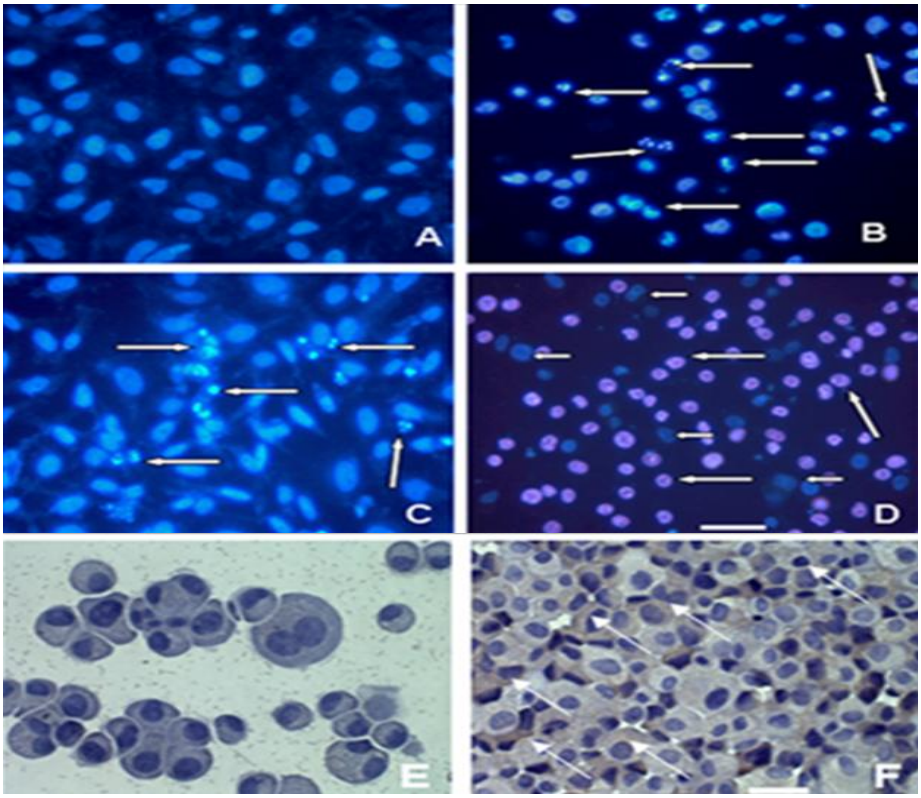

Figure 5. Inverted microscopy photos of transfected HeLa cells. (A) fluorescent microscope image of nuclei of untreated HeLa cells as a control, where formation of lifeless spots demostrate nuclei of non-apoptotic cells; (B) 400 $\mu g.ml^{-1}$ concentration of (CQ/PEI)/HeLa cells conjugate (stained with Hoechst 33342), where bright spots and dispersed nuclei (long arrow) indicate nuclei of apoptotic cells; (C) 200 $\mu g.ml^{-1}$ concentration of (CQ/PEI)/HeLa cells conjugate (stained with Hoechst 33342), where bright spots and dispersed nuclei (long arrow) indicate nuclei of apoptotic cells; (D) 400 $\mu g.ml^{-1}$ concentration of CQ/PEI)/HeLa cells conjugate (stained with Hoechst 33342 and propodium iyodide), where blue nuclei (short arrow showes) indicate non-necrotic cells and red nuclei (long arrow showes) indicated necrotic cells; (E) ınverted microscopy photos of non-apoptotic HeLa cells as a control group under visibile ligth (stained with caspas-3 immunostaining kit), where blue cytoplasmas of cells indicate non-apoptotic cells; (F) 400 $\mu g.ml^{-1}$ concentration of (CQ/PEI)/HeLa cells conjugate (stained with caspas-3 immunostaining kit), where brown cytoplasmas of cells image indicate the formation of apoptotic cells. All images were recorded with x400 magnification. Scale bar is 20 µm.

µg/ml concentrations. As observed in toxicity and apoptotic effect, the increase in quercetin concentration and incubation period led to an increase in the necrotic effect. Especially at 400 to 500 µg/ml concentration, the necrotic index was detected as 28% in HeLa cells. When CQ/PEI complex was applied in both types of cells, necrotic effect was observed to increase at active substance concentrations of 200 µg/ml and higher (Figure 5C), and it was detected as 18% in cancer cells. With the increase in concentration, necrotic effect was observed at higher rates compared to Q, CQ and PEI. Especially at 500 µg/ml concentration, necrotic effect was 51±2% in HeLa cancer cells (Table 4). Statistical difference ($P<0.05$) was found for necrotic activity between Q, CQ and CQ/PEI in different ratios and P values were 0.0157 and 0.0143 respectively. Statistical difference ($P>0.05$) was not found for necrotic activity between Q and CQ in different ratios and P values were 0.203.

DISCUSSION AND CONCLUSIONS

The effect of quercetin on HeLa and some other cancer cell lines were investigated *in-vitro* and *in-vivo*. The assessment was made by applying quercetin alone and in combination with cancer drugs used in cancer treatment. In addition, apoptotic and toxic effects were investigated by transferring quercetin into the cell at high concentrations by carriers like liposome (Wattanathorn et al., 2007). As reported in recent studies, quercetin is a natural substance that can be used as a cancer drug (Ren et al., 2003). However, poor solubility of quercetin in water prevents its penetration into the cell and causes a decrease in the therapeutic effect. The studies conducted so far focus on removing this inhibiting effect. To that end, *in-vivo* and *in-vitro* studies were designed by forming complexes with biocompatibility-enhancing polymers like quercetin or polyethyleneglycol (PEG), and positive results were obtained (Wattanathorn et al., 2007). Impressive results were obtained from *in-vivo* and *in-vitro* studies conducted with nano structures and especially nano and macro liposomes (Yuan et al., 2006). When quercetin toxicity is considered, the results of different studies contradict with one another. Kang and Liang (1997) detected that 20 µM quercetin killed 52.7% of the cells in HL 60 Leukemia cancer cell cultures. In another study carried out by So et al. (1996), it was reported that *in-vitro* application of quercetin killed 50% of the breast cancer cells at concentrations of 500 µM and higher. The apoptotic effect of quercetin was reported to be 17% at the end of 24-h incubation period when applied *in-vitro* to HeLa cells, and 37% when applied *in-vitro* to the same cell together with cisplatin (Jakubowicz-Gil et al., 2005). In another study, the effect of quercetin was investigated at high temperature, and it was found that FM3A caused 20% apoptotic effect on the mouse breast cell line and the toxic effect inhibited the proliferation of tumor cells to a rate of 100% at the end of 5-day incubation period at high temperature (Lee et al., 2004). In an *in-vivo* study conducted by charging quercetin on liposomes, a high rate of accumulation was reported in especially the liver, besides the pancreas, kidney and lung (Zhang et al., 2006).

Another study conducted again on liposomes reported higher rates of accumulation in tumor tissue compared to normal tissues, an increase in apoptosis, and the suppression of angiogenesis in the tumor tissue (Wei et al., 2006). In a study on its effect on gene expression, quercetin was reported to reduce P21, P53, MAP-kinase expression in tumor cells depending on time and dose (Mertenes-Talcott et al., 2005). In our study, similar to those mentioned above, quercetin failed to provide the desired effect due to its low solubility in water. Hence, quercetin was modified to enable its transfer into the cell by a carrying agent such as polyethyleneimine which is soluble in water. According to the obtained results, while quercetin alone was insoluble in water, its solubility in water increased by adding a carboxyl group to the structure by chloroacetic acid. Afterwards, quercetin gained complete solubility in water by the electrostatic complex formed between the amine groups of polyethyleneimine and the carboxyl groups of quercetin. Based on the apoptotic, necrotic and toxicity results, the desired goal was achieved by the formation of this complex.

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