

Antiproliferative effect of quercetin in mammary and epidermoid cancer cells

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Abstract

Background: Quercetin is a ubiquitous flavonoid displaying both anti- and pro-oxidant effects. We aimed to investigate the ability of quercetin to inhibit cell proliferation in two cancer cell lines with epithelial origin, SK-BR-3 mammary tumor cells and A-431 epidermoid tumor cells. Materials and methods: Modifications in cell viability, cell cycle progression and apoptosis were monitored to study the antiproliferative effect of quercetin. Results: Our results showed that quercetin had a biphasic effect on cell viability. Additionally, lower concentrations of quercetin (5 – 10 µM) induced G₀/G₁ arrest in A-431 cell line and increased concentrations (50 – 75 µM) were associated with S phase arrest in both investigated cell lines at 24 h of incubation. Moreover, the administration of quercetin induced early and late apoptosis in tumor cells at 72 h of incubation. Conclusions: Taken together these data suggested that first outcome of quercetin in displaying antiproliferative properties was associated with the cytostatic effect rather than with the cytotoxic activity.

Introduction

Quercetin (3,3',4',5,7-pentahydroxyl-flavone) is a secondary plant metabolite which can be found in onions, apples, grapes, green tea etc. (S.C. Bischoff [1], A.J. Vargas & al. [2], G.S. Kelly [3], F. Dajas [4]). Multiple studies reported the chemopreventive and chemotherapeutic effect of quercetin based on two essential activities, namely anti-oxidant and pro-oxidant properties. The anti-oxidant properties of quercetin were related to the flavonoid ability to donate electrons to reactive oxygen species (ROS) and those in turn to display reduced DNA injuring activity (A.J. Vargas & al. [2]). The anti-oxidant properties of quercetin were noticed at lower concentration of the flavonoid with a border at about 40 µM (A.J. Vargas & al. [2]) and were associated to anti-inflammatory activity, neuroprotective effects and cardiovascular protective effects (F. Dajas [4], H.R. Vasanthi & al. [5]). However, quercetin displayed a double activity and its pro-oxidant properties were connected to the ability to inhibit the proliferation of the tumor cells. The concentrations of quercetin required to hamper the tumor activity were appreciated to be in range of 40 – 250 µM (A.J. Vargas & al. [2]). Nevertheless, lower concentrations of quercetin (3 – 50 µM) were reported to have antitumor activity, as well (L. Gibellini & al. [6]).

The antiproliferative activity of quercetin was reported in various cancer cells (G. Wang & al. [7], K. Bishayee & al. [8], H. Zhang & al. [9], I. Baran & al. [10], S.U. Mertens-Talcott & al. [11], S.U. Mertens-Talcott & al. [12], A. Del Follo-Martinez & al. [13]), but not in healthy cells (J.H. Jeong & al. [14]). Previous reports showed that quercetin induced cell cycle arrest in G₂/M phase (K. Bishayee & al. [8]), G₀/G₁ phase (M. Yoshida & al. [15]), or S phase (H. Zhang & al. [9]). The diverging concentrations reported in correlation with the antitumor activity of quercetin and the variety of effects on cell cycle progression in tumor cells upon incubation with quercetin prompted us to investigate the effect of quercetin in epithelial cancer cells.

The present study was design to test the ability of quercetin to inhibit the tumor cell viability and to test two different mechanisms which could explain its behavior. First, we check the antiproliferative effects of quercetin by evaluating the cell cycle distribution and second, we evaluated the pro-apoptotic outcome of quercetin. The effect of quercetin was examined in two epithelial cancer cell lines, SK-BR-3 mammary cells and A-431 epidermoid cancer cells. Our data support both mechanisms, namely the ability of quercetin to interfere with the cell cycle progression and to have pro-apoptotic effects in dose and time dependent manner in SK-BR-3 and A-431 epithelial cancer cells. However, the effect of quercetin on cell cycle progression was cell line dependent.

Materials and methods

Chemicals and reagents

Quercetin, and dimethylsulphoxide (DMSO) were obtained from Sigma Aldrich. The water soluble tetrazolium salts 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) was purchased from Roche Diagnostics GmbH, Mannheim, Germany. Popidium iodide/RNase was from BD Biosciences, Annexin V-FITC from Beckman Coulter, Brea, CA and 7-AAD from BD Biosciences, San Jose, CA. Dulbecco's Modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin were from Sigma Aldrich and L-glutamine from Gibco – Life Technologies.

Cell culture

The human breast cancer cell line SK-BR-3 and the human epidermoid carcinoma cell line A-431 were obtained from the American Type Culture Collection (Rockville, MD) and were grown according to their specifications. Briefly, the tumor cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin in a 37 °C, 5% CO₂ and humidified atmosphere.

Viability assay

SK-BR-3 and A-431 cells were seeded in triplicate at a density of $7 \cdot 10^3$ cells/ sample in 96-well flat bottom microplates 24 h prior to the experiments. The treatment with various concentrations of quercetin for 24 h was carried out in serum free medium (H. van der Woude & al. [16]). The final concentration of DMSO in culture medium was 0.05%. The viability assay was performed in triplicate using cell proliferation reagent WST-1 based on the reduction of tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases. The absorbance of formazan was measured at 450 nm and corrected at 620 nm using a 96-well spectrophotometer (ELISA reader).

Cell cycle analysis

After flavonoid treatment (0, 5, 10, 50, and 75 μM quercetin) for 24 h in complete medium the cells were harvested by trypsinization, fixed in 70% ice cold ethanol and stored at $-20\text{ }^{\circ}\text{C}$. Ethanol was removed by centrifugation and the cells were washed in PBS followed by staining of 10^6 cells/ sample with propidium iodide/RNase for 15 minutes in the dark according to the manufacturer's instructions. The DNA content was analyzed using a FACSCalibur instrument (Becton Dickinson). Excitation at 488 nm was provided by an air-cooled argon-ion laser running at a power of 15 mW and data from 10000 events/ sample were collected with a 585-nm band pass filter. The experiments were done in triplicate. The results were analyzed for statistical significance according to the fraction of cells in the G_0/G_1 , S and G_2/M phases of the cell cycle.

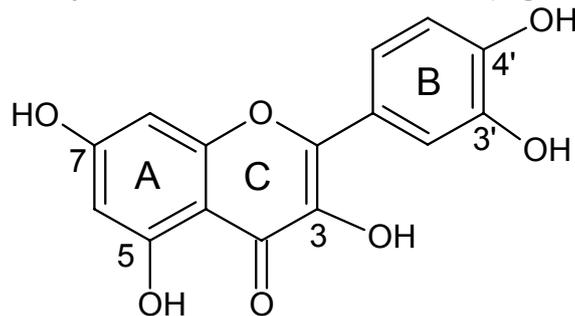
Apoptosis assay

The tumor cells were incubated with 0, 5, 10, 50, and 75 μM quercetin in complete medium for 24 and 72 h. Cells were collected by trypsinization, washed twice in PBS and 10^6 cells/ sample were doubled stained with Annexin V-FITC and 7-AAD according to the manufacturer's instructions. The samples were analyzed by flow cytometry within an hour after staining using a FACSCalibur instrument (Becton Dickinson, San Jose, CA). Both FITC and 7-AAD were excited at 488 nm (air-cooled argon-ion laser, 15 mW) and the emission was collected with a 530-nm band pass filter (FITC) and a 670-nm longpass filter (7-AAD). During data analysis Annexin V-FITC⁻/7-ADD⁻, Annexin V-FITC⁺/7-ADD⁻, Annexin V-FITC⁺/7-ADD⁺ and Annexin V-FITC⁻/7-ADD⁺ cells were considered to be viable, early apoptotic, late apoptotic and necrotic, respectively. The experiments were carried out in triplicate.

Results

Biphasic effect of quercetin in mammary and epidermoid tumor cell lines

Quercetin (Figure 1) was administrated to SK-BR-3 and A-431 tumor cells in various concentrations: 0, 2.5, 10, 20, 40, 80, 160 and 320 μM for 24 h. Higher concentrations of quercetin showed an inhibitory effect in both tumor cell lines (Figure 2).



quercetin (3,3',4',5,7-pentahydroxyflavone)

Fig 1. The chemical structure of quercetin (3,3',4',5,7-pentahydroxyflavone).

More sensitive to antiproliferative effect of quercetin at 24 h incubation was A-431 cell line compared to SK-BR-3 cell line. On the other side the lower concentrations of quercetin, particularly at about 2.5 μM , lead to a moderate increase in cell viability compared to control samples (Figure 2). The data could suggest a biphasic effect of quercetin in SK-BR-3 and A-431 tumor cell lines, depending on the concentration which was applied.

S-phase arrest of SK-BR-3 mammary tumor cell line at higher concentrations of quercetin

We were interested to identify the mechanism which was responsible for the inhibitory effect of quercetin in SK-BR-3 mammary tumor cell lines. In order to clarify this aspect we evaluated the effect of quercetin on DNA content measured by flow cytometry (Figure 3). The recorded histograms of DNA content were evaluated for cell cycle progression, namely for G₀/G₁, S and G₂/M phases. The incubation with 50 and 75 μM quercetin directed 64% ($p < 0.01$) and 78% ($p < 0.001$) of the SK-BR-3 cells in S phase compared to 30% in the control samples. The increase of S phase in SK-BR-3 cells was accompanied by significant decrease of cell population in G₀/G₁ phase from 63% in control samples to 6% for 50 and 75 μM quercetin ($p < 0.001$). Moderate effect on S phase arrest was noticed starting with 10 μM quercetin. Low concentration of quercetin (5 μM) did not display any significant changes in cell cycle distribution of SK-BR-3 cell line. As shown in Figure 5 the effect of quercetin on cell cycle arrest in S phase was dose dependent in SK-BR-3 cell line.

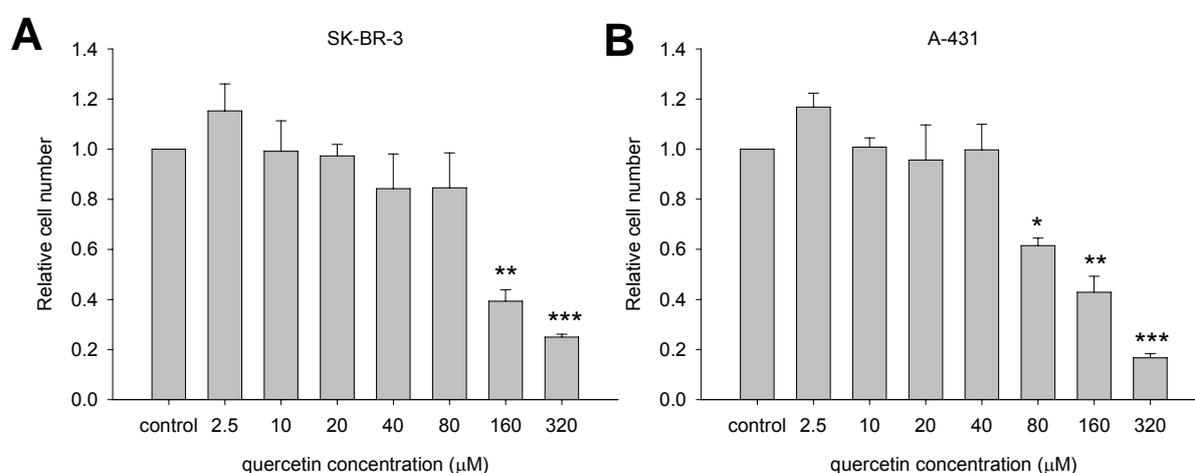


Fig 2. Inhibition of cell viability by quercetin in SK-BR-3 breast cancer cells (A) and A-431 epidermoid cancer cells (B). The tumor cells were incubated with various concentrations of quercetin for 24 h and the cell viability was evaluated by WST-1 assay. The data represent the mean \pm SEM of triplicate experiments. Significant differences from control: ** $p < 0.01$, *** $p < 0.001$.

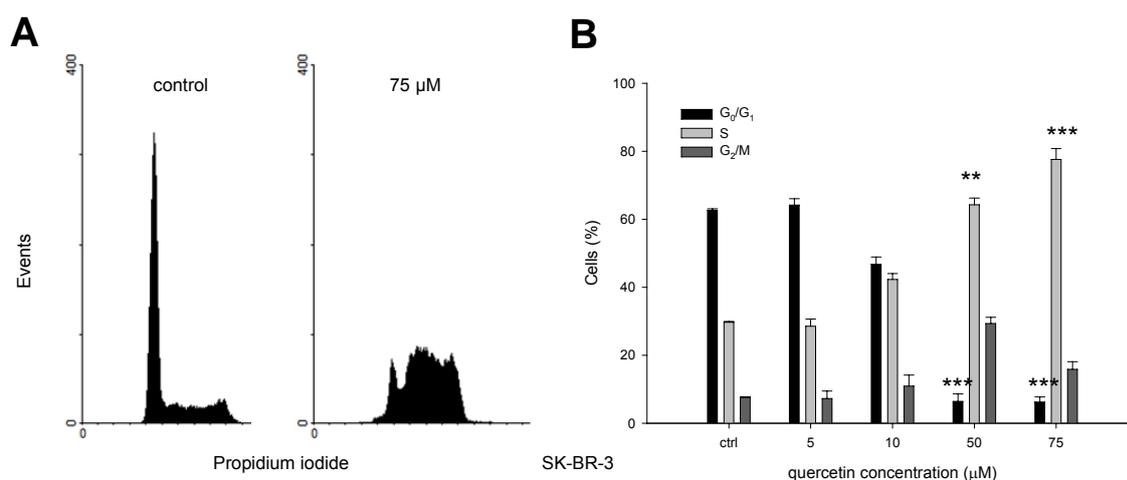


Fig. 3. Effect of quercetin on cell cycle in case of SK-BR-3 breast cancer cells. The tumor cells after the treatment with 0, 5, 10, 50, and 75 μM quercetin were stained with propidium iodide, followed by analyzing of DNA content by flow cytometry. (A) Representative histograms of DNA distribution for SK-BR-3 were shown. (B) Mean \pm SD of G₀/G₁, S, and G₂/M phases without and with quercetin treatment ($n = 3$); significant differences to the control samples: ** $p < 0.01$, *** $p < 0.001$.

G₀/G₁-phase arrest of A-431 epidermoid tumor cell line at lower concentrations of quercetin

Similarly we were interested to study the effect of quercetin on cell cycle progression in A-431 epidermoid cancer cell line (Figure 4).

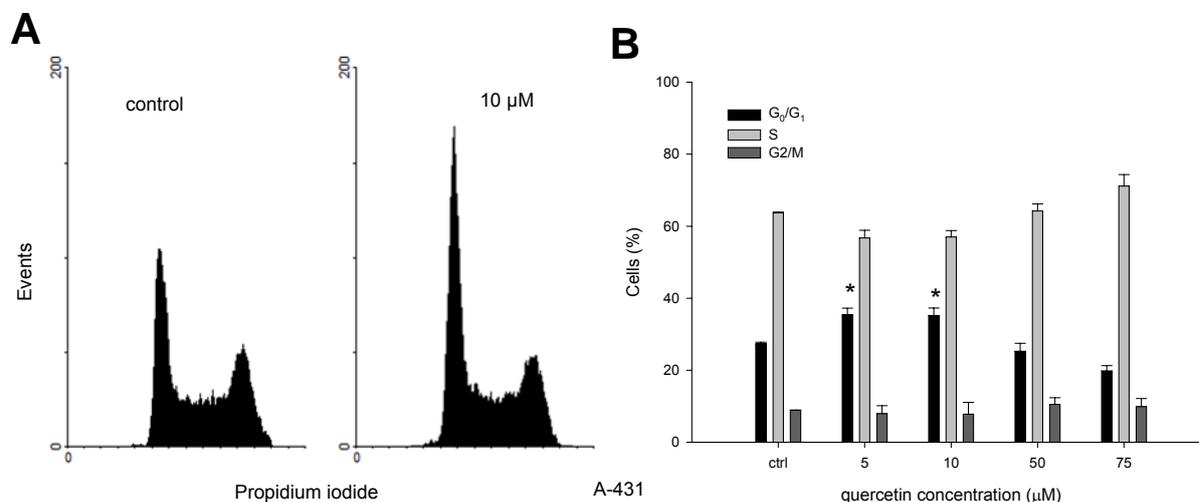


Fig. 4. Effect of quercetin on cell cycle distribution in A-431 epidermoid tumor cells. Ethanol fixed cells were stained with propidium iodide and measured by flow cytometry. (A) Representative histograms for tumor cells treated with 0 and 10 μM quercetin. (B) Data represent mean ± SD ($n = 3$) and asterisks indicate the significant differences compared to control samples, $*p < 0.05$

Interesting in case of this cell line the effect of quercetin depended on the applied concentration. Low concentrations of quercetin, in range of 5 – 10 μM induced cell cycle arrest in G₀/G₁ phase. Higher concentration of quercetin 50 – 75 μM led to moderate accumulation of tumor cells in S phase, in dose dependent manner. The results suggested again the dose dependent biphasic effect of quercetin in A-431 epidermoid tumor cell line.

Effect of quercetin on apoptosis in SK-BR-3 and A-431 cell lines

To investigate the effect of quercetin on apoptosis SK-BR-3 and A-431 tumor cell lines were stained with Annexin V-FITC and 7-AAD followed by flow cytometry measurements (Figure 5). The percentages of apoptotic cells were calculated from the sum of early (Annexin V⁺/7-AAD⁻) and late (Annexin V⁺/7-AAD⁺) apoptotic cells. Dose dependent and moderate effect of quercetin on generating apoptotic cells was noticed in both tumor cell lines. Incubation with 75 μM quercetin for 72 h induced an increase in apoptotic events to from 3% to 18% in SK-BR-3 and from 3% to 19% in A-431 tumor cells. Shorter incubation time (24 h) with quercetin did not produce any increase in the percentage of the apoptotic cells (data not shown).

Discussion

The present study provides the evidences that quercetin displayed anti-proliferative activities in two epithelial tumor cell lines. Notably quercetin showed biphasic features in both tumor cell lines. Our conclusions are supported by several lines of evidence. First, activity of the flavonoid on cell viability was evident at higher concentrations of quercetin, while lower concentrations increased the cell proliferation.

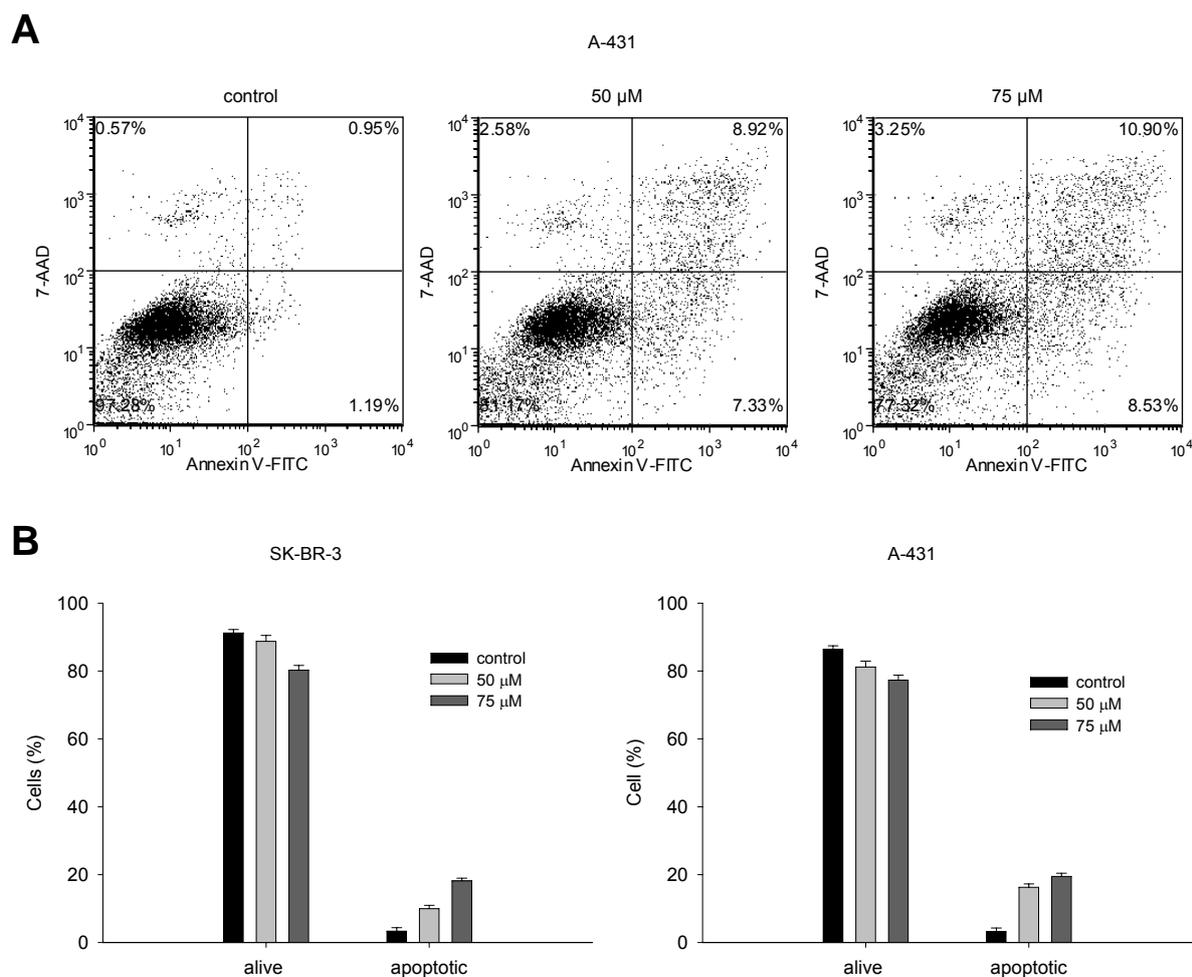


Fig. 5. Effects of quercetin on apoptosis in SK-BR-3 and A-431 tumor cells. The tumor cells after the treatment with quercetin for 72 h were stained with Annexin V-FITC and 7-AAD and measured by flow cytometry within one hour. (A) Representative dot plots from A-431 cells were shown. (B) Apoptotic cells, including both early (Ann V⁺/7-AAD⁻) and late (Ann V⁺/7-AAD⁺) apoptotic cells were presented in comparison with live cells (Ann V⁻/7-AAD⁻). Data represent mean \pm SEM of triplicate experiments.

These results were in line with other data from the literature, where the inhibition of cell viability induced by quercetin took place at 30 μ M for HCT-116 and 80 μ M for HT-29 colon cancer cells; on the opposite, low concentrations of quercetin increased cell proliferation by 20% in colon cancer cells (H. van der Woude & al. [16]). Second, the inhibition of cell viability might be based on two different mechanisms of action evident in tumor cells: i) quercetin displayed cytostatic effects by inducing cell cycle arrest in dose-dependent and cell line-dependent manner; ii) quercetin exhibited pro-apoptotic activity associated with higher doses and long term incubation. Taken together these findings support our central hypothesis that the treatment with quercetin will reduce the tumor cell viability by interfering in modulation of cell cycle progression and in promoting pro-apoptotic effects.

We demonstrated that quercetin could induce the inhibition of cell cycle close to the physiological concentrations (10 μ M). However, rather elevated concentrations of quercetin (50 – 75 μ M) applied for 24 h incubation have been required to induce the cell cycle arrest in case of both tumor cell lines. Several studies indicated that quercetin could induce cell cycle arrest in G₀/G₁ phase in leukemia cells (M. Yoshida & al. [15]), mammary cells (J.H. Jeong & al. [14]), and osteosarcoma cells (D.K. Suh & al. [17]). Previous reports indicated that the induction of cell cycle arrest in G₀/G₁ phase was associated with administration of lower

doses of quercetin, namely 5 and 10 μM (J.H. Jeong & al. [14]). Our data showed that lower concentration of quercetin (5 and 10 μM) caused the arrest of A-431 epidermoid cell line in G_0/G_1 phase. The lower concentrations of quercetin failed to produce similar effects in SK-BR-3 mammary cancer cell line. A possible explanation of these divergences could be based on differences in the incubation time used during the experiments: in the previous reported experiments the incubation time varied between 2 and 4 days (J.H. Jeong & al. [14]), while in our experiments the incubation time had been shorter (24 h). There are at least two possible mechanisms explaining the G_0/G_1 arrest by quercetin. First, quercetin could induce hypophosphorylation of retinoblastoma protein (Rb) which in turn may block the cell cycle transition through G_0/G_1 phase (J.H. Jeong & al. [14]). These effects were not noticed in normal MCF-10 mammary cells (J.H. Jeong & al. [14]). Second, Cyclins D and their partners, cyclin dependent kinases 4 and 6 (Cdk4 and Cdk6) are known to be regulators of G_1 phase (S. Lim & al. [18]). Recent line of evidences proved that quercetin inhibited the cyclin D and Cdk4 expression in glioblastoma cell lines (J. Michaud-Levesque & al. [19]). Very interesting, doses of 50 – 75 μM quercetin were associated with significant S phase arrest and moderate G_2/M blockage, particularly in SK-BR-3 mammary tumor cells. Earlier reports indicated the reduction in cyclin B and Cdk1 suggesting G_2/M arrest of mammary tumor cells (J.H. Jeong & al. [14]). Recent data demonstrated S phase arrest in LoVo human colon carcinoma cells, MCF-7 breast cancer cells (H. Zhang & al. [9]) and BGC-832 gastric cancer cells (P. Wang & al. [20]). Our studies corroborated the data from previous reports, but a unifying hypothesis would be difficult to state. The variation in the cell cycle results might be due to the specific cell type, the concentration of the flavonoid used, and the incubation time applied.

Quercetin induced apoptosis in both tumor cell lines SK-BR-3 and A-431 for concentrations starting with 50 and 75 μM . Recent reports indicated the ability of quercetin to induce apoptosis in HT1080 fibrosarcoma cells at concentrations of 60 μM (B. Sun & al. [21]). Disruption in cell cycle progression could eventually lead to cell death by apoptosis/ necrosis events. Various mechanisms could explain the ability of quercetin to induce apoptosis. It has been reported that apoptotic effect of quercetin in colon and breast tumor cells was related to the ability to generate reactive oxygen species which in turn could lead to DNA damage and consequently to cell death (H. Zhang & al. [9]). At the same time, the apoptotic machinery of BGC-832 gastric cancer cell lines challenged with 90 or 120 μM quercetin led to increase in caspase-3, Bax and reduction in Bcl-2 expression (P. Wang & al. [20]).

Conclusions

Taken together, the results of the present study suggest the dual role of quercetin associated with lower and respectively, higher concentrations of the flavonoid. In the light of the anti-oxidant and pro-oxidant properties quercetin may have biomedical applications in both, neurodegenerative and cancer diseases (F. Dajas [4]). The antiproliferative role of quercetin in mammary and epidermoid cancer cells was first correlated with the cytostatic effects followed by the cytotoxic outcomes. However, these aspects require to be further addressed by studies with combined plant secondary metabolites which showed antiproliferative abilities (A. Del Follo-Martinez & al. [13]).

Acknowledgements

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