Food and Chemical Toxicology 50 (2012) 3375–3383

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

In vitro effect of quercetin on human gastric carcinoma: Targeting cancer cells death and MDR

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ARTICLE INFO

Article history:
Received 18 March 2012
Accepted 24 June 2012
Available online 29 June 2012

Keywords:
Quercetin
Cytotoxcity
Apoptosis
P-glycoprotein
Drug resistance
Human gastric carcinoma cells

ABSTRACT

The benefits of plant polyphenols as chemotherapeutic agents are of great interest due to their possible anti-cancerogenic activities. Results available up to now suggest that flavonoid quercetin induces lethal effect in many types of tumours and may sensitize resistant cells to drugs. The aim of our study was to examine the effect of quercetin on human gastric carcinoma cells and to determine mode of its action. Parental EPG85-257P cell line and its daunorubicin-resistant variant EPG85-257RDB were used as cell models. Our data revealed that quercetin exerted antiproliferative impact on studied cells (with IC_{50} value of 12 μM after 72 h), mainly through induction of apoptosis. In sensitive cells cytostatic drug and flavonoid had synergistic effects, in EPG85-257RDB cells quercetin acted as a chemosensitizer. Its impact on resistance mechanism involved decrease of P-glycoprotein expression, inhibition of drug transport and downregulation of *ABCB1* gene expression. The results demonstrate that quercetin may be considered as a prospective drug to overcome classical resistance in gastric cancer cells.

1. Introduction

The effects of dietary polyphenols are still of great interest mainly due to their antioxidative and anticarcinogenic properties. Perspectives on the possible application of some biopolyphenols for the prevention of cancer have been widely discussed (Surh, 2003) also in our previous studies (Borska et al., 2003, 2004, 2010a). The most common group of plant polyphenols are flavonoids which include six subclasses: flavones, flavonols, flavanones, catechins, anthocyanidins and isoflavones (Ross and Kasum, 2002). One of the best described flavonol, quercetin, is found in large quantities in onions, apples, broccoli and berries (Erlund, 2004). Quercetin effects are multi-targeted. It has a wide range of biological activities including antiproliferative action against several cancer cells of human origin, induction of apoptosis and inhibition of cell invasion and angiogenesis (Tan et al., 2003). Moreover, quercetin as a pro-oxidant induces apoptosis in cancer cells and as antioxidant and chemopreventive agent protects normal cells against oxidative stress and mutagenesis (Bu et al., 2011; Gibellini et al., 2011; Ishisaka et al., 2011).

Multidrug resistance (MDR) phenomenon still remains an important impediment to successful chemotherapy. Daunorubicin is a drug involved in MDR. It may intercalate into the DNA and react with topoisomerase II. Therefore its maximal toxicity occurs in the S phase and G2/M phase of the cell cycle (Minotti et al., 2004). The classical MDR is characterized by overexpression of P-glycoprotein (P-gp) that mediates efflux of several cytotoxic compounds and decreases their intracellular accumulation. Among drugs which are substrates for the action of P-gp is daunorubicin. To date, mechanisms of MDR are fairly well understood (Salerno and Garnier-Suillerot, 2001; Wang et al., 2000).

Since the 1980s many drugs have been investigated for their ability to reverse MDR. Most of them turned out to cause dangerous side effects when trials were launched. Examples include verapamil, cyclosporine A, quinidine and others. Screening of new generations of MDR modulators is still work in progress (Colabufo et al., 2011; Szakacs et al., 2006). A number of studies demonstrate that quercetin may be a plausible agent to overcome MDR in many types of resistant cells. Among the mechanisms proposed to mediate resistance to drugs, overexpression of ABC transporters has received extensive investigation (Nabekura et al., 2005). These proteins function as ATP-dependent drug efflux pumps and some

Abbreviations: CAM, calcein AM assay; MDR, multi-drug resistance; NBDs, nucleotide binding domains; OD, optical density; P-gp, P-glycoprotein; P-line (P-cells), parental cells of human gastric carcinoma line EPG85-257P; RDB-line (RDB-cells), resistant cells of human gastric carcinoma line EPG85-257RDB; RF, resistance factor; RQ, relative quantification; TMDs, transmembrane domains.

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http://dx.doi.org/10.1016/j.fct.2012.06.035

of them were found to decrease cellular accumulation of cytotoxic substances. P-gp is the most known MDR-associated transporter (Leslie et al., 2005). An active protein contains two transmembrane domains (TMDs) which form substrate-binding sites and two nucleotide binding domains (NBDs) which are located in the interior of the cytoplasm and participate in ATP binding and hydrolysis (Lage, 2008). Quercetin as a P-gp inhibitor has been tested on a number of resistant cell lines and also on several animal models. Many of these studies gave positive results but they vary between cell lines and tumors. To date is known that quercetin may modulate drug efflux by directly interacting with ATP-binding site and/or the substrate-binding site (Cheng et al., 2010; Nagy et al., 2004).

The results so far are controversial, therefore we are convinced that each type of tumor should be considered individually. In our study, we investigated antiproliferative and pro-apoptotic action of quercetin and its potential to alter the expression and transport activity of P-gp in two human gastric cancer cell lines EPG85–257P and EPG85–257RDB.

2. Materials and methods

2.1. Cell culture

Daunorubicin-resistant, P-gp-positive gastric carcinoma cell line EPG85-257RDB (RDB-line, RDB-cells) and its parental line EPG85-257P (P-line, P-cells) were grown in Leibovitz's L-15 Medium (SIGMA, Germany) containing 10% FBS, 1 mM L-glutamin, 80 IE/l insulin, 6,25 mg/l fetuin, 2,5 mg/l transferrin, 1,1 g/l $NaHCO_3$, 1 g/l glucose, 1% minimal essential vitamins (SIGMA, Germany). RDB-cells were maintained in medium containing 2.5 μg/ml DB to assure the stability of resistance. The cell culture conditions were according to previous description (Dietel et al., 1990).

2.2. Estimation of IC_{50} and resistance index

For determination of IC_{50}-values of quercetin and daunorubicin (SIGMA, Germany), cells were incubated with increasing concentrations of drugs. The tests were based on a colorimetric technique with sulphorhodamine B (SRB) dye (Skehan et al., 1990). The cells were fixed with 50% trichloroacetic acid, stained with 0.4% SRB (in 1% acetic acid) and incubated with 10 mM tris(hydroxymethyl) aminomethane (pH 10,5). Optical density value (OD) was read after 72 h of drugs exposure at 562 nm using a microplate-reader (Infinite M200, TECAN, USA). IC_{50}-values were calculated from three independent experiments. Quercetin resistance factor (RF) was calculated using following formula: RF = IC_{50} for resistant cells / IC_{50} for sensitive cells (Kothan et al., 2004). The further study was performed using quercetin concentrations chosen after preliminary tests and with standard concentrations of DB.

2.3. Joint effect of quercetin and daunorubicin on cell viability

Proliferation tests were performed on 96-well plates for both cell lines treated with quercetin and/or daunorubicin. After 24 h of cells culturing, drugs were added for 72 h as follow: concentrations of quercetin: 3 μM (Q3), 6 μM (Q6), 12 μM (Q12); concentrations of daunorubicin: 0.043 μM (K1), 0.43 μM (K2), 4.3 μM (K3), where K2 (therapeutic dose) is the concentration of the cytostatic drug in patient's blood 2 h after administration, for daunorubicin equal to 0.25 mg/ml; combinations of quercetin and daunorubicin: Q3 + K1, Q3 + K2, Q3 + K3, Q6 + K1, Q6 + K2, Q6 + K3, Q12 + K1, Q12 + K2, Q12 + K3. As a solvent for quercetin was used ethyl alcohol in concentration $\leqslant$0.12%. Pre-tests showed that the solvent did not affect the results of experiments. Viability tests for daunorubicin and quercetin were based on a colorimetric technique with SRB dye (as described above). The absorbance was read at 562 nm using a microplate-reader (Infinite M200, TECAN, USA).

2.4. Apoptosis detection

Cells were cultured in 24-well plates, after 24 h drugs were added in concentrations used in viability test, omitting the combinations with the highest daunorubicin concentration K3. After 72 h cells were detached with 0.25% Tripsin-EDTA solution (SIGMA, Germany). Apoptosis was evaluated using comet assay (Yasuhara et al., 2003). The silver-staining was performed according to Cerda (Cerda et al., 1997). The cells were incubated 2 × 10 min in staining mixture of A + B solutions, 68 ml of A solution (100 mg ammonium nitrate, 100 mg silver nitrate, 500 mg wolframosilic acid, 250 μl formaldehyde – min. 37 in 500 ml H_2O) and 32 ml of B solution (12.5 g sodium carbonate in 250 ml H_2O). The staining reaction was stopped with 1% acetic acid. Apoptosis was measured in five randomly selected microscopic fields (magnification of 200×). Analysis was performed under a light microscope (Olympus BX41, Japan). The percentage of damaged nuclei was calculated for every drug combination in comparison with the control. The obtained results were categorized to 3 ranges: intact cell nuclei, intermediate damage (grades 1 and 2, in reference to Yasuhara et al.) and apoptotic cell nuclei (grades 3 and 4, in reference to Yasuhara et al.).

The apoptotic cells were detected by the TUNEL technique as well. Following 72 h of incubation with drugs, cells were fixed in a mixture of methanol and acetone (1:1) for 15 min, at the temperature of 4 °C. The damaged cell nuclei were detected using Apop Tag Peroxidase in situ Apoptosis Detection Kit (Intergen, USA) according to the manufacturer protocol and with 3,3′-diaminobenzidine (DAB + Chromogen, DakoCytomation, Denmark) as a chromogen. Number of apoptotic cell nuclei was counted under a light microscope (Olympus BX41, Japan; Analysis 3.2, Germany) as presented previously. The percentage of damaged nuclei was calculated for every drug combination in comparison with the control.

2.5. Immunocytochemical and immunofluorescence analysis

The cells were cultured on 8-well Merck Millicell® EZ slides (Merck Millipore, Germany). After 24 h of culturing, the tested substances were added as follow: quercetin: Q3, Q6, Q12; daunorubicin: K1, K2, K3; combinations: Q3 + K1, Q3 + K2, Q6 + K1, Q6 + K2, Q12 + K1, Q12 + K2. After 72 h of incubation cells were fixed in a mixture of methanol and acetone (1:1) for 15 min, 4 °C. Detection of P-gp expression in cell membrane were performed using monoclonal P-gp-specific antibody, clone C219 (Alexis Biochemicals, Germany) with 1:100 dilution and 1 h of incubation in room temperature. In immunocytochemical reaction were used LSAB + System-HRP kit (Dako Cytomation, Denmark) and DAB as a chromogen (DakoCytomation, Denmark). While in immunofluorescence method the primary antibody was detected with a 1:2000 dilution of Alexa Fluor® 594 Goat Anti-Mouse secondary antibody (Life Technologies), after 1 h of incubation. Analysis of results was conducted under a light and fluorescent microscope (Olympus BX41, Japan). The percentage of cells which manifested P-gp expression was calculated from five representative microscopic fields of each well (magnification 200×).

2.6. Western blotting analysis of P-gp expression

Changes in P-gp expression in RDB-line were examined after treatment with the Q12 concentration, as compared to the control (P-line). Cells were cultured in flasks for 24 h, then Q12 was added. After 72 h the cells were trypsinized, centrifuged, resuspended in PBS and scored in a Bürker haemocytometer. For each test 1.5–2 × 10^7 cells were washed in ice cold PBS and lysed on ice with RIPA buffer (50 mM Tris–Cl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Igepal CA-630 and 0.5% sodium deoxycholate) containing protease inhibitor cocktail (SIGMA) and 0.5 mM PMSF. The cell debris were removed by centrifugation at 12,000g, 10 min. Protein concentration was measured using BCA method (Thermo-Pierce). Cell extracts were mixed with SDS sample buffer (250 mM TRIS pH 6.8, 40% glycerol, 20% v/v β-mercaptoethanol, 100 mM DTT, 0.33 mg/mL bromophenol blue, 8% SDS) and incubated on ice for 30 min to avoid membrane protein precipitation. Protein samples (25 μg per gel lane) were separated in 7.5% TGX ready gels (Bio-Rad, USA) (Laemmli, 1970), and blotted onto PVDF membrane (Immobilon PSQ, Millipore). P-gp expression was detected after incubation with Pgp-specific monoclonal antibody C219 (Alexis Biochemicals, USA) using the chemiluminescence HRP detection substrate (Bio-Rad, USA) and visualized with Chemi-Doc XRS Molecular Imager (Bio-Rad, USA). Protein content in P-gp bands was normalized according to the β-actin AC-40 monoclonal antibody (SIGMA, Germany), blotted in SNAPiD apparatus (Merck Millipore, Germany). Secondary antibodies against mouse immunoglobulins conjugated with horseradish peroxidase were purchased from Jackson Immunoresearch. OD measurements of the protein bands in immunoblots were performed with the QuantityOne software (Bio-Rad, USA).

2.7. Analysis of P-gp activity

Alterations in transport function of P-gp under effect of quercetin were examined using calcein AM (CAM) assay. After 24 h of culturing RDB-cells were treated with Q for 72 h (Q3, Q6, Q12). Non-treated RDB-cells were left as a control. Retention of CAM was tested using Vybrant Multidrug Resistance Assay Kit (Molecular Probes, USA) according to the manufacturer protocol. The experiment was conducted in six replications per plate. The fluorescence was measured using a microplate-reader (Infinite M200, TECAN, USA) at 494/517 nm (Abs/Em). Values of fluorescence for P-line (not treated with quercetin or daunorubicin) were used to calculate retention coefficient of calcein (R_{CAM}) for every applied drug combination in RDB line, according to the following formula:

$$R_{CAM} \text{ for RDB-cells} = [\text{fluorescence of RDB-cells treated with quercetin} / \text{fluorescence of P-cells not treated with Q}] \times 100$$

2.8. RT-PCR relative quantification

ABCB1 gene expression in both gastric carcinoma cell lines were examined using real-time PCR and relative quantification (RQ) method. Cultures were conducted in 25 cm^2 flasks (2 per each line). Q12 was added to one of the flask leaving the other one as a control. After 72 h cells were trypsinized, then resuspended in PBS and cen-

trifuged (3×). RNA was isolated using the RNAqueous-4PCR kit (Ambion, UK) as described by the manufacturer. Reverse transcription was performed using High Capacity cDNA RT kit (Applied Biosystems, USA) and Thermal Cycler MJ Research PTC-200 (Bio-Rad, USA). *GAPDH* was applied as the normalizing gene. The PCR was provided in a 96-well optical plates (Applied Biosystems, USA). 20 μl of reaction mixture in each well included: 9 μl cDNA of each studied sample, 10 μl TaqMan Universal PCR master mix and 1 μl of primers and probes mix. All reagents were purchased from Applied Biosystems, USA. Real-time PCR was conducted using 7900HT Fast Real-Time PCR light-cycler and SDS 2.3 software (Applied Biosystems, USA). Thermal profile of reaction included polymerase activation – 2 min, 50 °C and initial denaturation – 10 min, 94 °C, followed by 40 cycles of alternating denaturation and synthesis, respectively 15 s, 94 °C and 60 s, 60 °C. Gene expression was analyzed by RQ Manager 1.2 software (Applied Biosystems, USA). Expression values of *P*- and RDB-cells non-treated with Q12, were used as calibrators (samples in relation which were compared the other samples in RQ method). The analysis was performed employing the formula: $RQ = 2^{-\Delta\Delta Ct}$, (where $\Delta\Delta Ct = \Delta Ct$ for the sample - ΔCt for the calibrator). RQ of the calibrator is always equal to 1. The graphs were made in the logarithmic scale using RQ Manager 1.2 software.

2.9. Statistical analysis

All the experiments were conducted in triplicates. The obtained results were subjected to statistical analysis using Prism 5.0 (GraphPad, La Jolla, CA, USA). Shaphiro-Wilk normality test was used for assessing the distribution of the variables. Mann–Whitney *U*-test, a non-parametric equivalent of the students t-test, was used to compare two groups of data. In cases, when more than two groups of data were analyzed, the Kruskall-Wallis test with post hoc analysis was utilized. The results were statistically significant when $p < 0.05$.

3. Results

3.1. Effect of quercetin and daunorubicin on cell viability and apoptosis

IC_{50} of quercetin for both studied cell lines was estimated at 12 μM (Fig. 1A and B). RF was amounted to 1. After administration of daunorubicin IC_{50} for P-line was almost equal to 1/2 K2 (Fig. 1C). A combined cytotoxic effect was stronger than of each drug alone (Fig. 2). In the case of P-cells it was particularly noticeable to Q + K1 combinations in comparison with effect of K1 itself (Fig. 2A and B). In the case of RDB-cells, quercetin significantly sensitized cells to action of daunorubicin (Fig. 2C and D).

Comet assay results showed that effect of quercetin and/or daunorubicin was pro-apoptotic in the case of P-cells (Fig. 3A). The effect was strongly enhanced after treatment of both substances. Differences between action of therapeutic dose K2 and combination K1 + Q12 were not statistically significant. The percentage of apoptosis increased in relation to the intermediate damage for combination K1 + Q12 and also K2 + Q12 as compared with action of daunorubicin without supplementation. RDB-line cells demonstrated significant apoptotic damage only after Q12 treatment and its combinations with daunorubicin vs. control (Fig. 3B). TUNEL technique confirmed this tendency (Fig. 4). A significant difference was estimated for Q12, Q12 + K1 and Q12 + K2 vs. control.

3.2. Change in expression of P-gp after exposure to quercetin

Evaluation of immunocytochemical and immunofluorescence reaction (Fig. 5) demonstrated that P-gp expression in RDB-cells decreased significantly after treatment of Q12 (Fig. 6). Other concentrations of quercetin did not cause any significant changes in protein expression. Expression of P-gp in P-line was not detected. Western blot analysis (Fig. 7) indicated that after treatment of Q12, in RDB-cells P-gp amount decreased by 20% (Fig. 8).

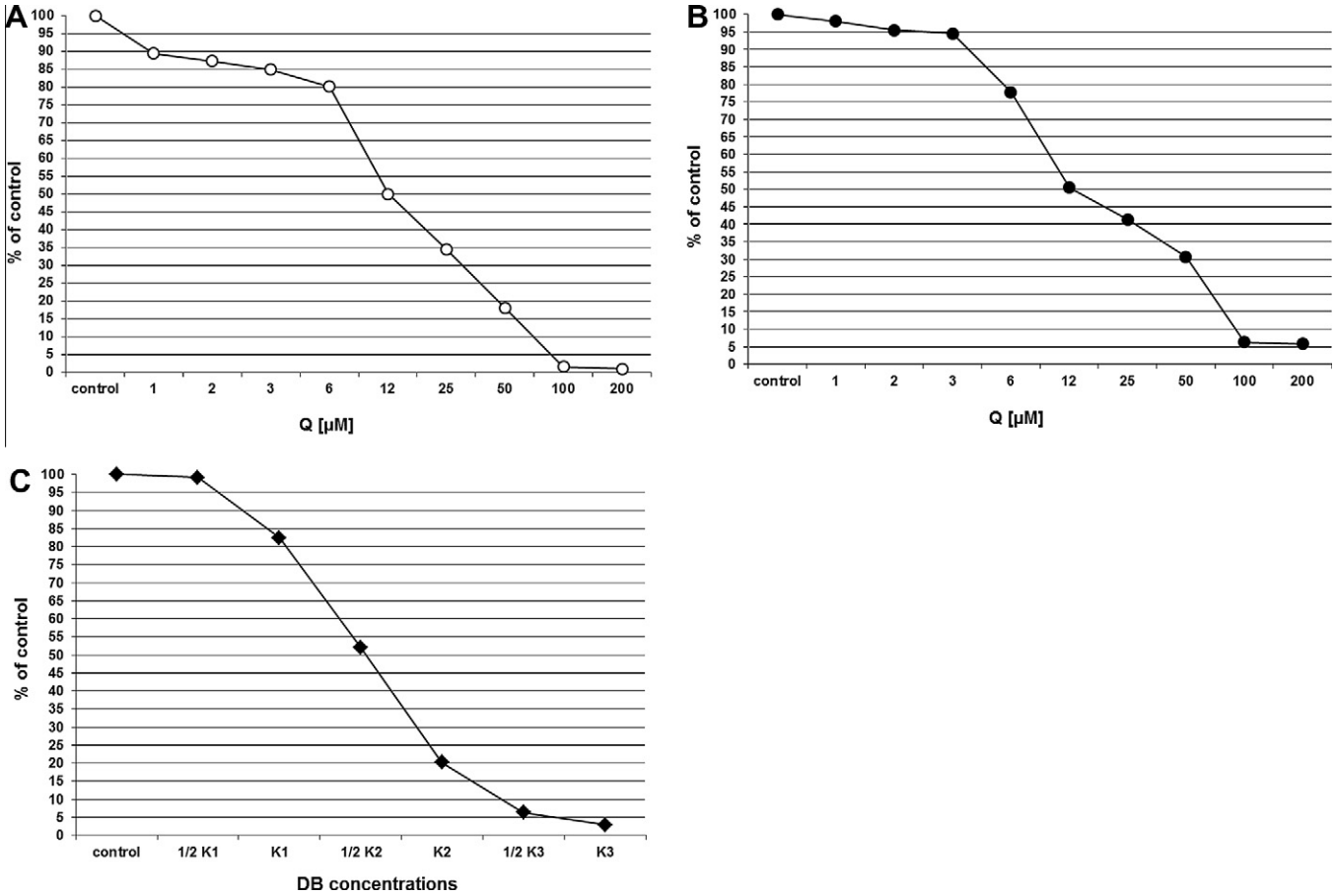

Fig. 1. Effect of various concentration of quercetin (Q) and/or daunorubicin (DB) on cell proliferation following 72 h treatment. Values represent mean of three independent experiments as % of control (C). (A) P-line supplemented with Q; (B) RDB-line supplemented with Q; (C) RDB-line supplemented with DB.

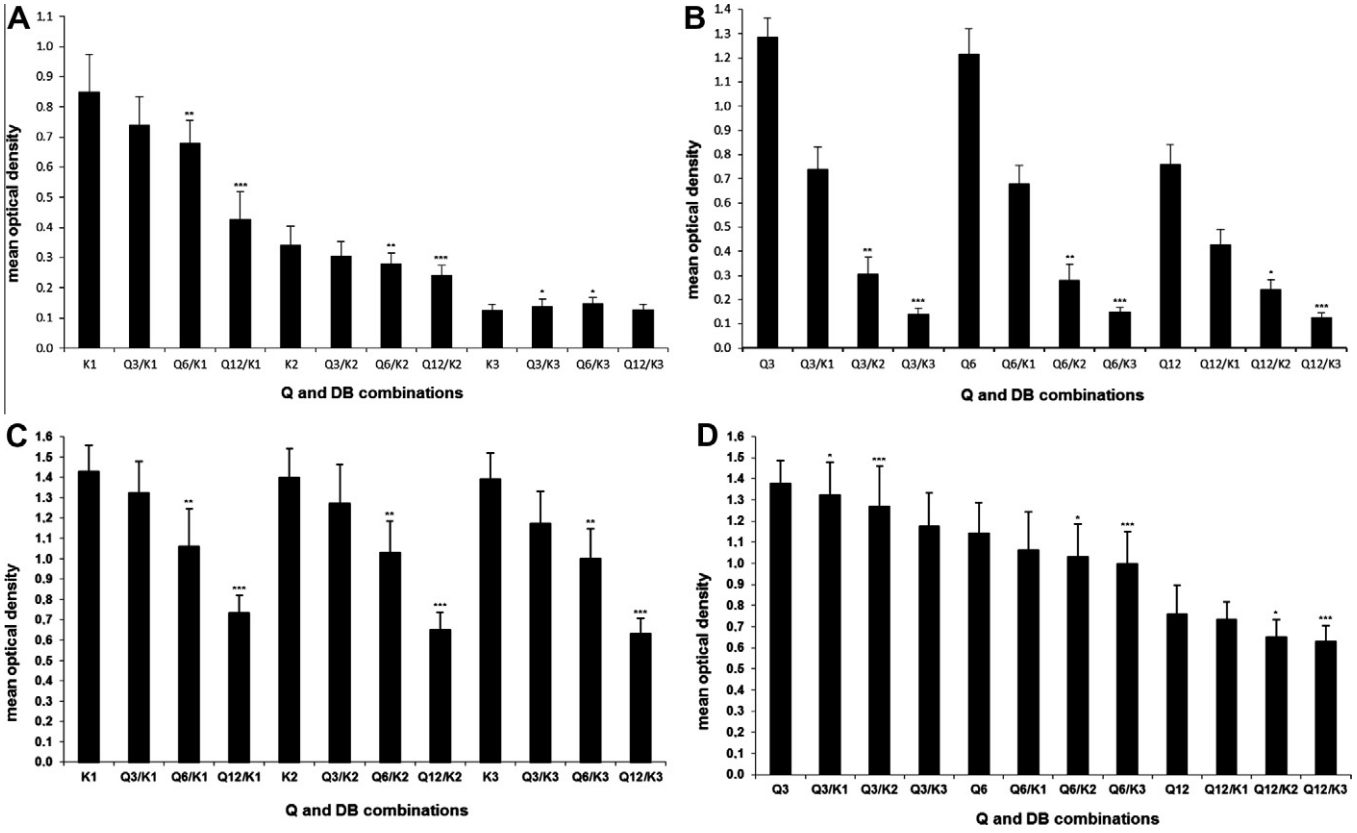

Fig. 2. Effect of various combination of quercetin (Q) and daunorubicin (DB) on optical density (OD) changes. Significant differences in mean values of OD are indicated by $^{*}p < 0.001$, $^{**}p < 0.01$ and $^{***}p < 0.05$ according to corresponding concentration of drug alone. (A) In comparison with the corresponding concentration of DB alone (P-line cells); (B) in comparison with the corresponding concentration of Q alone (P-line cells); (C) in comparison with the corresponding concentration of DB alone (RDB-line cells); (D) in comparison with the corresponding concentration of Q alone (RDB-line cells).

3.3. Effect of quercetin on P-gp function

In comparison with control, only the action of Q12 caused statistically significant difference in accumulation of calcein (Fig. 9). R_{CAM} increased by 130%. The differences between the effects of particular concentrations are also statistically significant, for Q3 and Q6 $p < 0.05$, for Q3 and Q12, Q6 and Q12 $p < 0.001$.

3.4. Effect of quercetin on ABCB1 gene expression

3.4.1. P-cells as a calibrator

Relative analysis of *ABCB1* expression indicated that in RDB-line it is 1029 times higher than in P line. After Q12 treatment this difference considerably decreased (Fig. 10A). Difference between *ABCB1* expression in P-cells before and after treatment of Q was not significant. RQ values of samples were as follow: $RQ_P = 1$, $RQ_{P+Q12} = 1.11$, $RQ_{RDB} = 1028.53$, $RQ_{RDB+Q12} = 245.77$.

3.4.2. RDB-cells as a calibrator

In comparison with expression in RDB line as calibrator it was noted that after exposure to Q12 expression of *ABCB1* in resistant cells decreased significantly (Fig. 10B). We indicated 76% inhibition in comparison with the RQ_{RDB}. RQ values according to the calibrator: $RQ_P = 0.0009$, $RQ_{P+Q12} = 0.001$, $RQ_{RDB} = 1$, $RQ_{RDB+Q12} = 0.24$.

4. Discussion

It is well known that quercetin has antiproliferative and pro-apoptotic potential especially in the case of cancer cells and thus may be considered as a therapeutic agent. Many *in vivo* and *in vitro* investigations (Xiao et al., 2011) and epidemiological studies (Murakami et al., 2008) confirmed this statement. Quercetin has been shown to inhibit enzymes involved in proliferation, signal transduction and cell death (Nguyen et al., 2004) in dose- and cell type-dependent manner. In the present study we investigated effect of quercetin on gastric cancer cells sensitive and resistant to daunorubicin. The results point out that studied cell models are very sensitive to cytotoxic action of quercetin, which equally inhibits growth of the parental and P-gp-expressing cells (for both cell lines $IC_{50} = 12\ \mu M$). Similar observations were done previously on other types of cancer e.g. many leukemia cell lines (Ishii et al., 2010). Moreover, our experiments revealed that quercetin and daunorubicin acted synergistically (mutual effect) in P-cells while in the case of RDB-cells polyphenol sensitized the cells to cytostatic drug (one-sided effect). Augmentation of many drugs impact by quercetin and/or synergy in action was described previously but its effectiveness varies depending on the concentration, exposure time, and above all, the type of cancer cells (Staedler et al., 2011). This work explains how quercetin acts on human gastric cancer cells in the case of two variants, sensitive and resistant to the cytostatic. It is particularly important that high cytotoxicity of plant polyphenols primarily concerns cancer cells, it seems that at low concentrations they do not affect many types of normal cells. Recent research shows that the strong quercetin action on transformed cells perfectly matches with the almost total absence of any damages for normal tissues (Bakheet, 2011; Choi et al., 2005). On the contrary, quercetin and its analogs are likely to be important in defending normal cells from oxidative attack and side effect of drugs and play important role in cancer chemoprevention (Jia et al., 2011). This was confirmed also in clinical trials (Ferry et al., 1996; Howells et al., 2010). Recent studies in Europe and

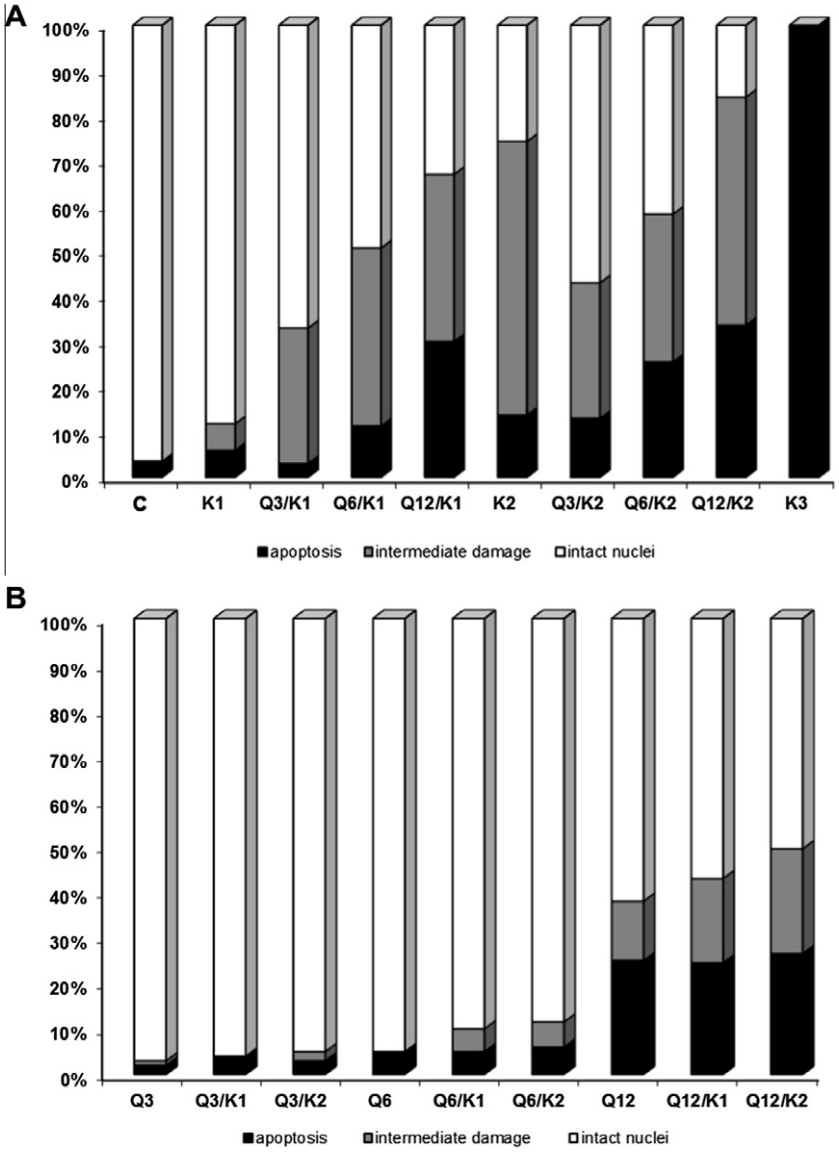

Fig. 3. Effect of quercetin (Q) and daunorubicin (DB) combinations on proportion of apoptotic and intact nuclei (%) examined using comet assay vs. control (C). A. P-line (comparison between Q + DB and DB); B. RDB-line (comparison between Q + DB and Q).

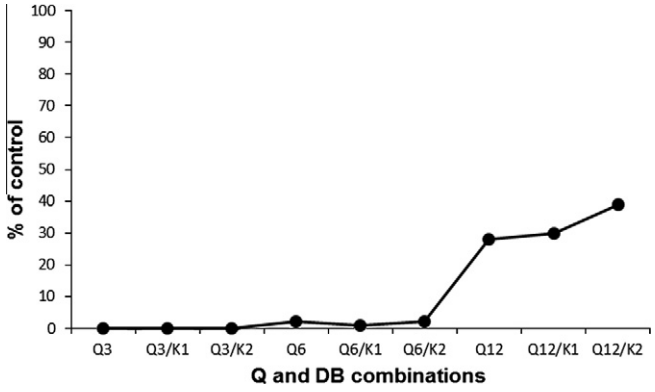

Fig. 4. Effect of quercetin (Q) and daunorubicin (DB) combinations on RDB-line cells in comparison with action of Q alone. (%) proportion of apoptotic and intact nuclei examined using TUNEL technique.

the U.S. provided evidence for beneficial effects of quercetin. Its safety upon human application is formally approved (Okamoto, 2005). However, there are some controversial reports which indicate genotoxic potential of quercetin (Harwood et al., 2007). Therefore, its beneficial effect should be considered individually for each cell type.

Pro-apoptotic action of quercetin on tumour cells is widely known and described. It induces cell death by various mechanisms e.g. by MAPK, ERK, PI3K, PKC kinases and corresponding cell signaling pathways (Granado-Serrano et al., 2006), by regulation of Bax and other proteins of Bcl-2 family expression (Lee et al., 2008), through inhibition of NFκB (Vidya Priyadarsini et al., 2010) and caspases (Niu et al., 2011). Induction of apoptosis by quercetin in cancer cells can be carried out by death receptors as well as type II estrogen receptors and via activation of the mitochondrial-dependent pathway. It is considered as a late type of apoptotic death with a peak after 48 h, which involves DNA damage and induction of p53 (Lee et al., 2011; Liang et al., 2011; Rosner et al., 2006). In studied gastric cancer cell lines quercetin induced apoptosis in dose-dependent fashion. P-cells death was observed after 72 h of treatment in all studied concentrations of quercetin. This effect was strongly enhanced after treatment with both quercetin and daunorubicin. It is noticeable that between the influence of therapeutic dose of daunorubicin

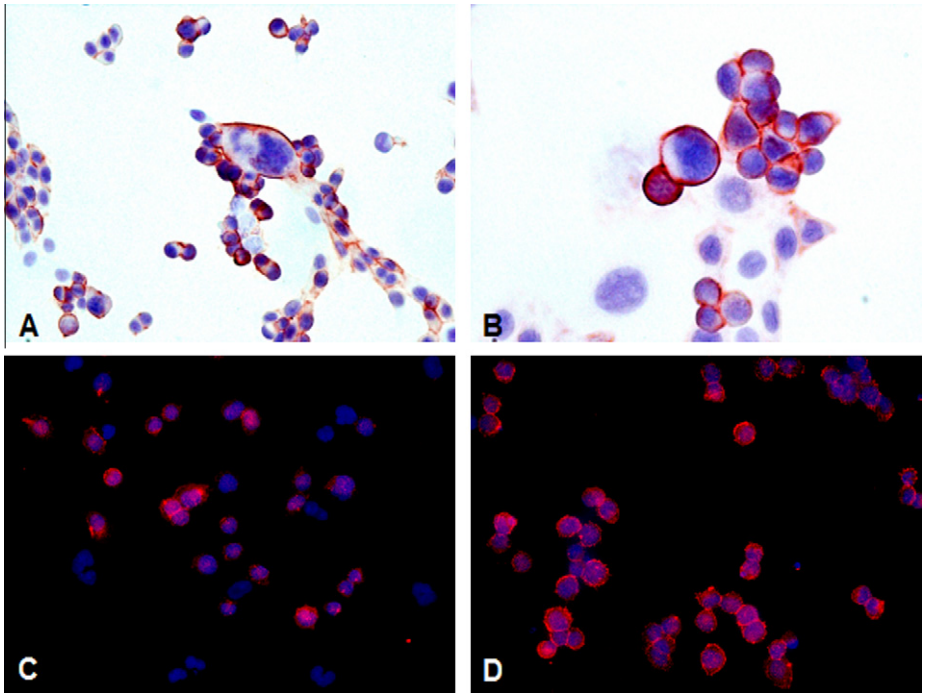

Fig. 5. Immunocytochemical and immunofluorescence membranous reaction of P-gp expression in cells of RDB-line. (A) RDB-line after 72 h exposure to Q12, magnification 200×; (B) RDB-line after 72 h exposure to Q12, magnification 400×; (C) RDB-line after 72 h exposure to Q12, magnification 200×; (D) RDB-line not treated with Q, magnification 200×.

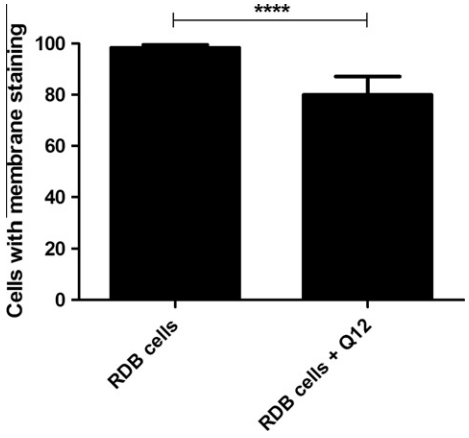

Fig. 6. Comparison between P-gp expression in membranes of RDB-cells not treated witch quercetin and after 72 h exposure to Q12. *P* value < 0.0001 is indicated by ****.

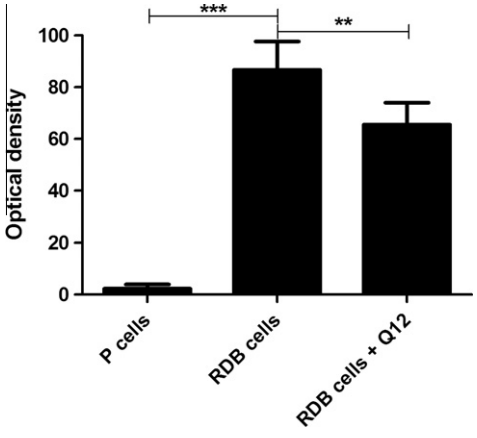

Fig. 8. Western blot analysis of P-gp expression. Comparison between optical density values in RDB-cells not treated with quercetin and after 72 h exposure to Q12. P-cells as a control. $^{**}p < 0.01$, $^{***}p < 0.001$.

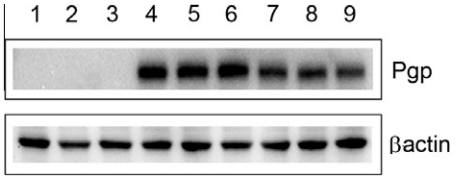

Fig. 7. Analysis of P-gp protein expression using Western blotting technique in RDB-line following 72 h treatment with Q12. P-gp-specific antibody C219 detected 170 kDa band in RDB-line cells non-treated with quercetin (lanes 4–9), revealing the decrease in P-gp expression level after incubation with Q12 (lanes 7–9). P-line cells (lanes 1–3) was used as negative control for P-gp synthesis, and beta-actin (42 kDa) staining was used as loading control.

(K2) and dose ten times lower (K1) in combination with 12 μM of quercetin (Q12) there was no significant difference. Such strengthening of cytostatic drug action allows its dose reduction what may help to minimize the side effects. In RDB-cells significant quercetin-induced apoptosis was detected after exposure to Q12. Additional supplementation with daunorubicin resulted in the highest fraction of cell nuclei with intermediate damage and had a stronger pro-apoptotic impact. This particular result points out that cell death of resistant cells is not only a result of quercetin action. It seems that studied flavonol sensitized RDB-cells to the cytostatic drug. Differences between results of viability tests and percentage of apoptotic damage might be due to possibility of decreasing proliferation activity, inducing non-specific apoptosis or non-apoptotic cell death e.g. mitotic catastrophe or autophagy. It was reported that a novel diterpenoid termed pharicin A from *Isodon pharicus* induces mitotic arrest of paclitaxel-sensitive and resistant tumor cells (Xu et al.,

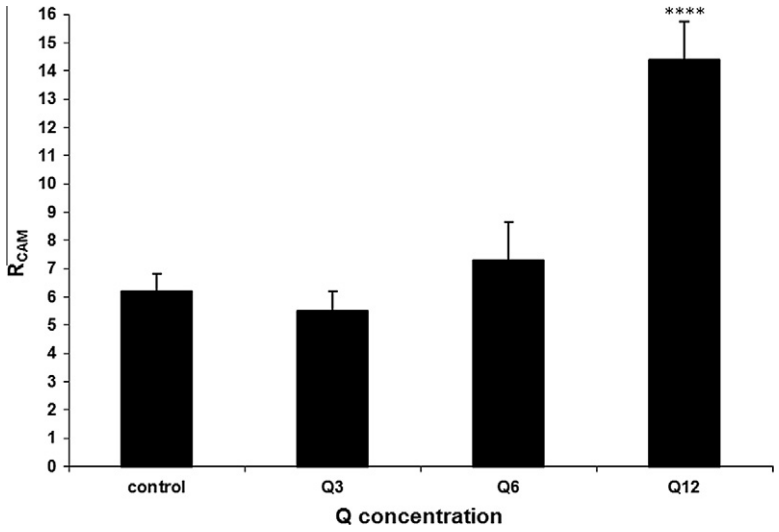

Fig. 9. Retention coefficient (R_{CAM}) values for RDB-cells after treatment with quercetin (Q3, Q6, Q12) in comparison with control. Statistically significant ($p < 0.001$) difference in calcein accumulation: Q vs. control is indicated by ****.

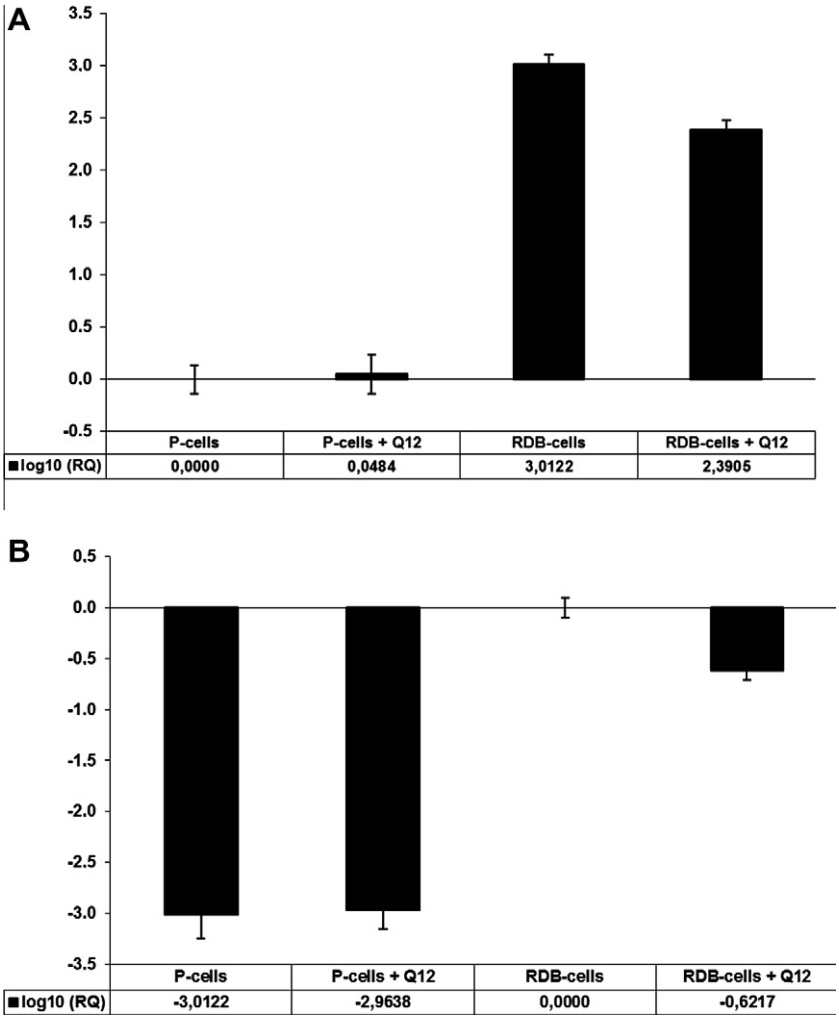

Fig. 10. Relative analysis (RQ) of *ABCB1* expression using real-time PCR in both cell lines treated and non-treated with Q12. (A) P-cells expression as a calibrator; (B) RDB-cells expression as a calibrator.

2010). Many chemotherapeutic agents, including daunorubicin, have a potential to induce mitotic catastrophe (Mansilla et al., 2006; Roninson et al., 2001). To date there were no reports indicating that quercetin may induce such type of cell death. However, since it is widely known that quercetin impacts so many intracellular processes and influences cell cycle controlling genes such as p53, this hypothesis cannot be rejected without further study.

In agreement with previous data (Seidel et al., 1995) we found that EPG85-257RDB cells overexpress both P-gp and its gene *ABCB1*. Considering the results of our study RDB-cells demonstrated significant reduction in P-gp expression after exposure to 12 μM of quercetin. This concentration also considerably affected the transport abilities of P-gp in RDB cells. Retention coefficient increased by 130%, as compared to non-treated cells. In the case of pancreatic cell lines we demonstrated as much as 200% increase in drug accumulation (Borska et al., 2010b). There are more and more examples of plant-derived compounds that are proven to down-regulate the P-gp expression and activity, e.g. curcumin (Hou et al., 2008), resveratrol (Quan et al., 2008) and quercetin (Choi et al., 2011; Wu et al., 2005). The latter may belong to a group of allosteric inhibitors of P-gp-mediated drug efflux which inhibits the late steps of transport (Nagy et al., 2004), probably in a manner strictly dependent on the concentration (Morris and Zhang, 2006). The most probable mechanism of quercetin effect on P-gp is inhibition of the ATPase activity by interaction with NBDs rather than competition for substrate binding site, as it is in the case of other modulators (Di Pietro et al., 2002). Our results seems to confirm this explanation. An important observation is that resistance factor for quercetin is equal to 1, thus indicating that it is rather not a substrate for P-gp in studied cancer cells.

Quercetin is also believed to be a potential modulator of MDR on DNA and/or mRNA level (Chieli et al., 2010). Regulation of P-gp expression is a complex process, and could include different mechanisms in normal cells compared with tumour cells. It is known that downregulation of *ABCB1* gene may enable the prevention of P-gp overexpression in cancer cells (Scotto, 2003). Our studies revealed that in resistant gastric carcinoma cell line the expression of *ABCB1* is 1028 times higher than in P line. After treatment with Q12 it significantly decreased, up to 76% as compared with non-treated RDB-cells. Some differences between mRNA and protein expression values suggest that translational and post-translational modifications appear to influence the expression levels of P-gp.

5. Conclusions

In summary, our research provided evidence that quercetin is an effective cytotoxic agent in the case of studied gastric carcinoma cell lines. It has not only a synergistic effect with daunorubicin on P-cells but also sensitize RDB-cells to toxic action of cytostatics. The analysis of P-gp activity and its expression at protein and mRNA levels, showed that quercetin may down-regulate this transporter. It pointed out that after quercetin supplementation classical resistance to drugs may be reversed.

However, it is known that *in vitro* studies are always questionable proof of the effectiveness of any substance as a drug. Therefore, before quercetin will be approached as a medicine to overcome MDR phenomenon it is a need to find answers to many questions about the appropriate use of current knowledge on this subject in clinical trials.

Conflict of Interest

None.

Acknowledgments

The work was supported by Ministry of Science and Higher Education Grant (research project No.: N N401 039537). We would like to thank H. Lage and M. Dietel (Humboldt University Berlin, Charité Campus Mitte, Institute of Pathology) for permission to use EPG85-257 cell lines. The authors are grateful to A. Jethon and M. Olbromski (Medical University in Wroclaw, Department of Histology and Embryology) for help with immunofluorescence microscopy. Special gratitude is due to B. Pula (Medical University in Wroclaw, Department of Histology and Embryology) and M. Sopel (Medical University in Wroclaw, Department of Pharmaceutical Biology and Botany) for carrying out statistical calculations.

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