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ORIGINAL ARTICLE

Apigenin, a dietary flavonoid, induces apoptosis, DNA damage, and oxidative stress in human breast cancer MCF-7 and MDA MB-231 cells

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Abstract

Apigenin is found in several dietary plant foods such as vegetables and fruits. To investigate potential anticancer properties of apigenin on human breast cancer, ER-positive MCF-7 and triple-negative MDA MB-231 cells were used. Moreover, toxicological safety of apigenin towards normal cells was evaluated in human lymphocytes. Cytotoxicity of apigenin towards cancer cells was evaluated by MTT assay whereas further genotoxic and oxidative stress parameters were measured by comet and lipid peroxidation assays, respectively. In order to examine the type of cell death induced by apigenin, several biomarkers were used. Toxicological safety towards normal cells was evaluated by cell viability and comet assays. After the treatment with apigenin, we observed changes in cell morphology in a dose- (10 to 100 μM) and time-dependent manner. Moreover, apigenin caused cell death in both cell lines leading to significant toxicity and dominantly to apoptosis. Furthermore, apigenin proved to be genotoxic towards the selected cancer cells with a potential to induce oxidative damage to lipids. Of great importance is that no significant cytogenotoxic effects were detected in normal cells. The observed cytogenotoxic and pro-cell death activities of apigenin coupled with its low toxicity towards normal cells indicate that this natural product could be used as a future anticancer modality. Therefore, further analysis to determine the exact mechanism of action and in vivo studies on animal models are warranted.

Keywords Apigenin · Breast cancer cells · Human lymphocytes · Cytogenotoxicity · Apoptosis · Anticancer effect

Introduction

Breast cancer is one of the most prevalent malignant diseases in women nowadays and its occurrence is rapidly increasing due to the stresses of modern life (Donepudi et al. 2014; Ferrini et al. 2015). A targeted therapy includes treatment with selective estrogen receptor modulators (SERMs), such as tamoxifen, in cells that express estrogen receptors (ER-positive cells) or with antibodies like trastuzumab (Herceptin) in human epidermal growth factor receptor 2 (HER2) expressing breast cancer cells (Chacón and Costanzo 2010). However, not all breast cancer cells express ER, progesterone receptor (PR), or HER2 receptor. This subclass of breast cancer cells, called triple-negative, responds poorly to therapy and is often associated with negative prognosis (Cleator et al. 2007; Hudis and Gianni 2011). Since surgical resection, radiation therapy, and chemotherapy are among the limited treatment options for breast cancer, there is a growing need to find new chemopreventive agents that may be effective in preventing and/or managing breast cancer. Several studies have shown that environmental and lifestyle factors such as diet play an important role in the genesis of cancer (Anand et al. 2008; Ferrini et al. 2015; Carruba et al. 2016). For instance, consumption of

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cruciferous vegetables reduces the risk of developing cancer and suppresses the progression of malignant tumors including breast cancer (Higdon et al. 2007; Abdull Razis and Noor 2013).

One of such natural phytochemicals is apigenin, a flavonoid, found in several dietary plant foods such as vegetables and fruits (Patel et al. 2007; Arango et al. 2013; Nabavi et al. 2015; Madunić et al. 2018). A large number of studies conducted over the past few years have shown that apigenin has potential antioxidant, anti-inflammatory, and anticancer properties (Shukla and Gupta 2010; Nabavi et al. 2015; Madunić et al. 2018). Therefore, apigenin has generated a great deal of interest as a possible therapeutic modality due to its low intrinsic toxicity and because of its striking effects on normal versus cancerous cells. Up to date, there is very little evidence suggesting that apigenin promotes adverse metabolic reactions in vivo when consumed as part of a normal diet and its possible cancer-preventive effects have increased owing to the reports of potent antioxidant and anti-inflammatory activities (Shukla and Gupta 2010). Although it has been found that apigenin possesses the ability to inhibit the cell cycle, diminish oxidative stress, improve the efficacy of detoxification enzymes, induce apoptosis, and stimulate the immune system, the results regarding these actions are still quite limited in the available literature (Naasani et al. 2003; O'Prey et al. 2003; Thiery-Vuillemin et al. 2005; Shukla and Gupta 2010).

Therefore, in the present study, we investigated a possible anticancer effect of apigenin on human breast cancer cells as well as its toxicological safety towards normal cells. The MCF-7 and MDA MB-231 cell lines were used as the in vitro tumor models. Although both lines are human breast cancer cell lines, MCF-7 is the "luminal" type, ER and PR-positive, whereas MDA MB-231 is the "basal" type and triple-negative (Cleator et al. 2007). The MCF-7 cell line is a widely studied model for hormone-dependent human breast cancer, and MDA MB-231 cells are highly invasive and metastatic cells (Anders and Carey 2009). The latter display their invasiveness by mediating the proteolytic degradation of the extracellular matrix (ECM), including the basement membrane and several mechanical barriers to ECM, through the increased expression of matrix metalloproteinases. Hence, we expected that these differences would affect sensitivity to apigenin.

The cytotoxic activity towards cancer cells was evaluated by the colorimetric MTT assay along with the evaluation of morphological changes and the type of cell death using light and fluorescent microscopy, flow cytometry, and Western blot analysis. Moreover, the effects of apigenin on membrane lipids and DNA molecule were evaluated using lipid peroxidation (LPO) and comet assays, respectively. In addition to cancer cells, the impact of apigenin towards normal human non-target cells was also evaluated to prove its toxicological safety. Human peripheral blood lymphocytes (HPBLs) were chosen as a cell model, as these cells are sensitive in vitro models for studying cytogenotoxicity. The cytogenotoxic effects of apigenin on HPBLs were evaluated by means of the cell viability and comet assays.

Materials and methods

Chemicals and cell media

Apigenin (5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange (AO), cisplatin (cDDP), Dulbecco's modified Eagle's medium (DMEM), ethidium bromide (EtBr), histopaque, low melting point (LMP) and normal melting point (NMP) agaroses, 1,1,3,3-tetramethoxy propane (TMP), thiobarbituric acid (TBA), bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), protease inhibitors (leupeptin, aprotinin, and sodium vanadate), sodium dodecyl sulfate (SDS), N,N,N′,N′-Tetrametil-etan-1,2-diamin (TEMED), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and nitro blue tetrazolium (NBT) were from Sigma (USA); FITC Annexin V Apoptosis Detection Kit I and heparinized vacutainer tubes were from Becton Dickinson (USA); fetal bovine serum (FBS) was from Gibco Life Technologies (UK); Coomassie Brilliant Blue G-250 was from Serva (Germany); ammonium persulfate (APS) and bromophenol blue were from BioRad (USA); acrylamide/bis-acrylamide was from Fisher Scientific (USA); rabbit-raised polyclonal anti-human poly(ADP-ribose) polymerase-1 (PARP-1) antibody (H-250: sc-7150) was from Santa Cruz Biotechnology (USA); secondary antibody, alkaline-phosphatase-labeled goat anti-rabbit IgG (GAR-AP) was from Jackson ImmunoResearch Laboratories (USA). All other reagents used were laboratory-grade chemicals from Kemika (Croatia).

Cell lines and treatment

Human breast cancer MCF-7 and MDA MB-231 cells (a kind gift from Dr. Sonja Levanat) were maintained as a monolayer culture in DMEM, supplemented with 10% FBS, in a humidified atmosphere at 37 °C and 5% CO_2. Cells were counted on a Z2 Coulter Counter (Beckman Coulter, USA) and seeded in Petri dishes and 96-well culture plates at an appropriate concentration. After overnight incubation, cells were treated with a range of concentrations (0 to 100 μM) of apigenin. At certain time point following the treatment, control (0.1% DMSO in DMEM) and treated cells were collected and analyzed. Each experiment was repeated at least two times.

Cytotoxicity (MTT) assay

The effect of apigenin on cell viability was determined using the MTT assay according to Mickisch et al. (1990). Briefly, 2.5×10^3 cells were seeded in 96-well plates and incubated at 37 °C for 24 h to allow attachment. The following day, the attached cells were treated with different concentrations of apigenin. Each concentration was tested in triplicate. After 72 h of treatment, DMEM was removed and an MTT solution (0.5 mg/mL) was added to each well. After 4 h of incubation at 37 °C, the formed formazan crystals were dissolved in DMSO (170 μL/well). At the end of the protocol, the plates were mechanically agitated for 10 min and the absorbance was measured at 570 nm using a microplate reader (Victor^3 1420 Multilabel Counter, Perkin Elmer, USA).

Morphological changes and the type of cell death

We used light and fluorescence microscopy to evaluate the effect of apigenin on cell morphology and to assess the type of cell death. 3×10^4 cells were first seeded in Petri dishes. After overnight incubation, they were treated with apigenin corresponding to ~ IC_{50} and 100 μM concentrations. cDDP (20 μM) was used as a positive control for apoptotic cell death (Eastman 1990). Cell morphology was analyzed under an epifluorescence microscope (Olympus BX-51, Japan) using bright-field (BF) illumination, while the type of cell death was determined by double-staining with AO/EtBr after 24 and 72 h treatment. Briefly, all cells were collected, centrifuged, and re-suspended in a small volume of medium. Ten microliters of cell suspension was mixed with 4 μL of AO (15 μg/mL in PBS) and 2 μL of EtBr (50 μg/mL in PBS) after which nuclear morphology was examined under the epifluorescence microscope. AO, a nucleic acid fluorescent cationic dye, enters both live and dead cells, intercalates in double-stranded DNA, and emits green fluorescence. At the same time, EtBr is taken up only by those cells that have lost membrane integrity so it stains dead cell nuclei orange. For this reason, live cells have green nuclei whereas apoptotic cells incorporate EtBr and exhibit condensed or fragmented orange chromatin, as EtBr signal overwhelms the AO staining. Conversely, necrotic cells stain orange, similar to dead cells, while having non-condensed chromatin resembling that of live cells (Kasibhatla et al. 2006). Images were taken with a digital camera (Olympus DP-70).

Analysis of FITC Annexin V/propidium iodide binding by flow cytometry

FITC Annexin V Apoptosis Detection Kit I was used for assessing the percentage of viable, early apoptotic, late apoptotic/necrotic, and necrotic cells. Cells were first treated with apigenin corresponding to ~ IC_{30} and ~ IC_{50} for 72 h and were afterwards washed twice in ice-cold PBS. They were then centrifuged for 5 min at 350×*g* at 4 °C. Cells were re-suspended in 1 × Annexin V Binding Buffer to a final concentration of 1×10^6 cells per 100 μL and then stained with 5 μL of FITC Annexin V and 5 μL of propidium iodide (PI). Samples were gently vortexed and incubated for 15 min at room temperature in the dark. After incubation, 400 μL of the Annexin V Binding Buffer was added to each Eppendorf tube and the samples were acquisited by flow cytometry (FACSCalibur, Becton Dickinson) using the BD CellQuest PRO software (BD Biosciences, USA). Data analyses were performed using FCS Express 3 (De Novo Software, USA).

SDS-PAGE and Western blot analysis

After 24 h of treatment with apigenin, cells were lysed in a Triton X-100 buffer (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 2 mM EDTA pH 8.0, 10% glycerol, 1% Triton X-100, 100 μg/mL PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin and 100 μg/mL sodium vanadate) and whole-cell extracts concentrations were determined by Bradford (1976). Subsequently, proteins were separated by SDS-PAGE in 10% acrylamide mini gels, and electrophoretically wet-transferred to nitrocellulose Immobilon membrane (Millipore, USA). The amount of protein per well was 60 μg. The membranes were blocked in a 5% milk protein-containing buffer, incubated at 4 °C overnight with a poly(ADP-ribose) polymerase-1 (PARP-1) antibody (1:1000), rinsed in an antibody-free blotting buffer, and incubated for 1 h in a blotting buffer containing 0.1 μg/mL of an alkaline-phosphatase-labeled goat anti-rabbit IgG (GAR-AP) antibody (1:1000). Afterwards, the membranes were rinsed and stained for the alkaline phosphatase activity using the BCIP/NBT assay.

Lipid peroxidation (LPO) assay

Peroxidation of membrane lipids was determined by measuring the level of malondialdehyde (MDA). MDA level was detected by high-performance liquid chromatography (HPLC) after reaction with TBA (Domijan et al. 2015). Briefly, MCF-7 (4×10^4/mL) and MDA MB-231 (2×10^4/mL) cells were seeded and incubated overnight. Afterwards, the cells were treated with different concentrations of apigenin for 24 h, collected and washed with PBS, and then re-suspended in ice-cold PBS to 2×10^5 cells/100 μL concentration. The supernatant was removed and cell pellets were placed at − 80 °C. Afterwards, the cell pellets were sonicated 3 × 3 s (Ultrasonic processor, Cole Parmer, USA) on ice in the presence of 5 mM butylated hydroxytoluene (BHT) in PBS to prevent sample oxidation. Then, 60 μL of the cell lysate was mixed with 400 μL of 0.1% H_3PO_4 and 100 μL of TBA and heated for 30 min at 100 °C. After heating, the samples were placed on ice to stop the reaction. MDA level was determined

on HPLC with a fluorescent detector (set at $\lambda_{ex} = 524$ nm and $\lambda_{em} = 551$ nm). The concentration of MDA in the sample was quantified by peak-area measurement using the linear regression curve obtained for MDA standard solutions prepared from the MDA standard (TMP). Results were expressed as nanomolars.

Genotoxicity (comet) assay

For the assessment of DNA damage, 2×10^5 cells/100 μL of MCF-7 and MDA MB-231 cells were prepared as described above ("Lipid peroxidation (LPO) assay" section). The alkaline comet assay was done according to Singh et al. (1988) with minor modifications (Gajski et al. 2014). After the treatment, 5 μL of cell suspension was embedded in an agarose matrix and the cells were lysed (2.5 M NaCl, 100 mM $EDTANa_2$, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide, pH 10) overnight at 4 °C. After the lysis, the slides were placed into an alkaline solution (300 mM NaOH, 1 mM $EDTANa_2$, pH 13) for 20 min at 4 °C and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in a 0.4-M Tris buffer (pH 7.5), stained with EtBr (10 μg/mL), and analyzed at × 250 magnification using an epifluorescence microscope (Zeiss, Germany) connected to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., UK). Fifty randomly captured comets from each slide were examined. The percent of tail DNA was used to measure the level of DNA damage.

Normal cell toxicity

The cytogenotoxic effect of apigenin during the 4- and 24-h periods at 37 °C was evaluated in HPBLs obtained from a young, healthy male, non-smoking donor. The subject gave informed consent to participate in this study. The study was approved by the Institutional Ethics committee and observed the ethical principles of the Declaration of Helsinki.

Cytotoxicity was determined by differential staining with AO/EtBr and by fluorescence microscopy (Duke and Cohen 1992). After the treatment, HPBLs were isolated by the histopaque density gradient centrifugation method. The slides were prepared by adding AO/EtBr to the HPBLs suspension. A total of 100 cells per repetition were examined with an epifluorescence microscope (Olympus BX-51). Quantitative assessments were made by determining the percentage of live and dead cells based on their appearance.

As for the genotoxicity assessment, after the treatment, 5 μL of whole blood was embedded in an agarose matrix and the alkaline comet assay was done according to Singh et al. (1988) with minor modifications (Gajski et al. 2014), as described above ("Genotoxicity (comet) assay" section). One hundred randomly captured comets from each slide were examined. The percent of tail DNA was used to measure the level of DNA damage.

Statistical analysis

The data were analyzed using descriptive statistics and the results are presented as means ± standard deviation (SD) or standard error (SE). Analyses were performed either using STATISTICA 13 (StatSoft, USA) or GraphPad Software (USA). The difference between the control and exposed samples were assessed either by the Student *t* test or by the Newman-Keuls test. The level of statistical significance was set at either $P < 0.05$ or $P < 0.01$.

Results

Sensitivity of MCF-7 and MDA MB-231 cells to apigenin

Human breast cancer MCF-7 and MDA MB-231 cells were exposed to various concentrations of apigenin and their viability was determined after 72 h of exposure. Apigenin inhibited the growth of both cell lines in a dose-dependent manner with MCF-7 cells being slightly more sensitive based on the IC_{50} values. The IC_{50} values were 38.03 ± 7.86 and 54.63 ± 11.05 μM for MCF-7 and MDA MB-231 cells, respectively, based on the MTT results (Fig. 1). Cell viability was additionally evaluated using XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) tetrazolium colorimetric assay, giving similar results (data not shown).

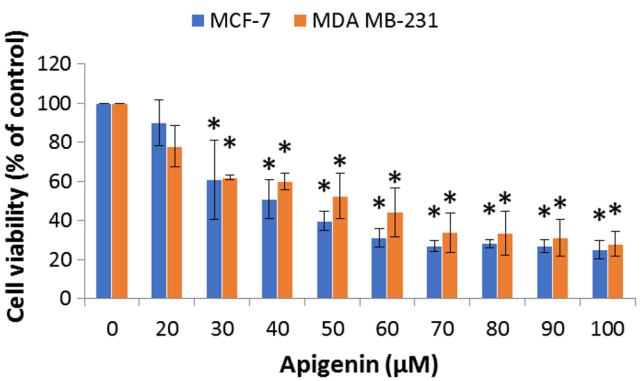

Fig. 1 Survival of human breast cancer MCF-7 and MDA MB-231 cells following treatment with apigenin. Cells were seeded in 96-well tissue culture plates and 24 h later apigenin was added. Cells were incubated for 72 h with different concentrations of apigenin (0 to 100 μM) and their viability was determined with a modified colorimetric MTT assay. The results are shown as mean values ± SD. *Statistically significant compared with the corresponding control ($P < 0.05$)

Apigenin induced morphological changes and apoptosis in MCF-7 and MDA MB-231 cells

The apigenin effects on cell morphology were analyzed using light microscopy. Cells were treated for 24 and 72 h with an apigenin concentration corresponding to ~ IC_{50}, as well as with 100 μM apigenin and 20 μM cDDP. The results showed that apigenin induced a dose-dependent morphology change with more pronounced effects in MDA MB-231 cells (Fig. 2a, b). After 24 h, both cell lines, particularly MDA MB-231, displayed elongated morphology with neuron-like projections which transformed to shrunken and granulated cells at higher dosages (Fig. 2a), features that became even more prominent after 72 h of treatment (Fig. 2b).

In order to define a type of cell death occurring in the apigenin-treated cells, we employed AO/EtBr double staining which revealed apoptosis as a prevailing type of cell death in both cell lines. The detection of cells dying by an apoptotic type of cell death is based on the observation that, unlike necrotic cells, apoptotic cells exhibit condensed or fragmented chromatin. The treated cells indeed displayed characteristic apoptotic features: fragmented and pyknotic nuclei with condensed orange-stained chromatin. These features were observed in the cells treated for 24 h with apigenin (Fig. 2c), becoming even more prominent after 72 h of treatment (Fig. 2d).

However, to quantify the percentage of apoptotic cells in the treated samples and to additionally explore the dose-dependent effect of apigenin on apoptosis, we preformed FITC Annexin V/PI staining. Following an incubation period of 72 h, the cells were stained and analyzed by flow cytometry. Percentages of viable, early apoptotic, late apoptotic/necrotic, and necrotic cells were illustrated as a histogram for MCF-7 (Fig. 3a) and MDA MB-231 cells, respectively (Fig. 3b). Both cell lines exposed to increasing concentrations of apigenin showed a dose-dependent decrease in cell viability and amplification of the apoptotic death mode. As a positive control, we used cDDP (20 μM). Since FITC Annexin V/PI staining discriminates between early and late apoptotic populations, we compared apigenin efficiency in the promotion of early/late apoptosis among the two breast cancer lines. The results showed that all applied concentrations of apigenin promoted the induction of early apoptosis in MDA MB-231 cells (40, 60, and 100 μM), while only the lowest concentration of apigenin (20 μM) was not sufficient to trigger statistically significant early apoptotic death of MCF-7 cells (Fig. 3c). Conversely, all concentrations of apigenin induced late apoptotic death in MCF-7 cells (20, 40, and 100 μM), while only higher concentrations (60 and 100 μM) triggered the same death mode in MDA MB-231 cells (Fig. 3d). It was noticed that apigenin was more efficient in killing MCF-7 cells by triggering late apoptosis/necrosis.

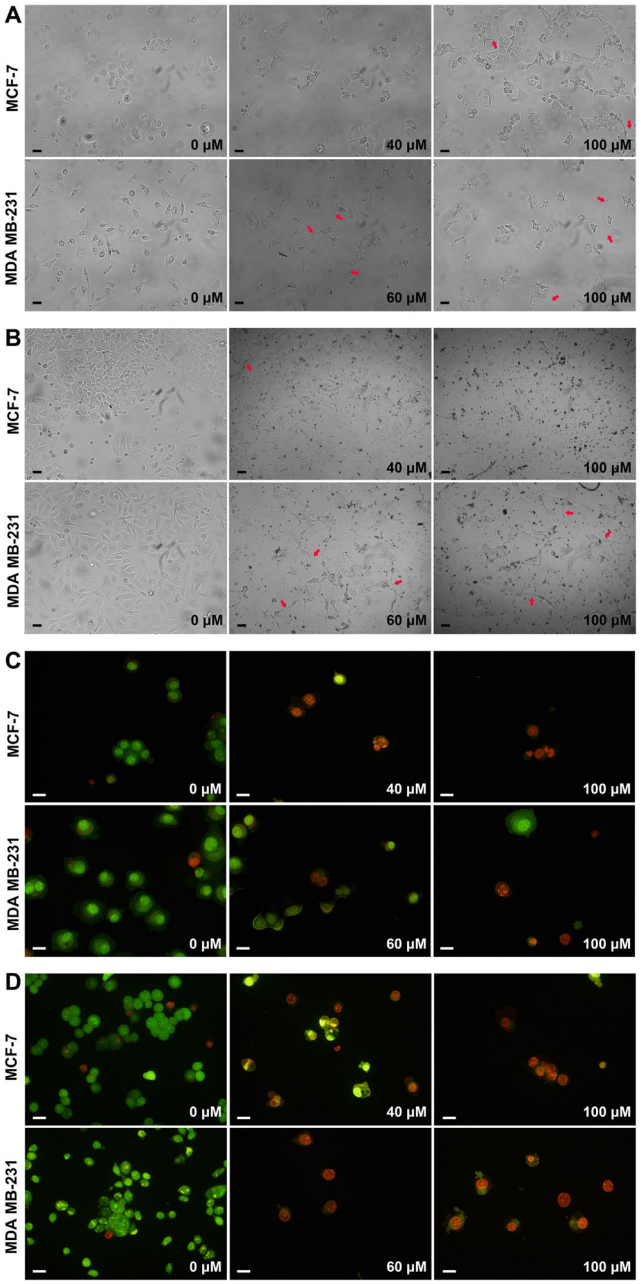

Fig. 2 Morphological changes and apoptosis induction in human breast cancer MCF-7 and MDA MB-231 cells after apigenin treatment. Morphology of MCF-7 and MDA MB-231 cells after 24 (**a**) and 72 (**b**) h treatment with indicated concentrations of apigenin was evaluated using light microscopy. Pictures were taken at magnification × 100, scale bar = 20 μm. Arrows are showing dendrite-like projections. Apoptotic nuclear morphology of MCF-7 and MDA MB-231 cells stained with AO/EtBr after 24 (**c**) and 72 (**d**) h treatment with indicated concentrations of apigenin evaluated using fluorescence microscopy. Pictures were taken at magnification × 200, scale bar = 40 μm (**b**)

Furthermore, we performed Western blot analysis to confirm apoptosis as a dominant type of cell death at a protein level in MCF-7 and MDA MB-231 cells after the 24-h apigenin treatment. PARP-1 is a polymerase whose cleavage is known to indicate apoptotic cell death (Gobeil et al. 2001).

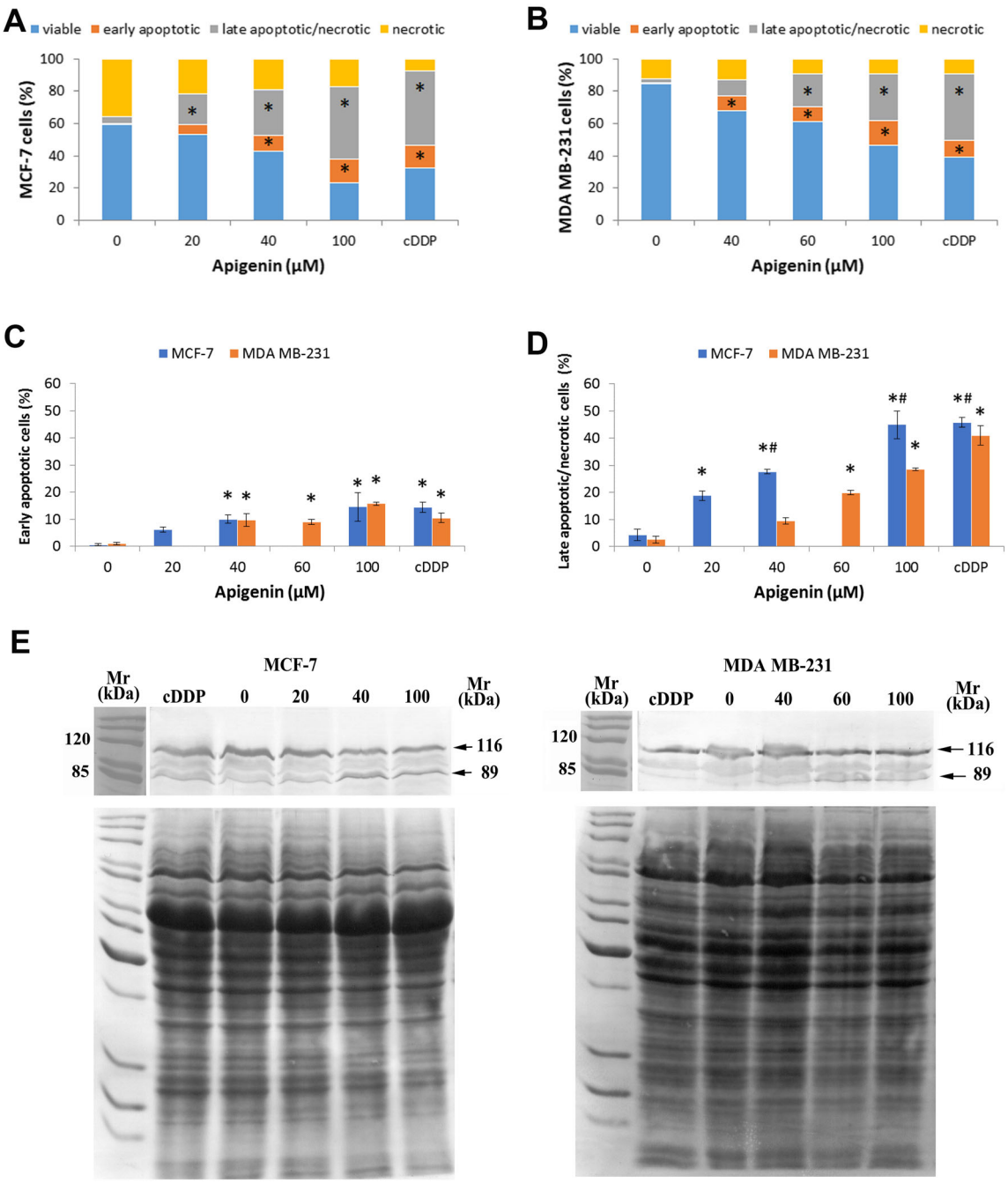

Fig. 3 Apoptosis induction in human breast cancer MCF-7 and MDA MB-231 cells after apigenin treatment. Using the appearance of phosphatidylserine on the extracellular side of membrane, evaluated with FITC Annexin V Apoptosis Detection Kit I using the flow cytometry method, the specific cell death type was analyzed after 72 h of treatment with indicated concentrations of apigenin. Percentages of viable, early apoptotic, late apoptotic/necrotic, and necrotic MCF-7 (**a**) and MDA-MB-231 (**b**) cells were illustrated as histogram. Moreover, percentages of early apoptotic (**c**) and late apoptotic/necrotic cells (**d**) were illustrated as a histogram. Cisplatin (cDDP) was used as a positive control (20 μM). Results are shown as mean values ± SD. *Statistically significant compared with the corresponding control ($P < 0.05$). #Statistically significant between the two cell lines ($P < 0.01$). By Western blot analysis (**e**), full length (116 kDa) form and C-terminal cleavage product (89 kDa) of PARP-1 were detected after the 24 h apigenin-treatment of MCF-7 and MDA MB-231 cells at various concentrations (0 to 100 μM) indicating apoptosis. Cells were also treated for 24 h with 20 μM cDDP as a positive control of apoptosis. Membranes stained with Coomassie Brilliant Blue were used as a loading control

In both cell lines, we detected a full length of PARP-1 (116 kDa) and its C-terminal cleavage product (89 kDa) indicating apigenin-mediated induction of apoptosis, which is consistent with previously described morphological changes and flow cytometry results (Figs. 2 and 3). As a positive control, the cells treated with a known inducer of apoptosis, cDDP

(20 μM), after 24 h revealed the same pattern of protein bands (89 and 116 kDa) (Fig. 3e).

Apigenin induced lipid peroxidation and DNA damage in MCF-7 and MDA MB-231 cells

To evaluate whether oxidative stress has a role in apigenin toxicity, the MDA level, as a marker of LPO, was determined in MCF-7 and MDA MB-231 cells. Results showed that apigenin induced LPO in both cell lines in a dose-dependent manner, which was more pronounced in MDA MB-231 compared to MCF-7 cells. A significant lipid-damaging effect was observed at 80 μM and above for MDA MB-231 cells whereas only the highest concentration was significant for MCF-7 cells (Fig. 4a).

The DNA damage in MCF-7 and MDA MB-231 cells after apigenin treatment was determined with the alkaline comet assay. Results showed that apigenin induced DNA damage in both cell lines in a dose-dependent manner, with MDA MB-231 cells being more sensitive to the genotoxic effect of apigenin compared to MCF-7 cells. A significant genotoxic effect was observed at 60 μM and above for MCF-7 cells whereas all apigenin concentrations induced significant DNA damage in MDA MB-231 cells (Fig. 4b, c).

Cytogenotoxic effects of apigenin on normal cells

Whole blood samples were exposed to apigenin and the cytogenotoxic effect was determined for HPBLs. Apigenin at the concentrations tested had no effect on HPBLs viability. After 4 h of treatment, the viability of cells was greater than 98.69 ± 2.16% and after 24 h viability was greater than 97.10 ± 3.30%, which was not significantly different from the corresponding controls (Fig. 5a). This indicate that apigenin up to 100 μM is not cytotoxic to HPBLs.

DNA damage in HPBLs after apigenin treatment was determined with the alkaline comet assay. There was no statistically significant difference in the amount of DNA strand breaks compared to the corresponding control samples for apigenin regardless of the concentration used and exposure time (Fig. 5b).

Discussion

In order to increase the effectiveness of cancer treatment, the interest has been recently focused on those drugs that have been used in traditional medicine (Efferth et al. 2007; Efferth et al. 2008; Singh et al. 2016). Flavonoids from food provide an essential link between diet and prevention of chronic

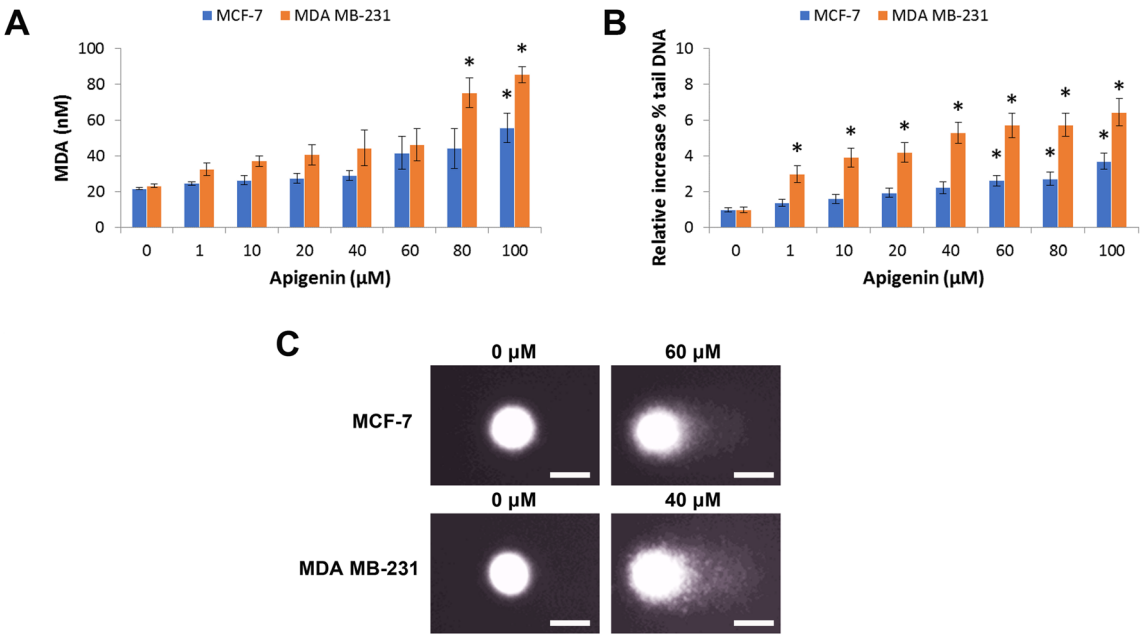

Fig. 4 Induction of lipid and DNA damage in human breast cancer MCF-7 and MDA MB-231 cells following the treatment with different concentrations of apigenin for 24 h. After the treatment with apigenin (0 to 100 μM), cell lysates were analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection and the concentration of malondialdehyde (MDA), as a lipid peroxidation (LPO) product, was determined. The results are shown as mean values ± SD (**a**). For detection of DNA damage, the alkaline comet assay was used and the results were expressed as a relative increase in % DNA in comet tail. The results are shown as mean values ± SE (**b**). The alkaline comet assay images represent undamaged nuclei from the unexposed population and damaged nuclei that have a comet appearance from the cell populations exposed to apigenin. Cells were stained with EtBr and the images were captured under epifluorescence microscope at × 250 magnification, scale bar = 20 μm (**c**). *Statistically significant compared with the corresponding control ($P < 0.05$)

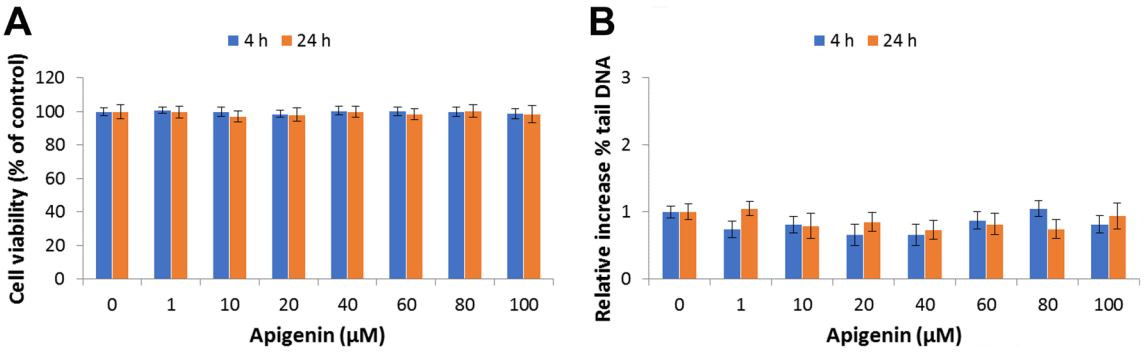

Fig. 5 The effects on the viability and DNA damage in human peripheral blood lymphocytes (HPBLs) after exposure to apigenin for 4 and 24 h. Cell viability was determined by differential staining with AO/EtBr. The results are shown as mean values ± SD (**a**). DNA damage was assessed with the alkaline comet assay and is expressed as a relative increase in % DNA in comet tail. The results are shown as mean values ± SE (**b**). There were no statistically significant differences between the treated samples compared to the corresponding control ($P < 0.05$)

diseases including cancer. Anticancer effects of these polyphenols depend on several factors such as their chemical structure and concentration but are also cell-specific and dependent on the type of cancer (Sharma et al. 2011; Romagnolo and Selmin 2012; Ravishankar et al. 2013; Sak 2014). Malignant cells from different tissues reveal somewhat different sensitivity towards flavonoids. This was the reason our study focused on the potential anticancer properties of apigenin in two different human breast cancer cell lines, ER/PR-positive and HER2-negative MCF-7 and triple-negative MDA MB-231 cells. Furthermore, toxicological safety towards HPBLs was also evaluated to determine its effects on normal non-target cells.

Firstly, cells were treated with increasing concentrations of apigenin and viability was assessed after 72 h of treatment. Results showed that apigenin reduced viability in both cell lines in a dose-dependent manner with MCF-7 cells being slightly more sensitive, based on the IC_{50} values (Fig. 1). This is in agreement with recent studies where apigenin exhibited cytotoxic activity in breast cancer cells (Cao et al. 2013; Hyuga et al. 2013; Bai et al. 2014; Lin et al. 2015; Scherbakov and Andreeva 2015; Seo et al. 2015a, b; Tseng et al. 2017), as well as in various other types of cancer cells (Seo et al. 2011; Budhraja et al. 2012; Harrison et al. 2014; Liao et al. 2014; Sak 2014; Sung et al. 2016; Zhou et al. 2016; Chen et al. 2016).

Furthermore, our results revealed that apigenin induced profound dose-dependent morphological changes that were more pronounced in MDA MB-231 cells. These changes included elongated morphology with neuron-like projections, which transformed to shrunken and granulated cells at higher dosages of treatment (Fig. 2). This effect on the morphology of MDA MB-231 cells was reported by other authors as well (Agrawal et al. 2006). Besides, Lu et al. (2010) observed similar morphological changes upon treatment of lung cancer cells with apigenin. Bai et al. (2014) reported changed morphology, reduced motility, and decreased intracellular communication in the apigenin-treated MCF-7 cells, which was the consequence of a disturbed structure and decreased amount of intracellular α-tubulin, an effect which could be closely related to the induction of apoptosis. Similarly, Lindenmeyer et al. (2001) attributed morphology changes in the apigenin-treated MDA MB-231 cells to the apigenin effect on microtubular dynamics and inhibition of MAPK activity. Moreover, observed morphological changes could be a consequence of apigenin-mediated suppression of the glutamate signaling pathway which might lead to the inhibition of motility, invasion, and proliferation as evidenced before in human melanoma cells (Zhao et al. 2017). Choudhury et al. (2013), on the other hand, reported that apigenin caused apoptosis in lung cancer cells by inhibiting tubulin polymerization into microtubules which was accompanied with evident morphology changes in apigenin-treated cells.

The morphological changes observed under light microscope and those observed using fluorescent double staining revealed apoptosis as a prevailing type of cell death in both cell lines. After the treatment with apigenin, we observed fragmented and pyknotic nuclei with condensed chromatin which became more prominent after a longer incubation period, the features typical of apoptosis (Kasibhatla et al. 2006). Cell death by apoptosis was also confirmed using Western blot analysis where cleaved PARP-1 in both cell lines was detected in addition to high population of apoptotic cells that were monitored by flow cytometry (Fig. 3).

Apoptosis is an evolutionary conserved and essential process which regulates development and homeostasis in organisms through the elimination of damaged, undesirable, or potentially harmful cells (Sun et al. 2004). Deregulation or mutation in the genes regulating apoptosis pathways (e.g., p53, Bcl-2, PTEN) leads to the development of numerous pathological conditions such as cancer (Sung et al. 2016). Therefore, apoptosis induction in cancer cells represents a highly specific and efficient approach in chemotherapy and chemoprevention (Sak 2014). Our results, which determine apoptosis as a dominant type of cell death in the apigenin-treated breast cancer cells, are consistent with those previously

reported by other studies. In these studies, authors observed that apigenin treatment in various breast cancer cells (including MCF-7 and MDA MB-231) led to cell cycle arrest accompanied by upregulation of p21 and p27 and subsequent downregulation of cyclin and CDK-1 expression (Choi and Kim 2009b; Harrison et al. 2014; Lin et al. 2015; Sung et al. 2016; Tseng et al. 2017). Similar effects were described in other types of cancer, including prostate, melanoma, lung, colon, hepatic, and pancreatic cancer cells (Caltagirone et al. 2000; Seo et al. 2011; Harrison et al. 2014; Sak 2014; Zhou et al. 2016). The cell survival pathway involving kinases PI3K and Akt/PKB is known to play a fundamental role in inhibiting apoptosis (Shukla and Gupta 2010). This could be the reason why apigenin-induced apoptosis was related to the suppression of Akt activation in breast cancer cells (Lee et al. 2008; Choi and Kim 2009a; Cao et al. 2013; Harrison et al. 2014; Lin et al. 2015), as well as in other cancer cell lines (Budhraja et al. 2012; Harrison et al. 2014). Cleavage of PARP-1, as one of the hallmarks of apoptosis in our cells, was also detected in the apigenin-treated BT-474, SKBR3 (Seo et al. 2015a, b) as well as MCF-7 and MDA MB-231 breast cancer cells (Cao et al. 2013; Lin et al. 2015). Furthermore, most of the studies using breast cancer cells report that apigenin-mediated apoptosis was related to the expression of p53, followed by the activation of caspases, increased levels of Bax and decreased levels of Bcl-2, release of cytochrome c from mitochondria, and overproduction of reactive oxygen species (ROS) (Agrawal et al. 2006; Choi and Kim 2009b; Chen et al. 2011; Cao et al. 2013; Bai et al. 2014; Lin et al. 2015; Sung et al. 2016). Other studies have shown similar p53-dependent apoptosis in the apigenin-treated lung cancer cells (Lu et al. 2010; Chen et al. 2016), macrophages (Liao et al. 2014), and leukemia cells (Budhraja et al. 2012).

There are numerous reports about the inclusion of oxidative stress as one of the first steps in apigenin cytotoxicity to different cancer cell lines (Morrissey et al. 2005; Shukla and Gupta 2008; Shukla and Gupta 2010; Liao et al. 2014). A recent study suggested that flavone-induced overproduction of ROS contributed to their selective killing of cancer cells (Khan et al. 2012). The cellular redox state is dependent on the net balance between the levels of ROS and endogenous thiol antioxidants, which shield the cells from oxidative damage. When ROS production surpasses the buffering capacity of antioxidants, oxidative stress accumulates, and the formed H_2O_2 (the most stable ROS) damages lipids, proteins, and DNA (Halliwell and Gutteridge 1989; Simon et al. 2000). Since there is information in the literature regarding ROS formation following the treatment with apigenin, we also measured the induction of LPO after apigenin treatment. Results showed that apigenin induced LPO in both cell lines in a dose-dependent manner, which was more pronounced in MDA MB-231 compared to MCF-7 cells (Fig. 4). These findings are in accordance with previous studies on breast cancer cells. A study by Chen et al. (2011) showed that apigenin strongly induced ROS production and reduced glutathione levels in MDA MB-231 cells. Similarly, Bai et al. (2014) observed overproduction of ROS, which lead to apoptosis in the apigenin-treated MCF-7 cells. Furthermore, low-dose apigenin was able to induce ROS production in MDA MB-468 breast cancer cells (Harrison et al. 2014). Similarly, other studies reported apigenin treatment causing loss of the mitochondrial transmembrane potential and elevation of ROS and Ca^{2+} production in leukemia and prostate cancer cells (Shukla and Gupta 2010), as well as in lung cancer cells (Lu et al. 2010; Lu et al. 2011).

Moreover, after apigenin treatment, we also observed a significant DNA-damaging effect which agrees with those previously reported (Lu et al. 2010; Arango et al. 2012). Our results showed that apigenin induced DNA damage in both cell lines in a dose-dependent manner with MDA MB-231 cells being more sensitive to the genotoxic effect of apigenin compared to MCF-7 cells. A significant genotoxic effect was observed at 60 μM and above for MCF-7 cells, whereas all apigenin concentrations induced significant DNA damage in MDA MB-231 cells (Fig. 4). This coincides with the LPO results and MDA MB-231 cells being more prone to apigenin-induced oxidative stress, since MDA, a product of LPO, is able to form mutagenic adducts and consequently damage DNA (Marnett 1999).

The observed differences in apigenin cytotoxicity in our cells may be due to biological and genetic variations between them. Based on their gene expression patterns, breast cancer cell lines are categorized in several subtypes. Therefore, MCF-7 cells belong to the basal subtype, a type of breast cancer cells that express ER/PR receptors and display a differentiated, non-invasive phenotype (Neve et al. 2006). This is consistent with the fact that patients with ER/PR-positive and HER2-negative breast cancer (primary tumors which share etymology with MCF-7 cells) have good prognosis with a high survival and low recurrence rates (Perou et al. 2000; Metzger-Filho et al. 2013; Inic et al. 2014). Conversely, MDA MB-231 cells are classified as triple-negative (ER/PR and HER2-negative) breast cancer (TNBC) cells, derived from a highly invasive and metastatic type of breast cancer associated with poor prognosis due to its non-responsiveness to hormonal therapy (Neve et al. 2006; Cleator et al. 2007; Anders and Carey 2009; Chacón and Costanzo 2010; Hudis and Gianni 2011; Abdal Dayem et al. 2016).

Considering the different DNA-damaging effect of apigenin in our cells, it is important to note that more than 80% of basal-like breast cancers, including MDA MB-231 cells (Neve et al. 2006), contain mutations in p53 leading to its high expression and impaired function. p53 is an important tumor suppressor, which acts as a checkpoint in the cell cycle and which protects the cell from DNA damage by facilitating

cell cycle arrest, damage repair, and/or apoptosis. Interestingly, King et al. (2012) recently reported on the ability of apigenin to promote cell cycle arrest and induction of apoptosis through p53-related pathways, even in cancer cells containing p53 mutations. Conversely, Sotiriou et al. (2003) found that only 13% of luminal tumors contained mutations in the *p53* gene. This might be a reason why TNBC cells have a high degree of genetic instability as numerous studies report (Cleator et al. 2007; Anders and Carey 2009; Hudis and Gianni 2011). Furthermore, these cells contain a great number of single nucleotide polymorphisms (SNPs), which is a trigger for genetic aberrations and could lead to a loss of DNA-repair and tumor suppressor genes (Wang et al. 2004). This all agrees with our finding that MDA MB-231 cells were more sensitive to apigenin. It is vital to mention that, even though TNBC cells share some molecular features with BRCA1 mutation-related breast cancers, MDA MB-231 cells (and MCF-7 for that matter) were not BRCA1-deficient (Inbar-Rozensal et al. 2009). Finally, these differences in cytotoxicity could be simply the result of apigenin acting differently on intracellular signaling in MCF-7 and MDA MB-231 cells, as was reported previously for other flavones (Jia et al. 2014).

It was already established that many natural products including apigenin have beneficial effects on estrogen-driven breast cancer, and it was reported that the apigenin's chemical structure is similar to estrogen (Bak et al. 2016). The ability to mimic estrogen could be one of the reasons why apigenin displayed a cytotoxic effect in ER-positive MCF-7 cells. Our results on MCF-7 cells are similar to the study of Sak (2014), who observed the apigenin effect on breast cancer cells' viability being dependent on the expression of ER. However, as we also observed that apigenin (IC_{50}) significantly reduced cell viability in MDA MB-231 cells, we cannot exclude the possibility that apigenin could also be a useful anticancer agent and/or adjunct supplement in the treatment of ER-negative tumors. Our findings are consistent with the observations of other authors who have demonstrated that apigenin has strong anticancer activities both in ER-positive and ER-negative breast cancer cells (Long et al. 2008; Mafuvadze et al. 2013; Scherbakov and Andreeva 2015; Seo et al. 2015a).

The beneficial health effects of dietary phytochemicals make them promising candidates for treatment and prevention of a large number of diseases. Nevertheless, cellular targets for dietary components remain largely unknown. Arango et al. (2013), by combining phage display with high-throughput sequencing, identified 160 human targets of apigenin and most of them fall into one of the three categories: GTPase activation, membrane transport, and mRNA metabolism/alternative splicing providing a perspective on how dietary phytochemicals function and what distinguishes their action from pharmaceutical drugs.

Although there are numerous chemicals that often show good results towards cancerous cells, there are always open questions regarding their potential toxicity on normal non-target cells and tissues making this kind of toxicity one of the greatest obstacles for the possibility towards actual remedy. Therefore, during the course of our study, we evaluated toxicological safety of apigenin towards normal human cells using widely accepted biomarkers for the evaluation of genome damage after exposure to different physical and/or chemical agents as well as to a wide range of natural products (Garaj-Vrhovac and Gajski 2009). HPBLs were chosen as a cell model, as these cells are considered to be sensitive in vitro models for studying cytogenotoxicity. Based on the observed results, apigenin at the concentrations tested had no effect on HPBLs viability which proves that apigenin up to 100 μM in the given time frame is not cytotoxic towards HPBLs. Moreover, the DNA-damaging effect was also determined with the alkaline comet assay indicating no significant difference in the amount of DNA strand breaks regardless of the concentration and exposure time used (Fig. 5). The lack of or lower cytogenotoxicity and even protective effect against oxidative DNA damage in normal human breast (MCF-10A) cells (Bai et al. 2014; Lin et al. 2015; Zhu et al. 2016; Wilsher et al. 2017), prostate cells (Gupta et al. 2001), and blood cells (Siddique et al. 2010; Rusak et al. 2010; Begum et al. 2012; Sharma 2013) was already reported for apigenin, although some studies also revealed a potential DNA damaging and pro-oxidative capacity of apigenin (Matsuo et al. 2005; Noel et al. 2006; Rusak et al. 2010). Studies showing no or little cytotoxic activity on normal human cells indicate that apigenin is less toxic to normal cells comparing with

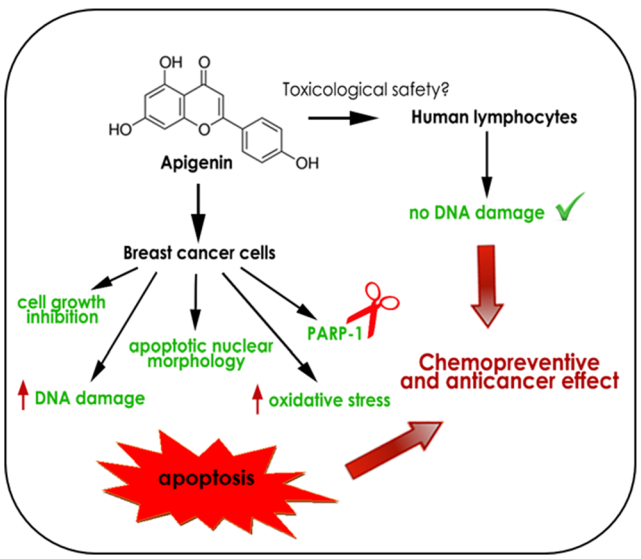

Fig. 6 Schematic diagram illustrating the mechanism of action of apigenin on breast cancer cells. Apigenin caused morphological changes in breast cancer cells followed by apoptosis induction evidenced by PARP cleavage. Moreover, apigenin induced oxidative stress and DNA damaging effect that contributes to its overall cytotoxic potential. At the same time, there were no observed cytogenotoxic effects in apigenin-treated human blood lymphocytes

cancer cells, implying its selective killing effects on tumor cells. Besides, it was suggested that the balance between the protection of DNA from oxidative damage and pro-oxidative effects is strongly dependent on the flavonoid concentration and the incubation period. Based on our results (Fig. 6) and those available from literature, it is to be presumed that apigenin could be safe for normal cells from the aspect of cytogenotoxicity.

Conclusions

The obtained results provide relevant new data on the responses of ER-positive MCF-7 and triple-negative MDA MB-231 breast cancer cells to apigenin treatment in vitro. Based on the results, it could be concluded that apigenin induces changes in cell morphology and lowers their viability leading to apoptotic cell death. Moreover, apigenin caused a significant lipid and DNA damaging effect that contributes to its overall cytotoxic potential. The observed low intrinsic toxicity and its prominent effects on normal versus cancerous cells, indicate that this natural and health-promoting flavonoid could be used as a potent chemopreventive and possible anticancer modality. Undoubtedly, therapeutic applications of apigenin are promising; however, further in vitro and in vivo experiments are warranted to resolve precise mechanisms responsible for its anticancer effects.

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Compliance with ethical standards

The study was approved by the Institutional Ethics committee and observed the ethical principles of the Declaration of Helsinki.

Conflict of interests The authors declare that they have no conflicts of interest.

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