ORIGINAL ARTICLE

Investigation of the role of quercetin as a heat shock protein inhibitor on apoptosis in human breast cancer cells

Ezgi Kıyga[1] · Aslıhan Şengelen[1] · Zelal Adıgüzel[2] · Evren Önay Uçar[3]

Abstract
High expression of heat shock proteins (Hsp) in breast cancer has been closely associated with tumor cell proliferation and thus a poor clinical outcome. Quercetin, a good Hsp inhibitor as a dietary flavonoid, possesses anticarcinogenic properties. Although there are many studies on the effects of quercetin on Hsp levels in human breast cancer cells, research on elucidation of its molecular mechanism continues. Herein, we aimed to investigate the effect of quercetin on Hsp levels and whether quercetin is a suitable therapeutic for two breast cancer cell lines (MCF-7 and MDA-MB-231) representing breast tumors which differed in hormone receptor, aggressiveness and treatment responses. To examine the response to high and low doses of quercetin, the cells were treated with three doses of quercetin (10, 25 and 100 μM) determined by MTT. The effects of quercetin on Hsp levels, apoptosis and DNA damage were examined by western blot analysis, caspase activity assay, comet assay and microscopy in human breast cancer cells. Compared to MDA-MB231 cells, MCF-7 cells were more affected by quercetin treatments. Quercetin effectively suppressed the expression of Hsp27, Hsp70 and Hsp90. While quercetin did not induce DNA damage, it triggered apoptosis at high levels. Although an increase in NF-κB levels is observed in the cells exposed to quercetin, the net result is the anticancer effect in case of Hsp depletion and apoptosis induction. Taken together our findings suggested that quercetin can be an effective therapeutic agent for breast cancer therapy regardless of the presence or absence of hormone receptors.

Keywords Quercetin · Breast cancer · Heat shock protein (hsp) · Apoptosis · DNA damage

Abbreviations

EMS	Ethyl methanesulfonate
ER	Estrogen receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HER2	Human epidermal growth factor receptor 2
HSF	Heat shock factor
Hsp	Heat shock protein
IC$_{50}$	The half-maximal inhibitory concentration
NF-κB	Nuclear factor kappa-B
PARP	Poly (ADP-ribose) polymerase
PR	Progesterone receptor

✉ Evren Önay Uçar
evrenonay@istanbul.edu.tr

1 Department of Molecular Biology and Genetics, Institute of Graduate Studies in Sciences, Istanbul University, Istanbul, Turkey

2 Basic Medical Sciences Department of Molecular Biology and Genetics, School of Medicine, Koç University, Istanbul, Turkey

3 Department of Molecular Biology and Genetics, Faculty of Science, Istanbul University, Vezneciler, 34134 Istanbul, Turkey

Introduction

Breast cancer is the most commonly diagnosed cancer and one of the major causes of death in women worldwide. It accounts for 11.6% of all cancer and has the highest incidence in the world after lung cancer [1]. Despite the fact that there are several chemotherapeutic agents available for breast cancer treatment, they usually are associated with a number of side-effects, and the cure rates are not satisfactory. In addition to destroying fast-dividing cancer cells, chemotherapy also damages normal fast-dividing cells such as bone marrow and hair follicles. Hormone therapy, which is used as adjuvant therapy, is an important approach to treat breast cancer patients [2]. However, it is effective in only one-third of breast cancer patients and has been reported to cause DNA damage to the endometrium [3, 4]. A natural

drug could overcome the side effects in restricting the clinical applications for cancer patients.

Evidence suggests that flavonoids, a group of natural compounds, have anticarcinogenic properties and potential for cancer prevention and therapy. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is an important member of the flavonoid family and found in many vegetables and fruits. It has been shown to possess potentially beneficial biological activities on human health [5]. The anticancer effect of quercetin has been extensively studied and its protective and preventive effects against cancers such as brain, breast, cervix, colorectum, lung and prostate have been reported in many in vivo and in vitro studies [6]. Particularly, it is widely known for its pro-apoptotic effect in various tumor cells including breast cancer cells [7–10]. Quercetin is also a very good inhibitor for heat shock proteins (Hsp) that closely associated with tumor cell proliferation and apoptosis inhibition [5, 10]. They constitute a large family of proteins with conserved structures and are essential proteins that play a key role in cell survival via the protective mechanisms. Moreover, especially high levels of Hsp27, Hsp70 and Hsp90 have been observed in breast carcinoma compared to normal cells, and their overexpression has been correlated with a poor clinical outcome [11]. Therefore, Hsps have been presented as possible therapeutic targets in the treatment of breast cancer. Although quercetin is a good Hsp inhibitor, its effect on Hsp expression in breast cancer has not been studied sufficiently. More research is needed to clarify the effect of quercetin on the expression of Hsps in breast cancer.

The present study aimed to examine the effects of quercetin on Hsp levels and apoptosis induction in human breast cancer cells. The two cell lines MCF-7 [(positive for estrogen receptor (ER$^+$) and progesterone receptor (PR$^+$) and negative for human epidermal growth factor receptor 2 (HER2$^-$)] and MDA-MB-231 (triple negative) were chosen because of their differences in terms of hormone receptors, aggressiveness and treatment responses. Another important reason for cell lines selection was the absence of a study showing the effect of quercetin on Hsp27 levels in MCF-7 cells and on Hsp90 levels in MDA-MB-231 cells. We

investigated the effects of quercetin on cell viability, expression levels of Hsps (Hsp27, Hsp70 and Hsp90), apoptosis induction and DNA damage. Our findings demonstrated that quercetin treatment caused a decreased Hsp expression and induced cell death in both cell lines. These findings emphasize the potential use of quercetin as an effective therapeutic in the treatment of breast cancer regardless of the presence or absence of hormone receptors.

Materials and methods

Experimental reagents and antibodies

Quercetin was from Santa Cruz Biotechnology (sc-206089B) and dissolved in dimethyl sulfoxide (DMSO). Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture (DMEM/F12) was from Sigma. Fetal Bovine Serum and other standard tissue culture reagents were obtained from Gibco. EDTA-free protease inhibitör cocktail was from Roche. SMART™ BCA Protein Assay Kit was from iNtRON Biotechnology. PVDF membrane and SuperSignal West Pico PLUS Chemiluminescent Substrate were purchased from Thermo Fisher Scientific. RayBio® Caspase-3 Colorimetric Activity Assay Kit was purchased from Ray Biotech. Ethyl methanesulfonate (EMS) used as a positive genotoxic control in the comet assay was from Merck-Millipore. All the other chemicals were obtained from Sigma. The antibodies used in the study are given in Table 1.

Cells and culture conditions

The human breast cancer cells MCF-7 and MDA-MB-231 were obtained from Istanbul University Cell Culture Collections. The cells were grown in a 1:1 mixture of DMEM/F12 supplemented with 10% FBS, 1% antibiotic-antimycotics solution (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B) in 5% CO_2 at 37 °C.

Table 1 Antibodies used for Western blotting

Antibody	Host	Dilution	Catalog number	Company
Anti-Hsp27	Mouse	1:1000	ADI-SPA-800	Enzo Life Sci
Anti-Hsp70	Mouse	1:1000	ADI-SPA-810	Enzo Life Sci
Anti-Hsp90	Mouse	1:400	sc-69703	SantaCruz
Anti-NF-κB	Rabbit	1:1000	PA5-17654	Invitrogen
Anti-caspase-3 (pro and cleaved)	Mouse	1:1000	NB100-56708	Novus
Anti-PARP-1 (cleaved)	Rabbit	1:1000	M00122-1	Boster
Anti-GAPDH	Mouse	1:2500	MA5-15738	Invitrogen
Anti-mouse IgG	Goat	1:2500	31,430	Invitrogen
Anti-rabbit IgG	Goat	1:2500	31460	Invitrogen

Cell viability assay

The effect of quercetin on cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) as previously described [12]. MCF-7 and MDA-MB-231 cells were seeded in 96-well plates (1×10^4 and 1.5×10^4 per well, respectively) 24 h prior to treatment. The cells were treated with increasing concentrations of quercetin (1, 5, 10, 25, 50, 100 and 200 μM) for 24, 48 and 72 h. Then, 30 μL of MTT (5 mg/mL in D-PBS) was added, incubated for 4 h, and 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance of the samples was measured at 540 nm using a microplate reader (EON, BioTek Instruments Inc.). All experiments were performed in triplicate.

Quercetin treatments

MCF-7 and MDA-MB-231 cells were plated in 6-well plates (3×10^5 and 4.5×10^5 per well, respectively) and incubated overnight. According to cell viability results, both the cell lines were treated with fixed concentrations (10, 25 and 100 μM) of quercetin for 48 h. The final concentration of DMSO in the culture medium did not exceed 0.1% (v/v).

Morphological cell changes

In order to examine the effect of quercetin on the cell morphology, MCF-7 and MDA-MB-231 cells were plated in 12-well plates (1.3×10^5 and 1.9×10^5 per well, respectively) and exposed to quercetin (10, 25 and 100 μM) or DMSO (0.1%) for 48 h. After then, the cells were observed and photographed under an inverted phase-contrast microscope (Nikon Eclipse Ti-E).

Western blot analysis

The protein levels of Hsp27 (HspB1), Hsp70 (HspA1), Hsp90 (HspC1), NF-κB (nuclear factor-kappa B), pro- and cleaved forms of caspase-3, cleaved product of PARP-1 (poly(ADP-ribose) polymerase 1) in the cells were analyzed by Western blotting using standard procedure as previously described [13]. Protein concentration was determined using BCA kit, equal amounts of protein (30 μg/well) were separated by SDS-PAGE and transferred to PVDF membranes using a wet tank transfer system (Bio-Rad). Primary antibodies (overnight at 4 °C) and secondary antibodies (2 h at RT) indicated in Table 1 were used. The enhanced chemiluminescent (ECL) detection systems were used to protein detection, and quantification was performed using ImageLab 5.2.1 software (Bio-Rad).

Caspase activity assay

After treatment, a colorimetric assay kit was utilised to detect DEVDase (caspase-3/7) activity according to the manufacturer's protocol. Briefly, cells were resuspended in cell lysis buffer, incubated on ice for 10 min, and centrifuged at $10,000 \times g$ for 1 min at 4 °C. The supernatant was placed in the microplate containing reaction buffer. DEVD-pNA substrate (200 μM final concentration) was added to each well and incubated at 37 °C for 2 h. The caspase-3 activity was measured at 405 nm in a microplate reader. Three independent assays were performed.

Comet assay

The effect of quercetin on DNA damage was detected using the single-cell gel electrophoresis (comet) assay as previously described [14]. 40 mM EMS (1 h) was used as the positive control. At the end of treatments, cells were detached using trypsin–EDTA. The cell suspensions (1.6×10^4 cells/mL in PBS) were mixed with 1.2 mL 1% low melting point agarose, rapidly pipetted, and spread onto the 1% normal melting point agarose-covered surface of the slide. After solidification of the layer, slides were incubated in freshly prepared cold lysis buffer [1.2 M NaCl, 100 mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26 M NaOH (pH > 13)] for overnight at 4 °C. Subsequently, the slides were washed three times (20 min) with electrophoresis solution (0.03 M NaOH, 2 mM Na_2EDTA (pH ≥ 12.3) at RT, and then electrophoresis for 25 min at 0.6 V/cm were performed under alkaline conditions at 4 °C. Following washing three times with cold ddH_2O, slides were stained with 10 μg/mL of propidium iodide (PI) for 20 min. Analysis of comet appearance was carried out with a fluorescence microscope (Leica DMI 6000) at a ×40 magnification and $\lambda_{ex}/\lambda_{em}$ = 535/617 nm. In order to evaluate the DNA damage "head part" and "tailing part" density areas were marked using NIH Image J software, and the results obtained were calculated by using the following equation: % tailing DNA = $100 \times$ (tailing DNA density)/(cell DNA density). For each experiment, 100 individual cells were analyzed, and three independent assays were performed.

Statistical analysis

All data were presented as mean ± standard deviation (SD) of at least three independent experiments. Nonlinear regression analysis of sigmoidal dose–response curve to calculate IC_{50} (50% inhibition of cell growth) value was performed. Statistical analysis and graph generation were performed

using the GraphPad Prism Software version 7.00. For multiple comparisons, Tukey post-hoc test was used after one-way analysis of variance (ANOVA). Values at $P < 0.05$ were considered as significant.

Results

Quercetin treatment blocks proliferation of human breast cancer cells in a dose- and time-dependent manner and induces morphological changes in the cells

MTT assay showed that quercetin treatments blocked cell proliferation in a dose- and time-dependent manner, especially in MCF-7 cells (Fig. 1). MCF-7 cells were shown to be more sensitive than MDA-MB-231 cells against quercetin treatments. When all treatment periods were considered, only 58% cytotoxicity was observed in MDA-MB-231 cells, even at the highest dose quercetin (200 μM), while cytotoxicity reached 88% in MCF-7 cells. Additively, in MDA-MB-231 cells time-dependent statistical differences were observed for only 200 μM dose of quercetin (Fig. 1b, d). For MCF-7 cells, the IC_{50} value was less than 100 μM in 3 different treatment periods, whereas this was the

Table 2 Time-dependent IC_{50} values of quercetin in human breast cancer cells

Cell line	IC_{50} (μM) based on Qu treatment time		
	24 h	48 h	72 h
MCF-7	89.79 ± 1.54	64.62 ± 1.15	65.31 ± 1.14
MDA-MB-231	197.89 ± 5.20	146.26 ± 2.42	157.70 ± 2.17

Values represent the mean $\pm$ SD of at least three individual experiments performed

opposite for MDA-MB-231 cells (Fig. 1a, c, Table 2). We restricted our study to 48 h at which the lowest IC_{50} value was obtained for both the cell line. To evaluate the effects of high and low doses of quercetin, three doses (10, 25 and 100 μM) were chosen based on dose–response curves as test concentrations.

We also presented the effects of selected doses of quercetin on cell proliferation and morphology in MCF-7 and MDA-MB-231 cells in Fig. 2. Cells treated with quercetin or DMSO were viewed in an inverted microscope under phase-contrast. DMSO (the final concentration in the culture medium did not exceed 0.1%) or 10 μM quercetin for 48 h showed no statistically significant difference in cell viability compared to the untreated group in both

Fig. 1 Effects of quercetin on the viability of human breast cancer cells MCF-7 and MDA-MB-231. Quercetin treatments were associated with an increase in cell death in a dose- and time-dependent manner. **a** and **b** are dose–response curves, **b** and **d** are statistical expression of the dose and time dependent effect of quercetin treatment. * and #Comparison based on quercetin dose and administration time, respectively. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$
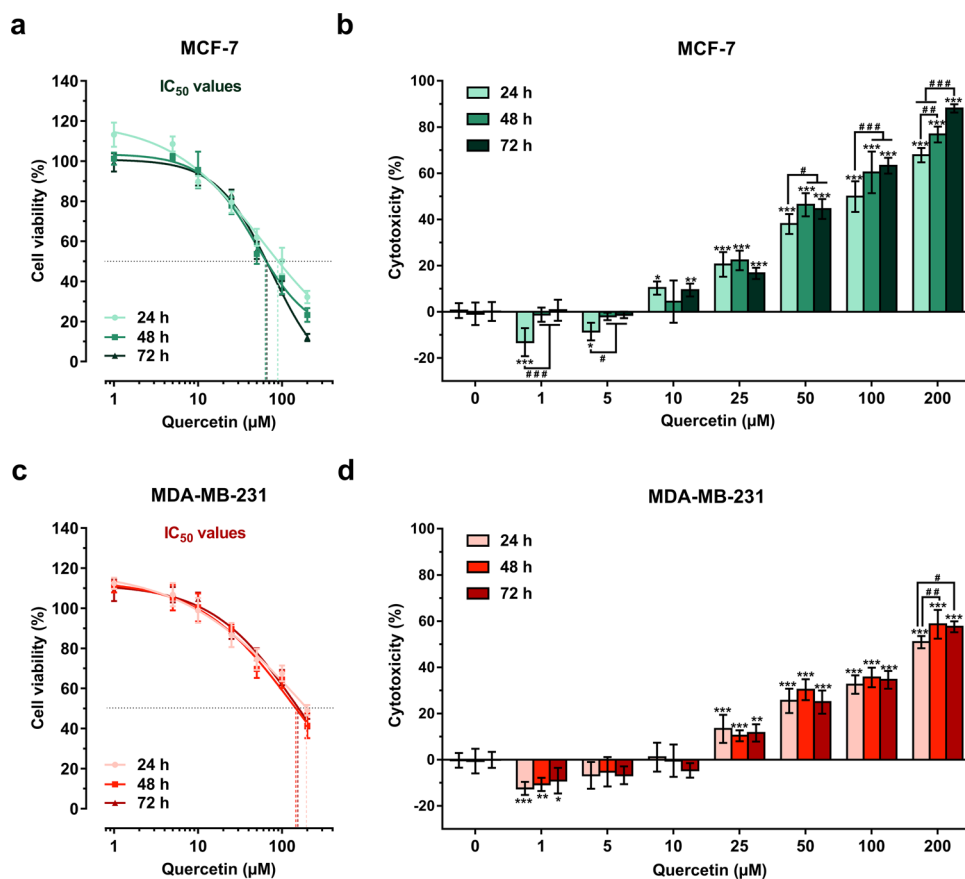
Fig. 2 Effects of selected doses of quercetin on cell proliferation ▶ and morphology in human breast cancer cells. **a** The cells either left untreated (C, control), or treated with 0.1% DMSO (D) or 10, 25 and 100 µM quercetin (Qu). After 48 h, MTT analysis showed that MCF-7 cells were more susceptible to Qu treatment than MDA-MB-231 cells for the same dose administration. ***$P < 0.001$ versus MCF-7 cells control group. #$P < 0.05$ and ###$P < 0.001$ versus MDA-MB-231 cells control group. &$P < 0.05$ and &&&$P < 0.001$, MCF-7 cells versus MDA-MB-231 cells. **b** After treatments, the cell morphological alterations were observed and photographed at ×200 magnification with inverted phase-contrast microscope

cell lines. In contrast, a statistically significant difference was determined for the 25 and 100 µM doses. In MCF-7 cells 25 µM and 100 µM doses of quercetin reduced cell viability by 22.24% and 57.85%, while in MDA-MB-231 cells decreased by 10.35% and 35.57%, respectively. The viability difference between the two cell types due to quercetin treatment was determined to be statistically higher at 100 µM quercetin concentration ($P < 0.001$). The untreated control cells and the cells treated with 0.1% DMSO or 10 µM quercetin exhibited normal morphology (well spread and flattened), whereas changes in the shape and adhesion capacity of the cells were observed in groups treated with 25 and 100 µM quercetin. These doses were caused to cell shrinkage, cellular irregularity in shape and detachment. Both MTT assay and microscopic imaging revealed that MCF-7 cells were more affected by quercetin according to MDA-MB-231 cells for the same dose applications.

Quercetin treatment leads to a reduction in the expression of Hsps in a dose-dependent manner in human breast cancer cells

Western blot analyses (Fig. 3) were performed to evaluate the effect of quercetin treatment on Hsp27, Hsp70 and Hsp90 expression in both MCF-7 and MDA-MB-231 cells. Only 10 µM quercetin showed no statistical difference in Hsp70 and Hsp90 expression in MCF-7 cells. However, this dose significantly reduced Hsp27 expression in the same cell line, and increased doses of quercetin caused a further decrease in the protein levels. In 25 and 100 µM quercetin treated MCF-7 cells, the levels of Hsp70 and Hsp90 significantly decreased compared to the untreated cells (control group). The Hsp90 levels were nearly exhausted by about 93% decrease. Similarly, inhibition rates of Hsp27, Hsp70 and Hsp90 increased in a dose-dependent manner in MDA-MB-231 cells. Even low-dose quercetin (10 µM) reduced Hsp27 expression by 75%. While Hsp27 and Hsp90 levels were almost depleted due to high-dose quercetin (100 µM) treatment, Hsp70 levels were reduced by 65%.
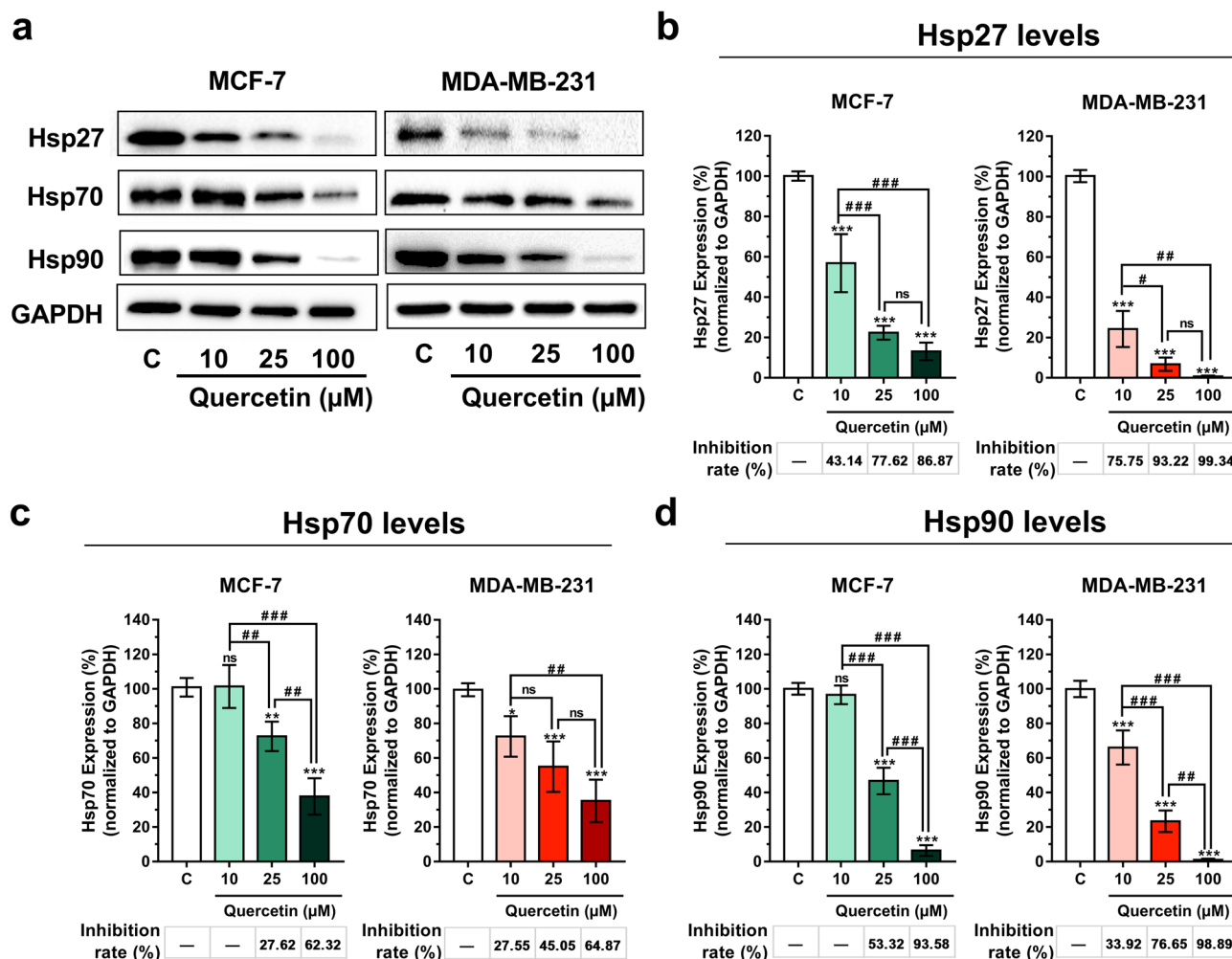
a

b
Hsp27 levels

c
Hsp70 levels

d
Hsp90 levels
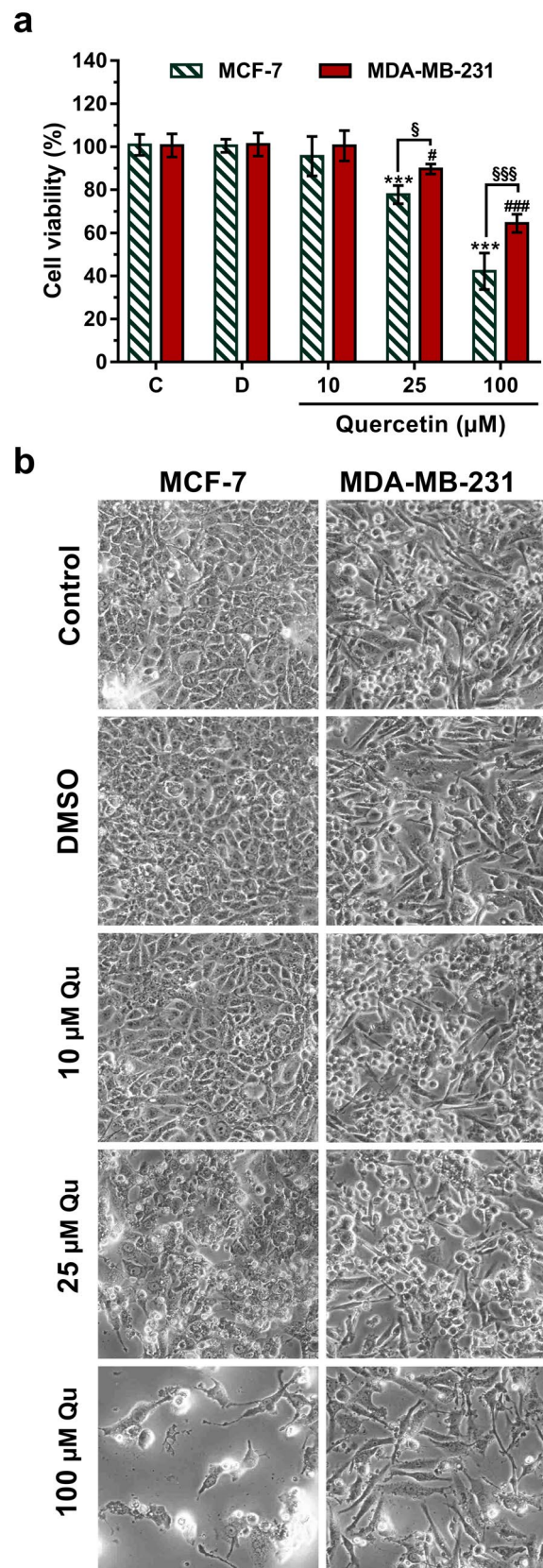
Fig. 3 Expression of Hsps in response to quercetin treatment in human breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with 10, 25 and 100 μM of quercetin for 48 h. Western blot analysis **a** showed that quercetin caused a reduction in the expression of **b** Hsp27, **c** Hsp70 and **d** Hsp90 in a dose-dependent manner. GAPDH was used as a loading control. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus untreated control (C) group. ##$P < 0.01$ and ###$P < 0.001$, which show multiple comparisons between different groups. *ns* – not significant

The effect of quercetin treatment on apoptotic cell death, NF-κB activity and DNA damage in human breast cancer cells

The induction of apoptosis due to quercetin treatment in MCF-7 and MDA-MB-231 cells was first evaluated by determining the activity of DEVDase (caspase-3/7). Based on this analysis, the results showed that apoptosis was induced in treated cells with 25 and 100 μM quercetin. A higher level of caspase-activity was detected in MCF-7 cells according to MDA-MB-231 cells. The highest apoptosis rate for MCF-7 cells was calculated to be a 3.53-fold increase compared to untreated cells (control group), whereas it was 1.84 in MDA cells (Fig. 4a). To confirm caspase-dependent cell death after quercetin treatment in the cells, we detected the levels of caspase-3 and PARP-1 (Fig. 4b–d). Herein, we confirmed

that pro-caspase-3 was expressed in the MCF-7 cell line but could not be cleaved into the activated form [15]. Besides, treatment with quercetin triggered PARP-1 cleavage (up to ~ 20-folds) in the MCF-7 cells. In MDA-MB-231 cells treated with 25 and 100 μM quercetin, cleaved caspase-3 and PARP-1 levels were found to be increased compared to untreated cells (control group). The most triggered caspase-3 and PARP-1 cleavage were in 25 μM quercetin treated cells with a 6.29- and an 8.45-fold increase compared to control.

Here, we also investigated the activity of NF-κB by Western blot method. As shown in Fig. 4e and f, we found that the levels of NF-κB p50 subunit increased in both cell lines as a result of quercetin treatment (except for 10 μM quercetin-treated MCF-7 cells). Fold increase of NF-κB p50 in quercetin (25 ve 100 μM) treated MCF-7 cells was determined by 15.4 and 20.9, respectively. In MDA-MB-231 cells, p50
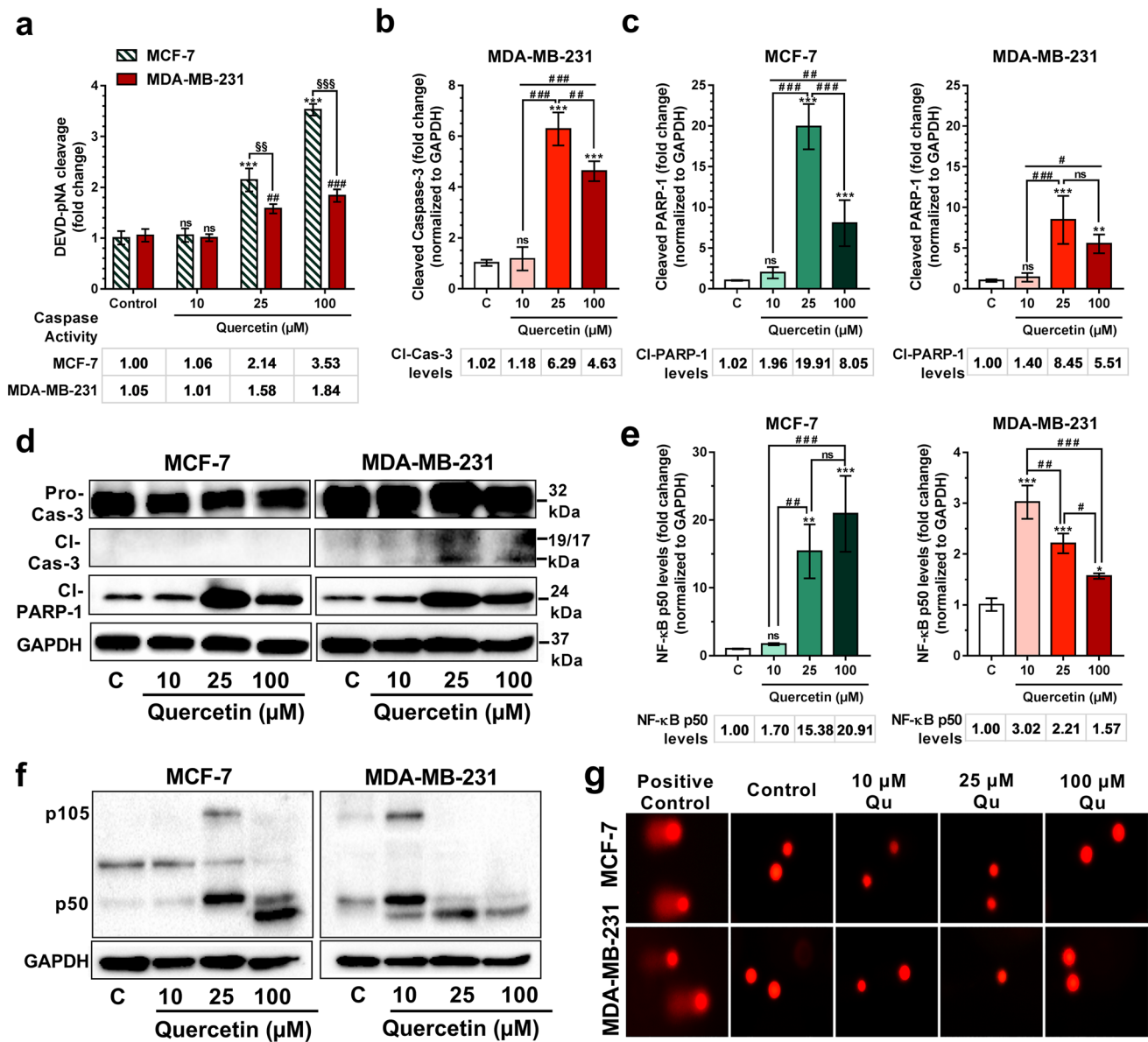
Fig. 4 Effects of quercetin treatment on apoptotis, NF-κB activity and DNA damage in human breast cancer cells. Quercetin induced apoptotic cell death, but did not cause DNA damage in the cells. In addition, it led to an increase in NF-κB p50 levels. **a** DEVDase (caspase-3/7) activity assay. ***$P < 0.001$ versus MCF-7 control group. ##$P < 0.01$ and ###$P < 0.001$ versus MDA-MB-231 control group. &&$P < 0.01$ and &&&$P < 0.001$, MCF-7 cells versus MDA-MB-231 cells. **b–d** Western blot analysis of caspase-3 and PARP-1 **e** and **f** Western blot analysis of nuclear factor kappaB (NF-κB) p50 subunit. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus control (C) cells. #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$, which show multiple comparisons between different groups. *ns* not significant. GAPDH was used as a loading control. **g** Evaluation of the genotoxic effect of quercetin was performed by Comet assay

levels were increased by 3-, 2.2- and 1.5-fold in treatment groups with 10, 25 and 100 µM quercetin, respectively.

Evaluation of the genotoxic effect of quercetin was performed by comet assay test (Fig. 4g), which is a sensitive method for quantifying and analyzing DNA damage based on single-cell gel electrophoresis. When a genotoxic effect occurs on the cell, the resulting image is similar to

a comet composed of a head (represents intact DNA) and tail (shows strands of degraded DNA). In our analysis, while untreated cells were used as the control group, EMS served as positive control. Upon treatment with quercetin (10, 25 and 100 µM), a significant difference between treated and untreated cells was not observed. DNA fragmentation in the treated cells was not detected.

Discussion

This study is based on investigating the effects of quercetin on Hsp levels and apoptosis induction in human breast cancer cells MCF-7 (ER$^+$, PR$^+$, HER2$^-$) and MDA-MB-231 (triple negative). Herein, our results indicated that quercetin significantly inhibited cell growth in a dose- and time-dependent manner. According to MTT assay results, MCF-7 cells were more sensitive to the cytotoxic effects of the quercetin, compared to MDA-MB-231 cells. This result indicates the cytotoxicity effect of quercetin in ER presenting cells. Because quercetin (2 hydroxyl groups and phenolic ring), which resembles the structure of estrogen, potentially acts as a phytoestrogen that modulates cell proliferation by binding to ER and exhibits anticancer effect in a dose-dependent manner [16]. The dose-dependent effect of phytoestrogens is a dual effect that reflects growth stimulation at low concentrations and growth inhibition at high concentrations [17]. This explains why low-dose quercetin we tested causes proliferative action in cells (negative directional columns Fig. 1b, d). Besides, IC$_{50}$ values of quercetin in MCF-7 cells were lower than 100 µM for 24, 48 and 72 h treatment periods, however, IC$_{50}$ values of MDA-MB-231 cells were twofold higher. This result was inevitable due to the more aggressiveness of MDA-MB-231 cells than MCF-7 cells [18]. Hence, to evaluate the difference in cellular response on quercetin treatment, we used fixed three doses (10, 25 and 100 µM) of quercetin and 48 h treatment time for both the cell lines. Morphological analyzes with selected doses also confirmed that MCF-7 cells were more sensitive to quercetin in a dose-dependent manner compared to MDA-MB-231 cells (Fig. 2).

Hsps are closely associated with tumor cell proliferation and apoptosis inhibition. They are often overexpressed in breast cancer. Especially high levels of Hsp27, Hsp70 and Hsp90 have been correlated with a poor clinical outcome [11]. Although quercetin is a very good Hsp inhibitor by preventing activation of heat shock factors (HSF), there are limited studies showing its effect on Hsp expression in breast cancer. In a study conducted by Yang et al. [19], it has been shown that quercetin inhibits Hsp70 and Hsp90 expression by decreasing the levels of HSF1, which is the common transcription factor of Hsps, thus apoptosis is induced in breast cancer. In another study, quercetin has been shown to reduce Hsp27, Hsp70 and HSF2 levels in breast cancer cells [20]. Here, we investigated the effect on these three important Hsps (Hsp27, Hsp70 and Hsp90) expression in MCF-7 and MDA-MB-231 cells. Also, the effect of quercetin on Hsp27 and Hsp90 levels in MCF-7 and MDA-MB-231 cells, respectively, was presented in this study for the first time. As shown in Fig. 3 Western

blot results, quercetin caused increased Hsp silencing due to dose increase in both cell lines (except for the only 10 µM quercetin effect on Hsp70 and Hsp90 expression in MCF7 cells). The decrease in expression levels of all Hsps analyzed as a result of quercetin treatment may be associated with the downregulation of HSF1 and/or HSF2. Moreover, Hsp90, as a molecular target for anti-cancer drug/inhibitor development in the fight against breast cancer, was nearly completely silenced in both breast cancer cell lines as a result of quercetin treatment in this study. The results of this study suggest that quercetin may be an effective agent or adjuvant for the treatment of breast cancer due to leading a significant reduction in the three important levels of Hsps that are effective in poor response to treatment.

In many cancer cells including breast cancer, quercetin is widely known to induce apoptosis [6, 8, 21]. Apoptosis is a primary and highly conserved way of cell death and is important for the suppression of oncogenesis. Among the family of caspase (cysteine-dependent aspartate-specific protease), caspase-3 is a critical downstream effector protease, and its cleavage product is a typical hallmark of apoptosis [22]. Herein, we examined the effects of quercetin on apoptosis in human breast cancer cells. First, we checked the caspase activity by using a colorimetric assay kit. DEVDase (caspase-3/7) activity was detected in both MCF-7 and MDA-MB-231 cells treated with 25 and 100 µM quercetin, and caspase activity was observed to be higher in MCF-7 cells when two cell lines were compared (Fig. 4a). Secondly, we evaluated the level caspase-3 in both cell types by western blotting (Fig. 4b, d). Pro and cleaved forms of caspase-3 were detected in MDA-MB-231 cells, while we have demonstrated that pro-caspase-3 is expressed in MCF-7 cells but could not be cleaved into the activated form. As stated in the results of this study and in the literature [15], there is no caspase-3 activity in MCF-7 cells. However, caspase-7 is highly homologous to caspase-3 and has very similar substrate specificity. Therefore, it may be capable of functionally substituting for caspase-3 [23]. In this case, the caspase activity seen in MCF-7 cells in this study is the effect of caspase-7.

During apoptosis, PARP-1 which has many functions including regulation of DNA repair, cell death, transcription and inflammation is cleaved by caspases 3 and 7, thereby forming two enzymatically inactive fragments. This is another indicator of apoptosis [24]. As shown in Fig. 4c and d, quercetin treatment (both 25 and 100 µM) resulted in the cleavage of PARP-1 in both cell types at a statistically significant level. An increase of about 20-fold in MCF-7 cells and about ninefold in MDA-MB-231 cells compared to control were determined. This showed that quercetin is a good agent for apoptosis induction in breast cancer. Consistent with caspase activation results,
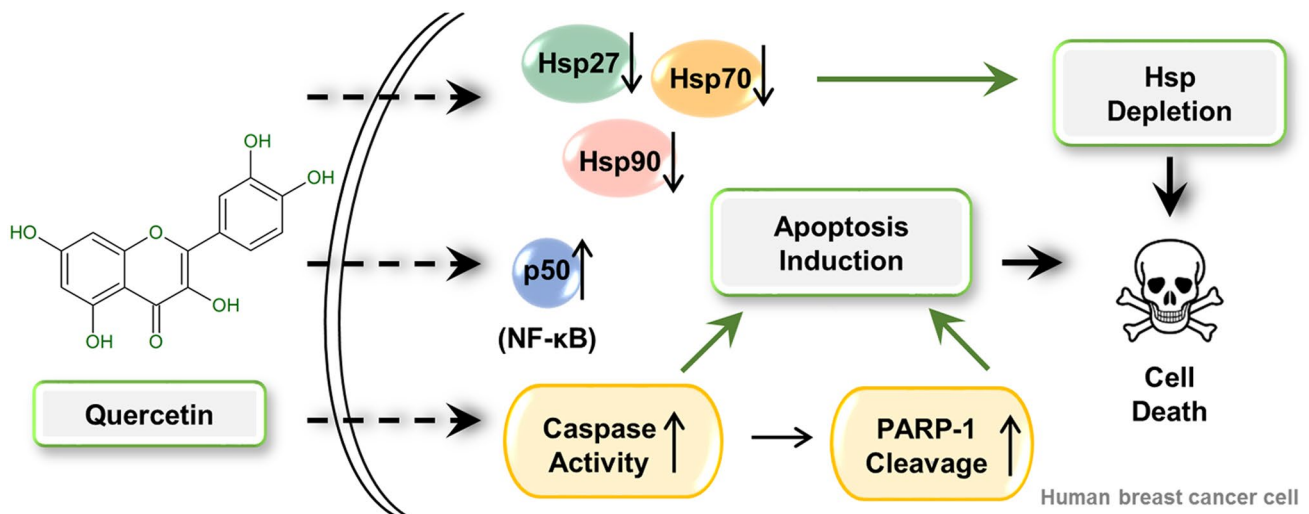
Fig. 5 Overview of the study outputs. Our findings suggested that quercetin, as an Hsp inhibitor, can be an effective therapeutic agent for breast cancer therapy. Quercetin has caused nearly depletion of Hsp27, Hsp70 and Hsp90, which is closely associated with a poor clinical outcome in breast cancer, thus leads to apoptosis of cells

quercetin caused higher PARP-1 cleavage in MCF-7 cells compared with MDA-MB-231 cells. This suggests that quercetin can function as an anti-breast cancer agent with higher potential in ER$^+$ breast cancer than compared to ER$^-$ breast cancer cells.

NF-κB is a transcription factor that plays a crucial role in the regulation of cell survival and cell death. Mainly, both p65 and p50 subunits reside in the cytoplasm in an inactive form bound to inhibitor IκB, and when NF-κB activated, stimulates transcription of the various antiapoptotic genes, enabling cell survival [25, 26]. Here, we found that NF-κB p50 subunit increased upon quercetin treatment in both cell lines (except for 10 μM quercetin-treated MCF-7 cells). (Fig. 4e, f) Interestingly, while the results we obtained and presented above are against cancer, NF-κB activity results seems to benefit the progress of cancer. On the contrary, the result is likely to be different than it appears. Because, the decrease of NF-κB due to Hsp70 up-regulation [27] or increase of NF-κB due to Hsp70 down-regulation has been shown [28]. Herein, the increase in NF-κB p50 may be caused by decreased Hsp70 levels due to quercetin treatment. In addition, despite increased NF-κB subunit levels, NF-κB may not have been translocated to the nucleus and failed to function as a transcription factor. Further analysis is needed to clarify this, and our future research will focus on this. According to some sources, while triggering apoptosis (during caspase and PARP cleavage), activation of the NF-κB signaling pathway provides the cells with a balancing mechanism to re-evaluate whether the cell should survive or die [29–31]. The evidence shows that in the case of depletion of Hsp70 and induction of apoptosis, the net result is an anti-cancer effect.

Although quercetin is well known to cause DNA damage and to trigger apoptosis [32, 33], the effect of quercetin on DNA damage is controversial because of its protective role on DNA has also been documented [34–36]. We examined the effects of quercetin on DNA damage in human breast cancer cell lines by comet assay. Interestingly, we found that quercetin treatment did not cause DNA damage (Fig. 4g). In support of our results, it has been shown that quercetin treatment in breast cancer cells does not cause DNA damage [36]. While our results were providing evidence for apoptosis due to quercetin treatment in both cell lines, the absence of DNA damage may be indicative of early apoptosis [37].

In conclusion, the present study is the first to report that quercetin suppresses Hsp27 and Hsp90 expression in MCF-7 and MDA-MB-231 cells, respectively. Furthermore, this study showed that quercetin causes nearly depletion of Hsp27, Hsp70 and Hsp90 levels, which are the three important Hsps leading to poor prognosis in breast cancer and leads to induce apoptosis at high levels in both cell lines. Western blot analysis revealed that caspase-3 activation and PARP cleavage occurred upon quercetin treatment. Compared to MDA-MB231 cells, MCF-7 cells were more affected by quercetin treatment for the same dose applications, and more apoptotic activity was determined in these cells. Although apoptosis was observed in the cells, DNA damage did not be detected as a result of the comet assay, which may be indicative of early apoptosis. Even if an increase in NF-κB levels is observed in the cells exposed to quercetin, the net result of caspase activity and PARP-1 cleavage is induction of cancer cell apoptosis. An overview of the study outcomes is summarized in Fig. 5. Data from this study show that quercetin has an anticancer effect in

both MCF-7 and MDA-MB-231 cell lines (two important models for breast cancer in terms of hormone receptor, aggression, and treatment response differences). This emphasizes that quercetin is a potential agent for breast cancer treatment. Nevertheless, further research will be needed to determine the mechanisms by which quercetin is effective in modulating Hsp expression in breast cancer. Therefore, similar studies should be repeated comparatively on healthy cells and breast cancer cells. Our further studies will focus on this subject.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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