ORIGINAL ARTICLE

Effects of Quercetin and Hesperetin on MCF-7 Cell Proliferation by Using Real-Time Cell Analyzer

Gülsüm Tekin, Bahadır Ozturk

Department of Biochemistry, Faculty of Medicine, Selcuk University, Alaeddin Keykubad Campus, Turkey

ABSTRACT

Background: Plant-derived flavonoids have recently interested for the researchers, due to antioxidant and anti-proliferative properties. Cell culture and experimental animal studies have shown to cancer-preventive effects of flavonoids in carcinogenesis. However, some of the biological activities of flavonoids are still unclear. Quercetin and Hesperetin are flavonoid compounds which are evaluated as inhibitors of proliferation of various cancer cells. In our study, we investigated the mechanisms for the effects of Quercetin and Hesperetin on with different concentrations and durations to cell proliferation in MCF-7. Although common methods were used in previous studies the microelectrode based method was used in our proliferation analyze. **Materials and Methods:** A real-time cell analyzer was used to assess the effects of various doses of Hesperetin (10-175 μM) and Quercetin (125-250 μM) on the proliferation of MCF-7. Changes in the number of cells were observed continuously every 15 minutes during the experimental period in special cell culture flasks that containing micro-electrodes. **Results:** Hesperetin (175 μM) inhibit the MCF-7 cells proliferation rate of 52.8% compared to the control for 72 h and inhibit cells rate of 60.6% for 96 h after the treatment (P<0.05). Quercetin (250 μM) inhibit MCF-7 cells rate of 70.4% compared to control for 72 h and inhibit cells rate of 81.1% for 96 h after the treatment (P<0.05). **Conclusions:** As a result, Hesperetin and Quercetin inhibit the human breast cancer cells dose-time dependent manner by real-time cell analyzer. xCELLigence is more comfortable, useful and higher of specificity than the other cell viability analyze methods. As a result, hesperetin and quercetin may be useful therapy or prevention on breast cancer prognosis.

Key words: Real-time cell analyzer, hesperetin, MCF-7, proliferation, quercetin

Correspondence:
Dr. Gülsüm Tekin,
Department of Biochemistry,
Faculty of Medicine, Selcuk University,
Alaeddin Keykubad Campus,
42075 Selcuklu/Konya, Turkey.
E-mail: tekinglsm@gmail.com

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INTRODUCTION

Breast cancer is the most common malignancy in women, and is a leading cause of cancer death in women worldwide according to International Agency for Research on Cancer.[1] Several studies report that food products containing flavonoids reduce risk for various cancers including breast cancer. Flavonoids are a group of naturally-occurring, low molecular weight compounds that are widespread in plants or in many foods.[2,3] They are prominent components of citrus fruits and other food sources and are in many countries regularly consumed in a diet. Flavonoids are found in fruits, vegetables, herbs, spices, aromatic plants, tea, red wine[4] as well as stems and leaves of plants.[5]

Flavonoids subdivided into six classes including flavonols, flavones, flavanones, flavan-3-ols, anthocyanidins and isoflavones. Hydroxyl groups which are active in flavonoids, give rise to subdiving of flavonoids and provide characteristic property of them.

These compounds, which have therapeutic effects,[6] may act as natural antitumor agents as chemical inhibitors.[7] Several mechanisms of action have been proposed to explain *in vivo* and *in vitro* flavonoid antiproliferative actions such as induction of apoptosis and cell cycle, inhibit cell proliferation, kinase activity and differentiations of cell[3,8-14] and flavonoids may reduce the risk of developing cancer and cardiovascular disease[7,8,15]. Previous studies showed that flavonoids have affected anticancer activities including effective for colon, breast, lung, leukemia, prostate cancers.[1,8,16-21]

Hesperetin (3',5,7-trihydroxy-4'-methoxyflavone) is a flavonone subgroup of flavonoids. Hesperetin is the aglycone of hesperidin (hesperetin-7-O-rutinosit) in nature and it is found in citrus species[8,18] and is consumed in many countries regularly, especially in Turkey which is Mediterranean country. This polyphenol were observed to inhibit the proliferation human breast cancer cells because of antiestrogenic effect in culture medium[8] Quercetin (3,3',4',5,7-pentahidroksiflavon) a member of polyphenolic flavonoids, is one of the most prominent dietary antioxidants and covers a large class of flavonoids.[22] It is found in various vegetables and fruits especially apple and onion, red wine, grapefruit, black tea, raspberries, blueberries, cherries.[5,6] Several epidemiological studies have supported that consumption of a diet rich in containing low-fat and high-fiber of whole grain foods, vegetables and fruits may be associated with a reduced risk of[7,15] various type of cancers.[1,16,17] The major purpose of the present study is to investigate the possible anti-carcinogenic and anti-estrogenic effects of hesperetin and quercetin with various concentrations and durations through the inhibition of cell proliferation in human breast cancer cell line MCF-7 by a real-time cell analyzer which is a different and more reliable method than the others. Therefore, developing new strategies for therapy of breast cancer progression and overcoming drug resistance represents a major challenge.

METHODS AND MATERIALS

Cell culture

The breast cancer cell line MCF-7 was obtained by Dr. Abdulkerim Bedir, Medical Faculty of Ondokuz Mayıs University, Samsun, Turkey. The cells were placed into tissue culture flasks under a humidified 5% CO_2 and 95% air maintained at 37°C atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, v/v), 1% penicillin (100 U/ml)-streptomycin (100 mg/ml) and 1% glutamine (100 mg/ml). The cells were passaged with 0.25% trypsin and 0.1% ethylene diamine tetra acetic acid (EDTA) after reaching 80% of confluency.

Cell proliferation analyzes

Effect of hesperetin and quercetin on MCF-7 cell proliferation was determined by real time cell analyzer (xCELLigence, Roche Diagnostics GmbH, Penzbeerg, Germany) After seeding 200 μl of

Cite this article as: Gulsum T, Bahadır O. Effects of Quercetin and Hesperetin on MCF-7 Cell Proliferation by Using Real-Time Cell Analyzer. J Basic Clin Pharm. 2017;8:121-126.

the cell suspensions in DMEM containing 10% FBS into the wells (10.000 cells/well) of the E-plate 16. Cells were allowed to adhere to the E-plate for 24 h and media were removed from the well. A 200 µM stock solution of hesperetin was prepared in DMSO (0.1%). The cells were treated with different doses of hesperetin (10, 50, 100, 150, 175 µM). Then the hesperetin was added in various concentrations to cells at following every 48 h. The changes of MCF-7 cell proliferation were monitored every 15 min for a period of 144 hours by xCELLigence device. A 250 µM stock solution of quercetin was prepared in DMSO (dimethyl sulfoxide 0.1%). The same pre-treatment was made for different dose groups of quercetin (125, 150, 175, 200, 225, 250 µM). The quercetin was added in various concentrations to cells at following every 48 h. The changes of MCF-7 cell proliferation were monitored every 15 min for a period of 144 hours. Cell proliferation experiments were performed triplicate.

Cell viability analyze by xCELLigence system

The xCELLigence system (xCELLigence, Roche Diagnostics GmbH, Penzbeerg, Germany) was performed to evaluate cell viability. The xCELLigence system comprises 4 components: the impedance based real-time cell analyzer (RTCA), the RTCA a device station, the RTCA computer with integrated software, and three disposable E-plates 16. Electrical impedance are measured across combined with micro-electrodes integrated on the bottom of tissue culture E-Plates.[23] The xCELLigence system allows for label-free and dynamic monitoring of cellular phenotypic changes in real time using impedance.[24] Impedance measured between electrodes in an individual well depends on electrode geometry, ionic concentration in the well, and whether there are cells attached to the electrodes.[25,26] The impedance measurement, which is displayed as cell index (CI) value, provides quantitative information about the biological status of the cells, including cell number, viability, and morphology. Therefore, we have preferred the xCELLigence system, because it allows for maximal sensitivity for the detection of the cells with relatively uniform distribution of the electric field.

Apoptosis analyzes

Cells were cultured in 25 cm^2 Petri dishes, after 24 h flavonoids were added in IC_{50} concentrations (hesperetin IC_{50}:115 µM; Quercetin IC_{50}:200 µM) which are used in viability test. After 96 h cells were detached with 0.25% Tripsin-EDTA solution. MCF-7 cell apoptosis was analysed using a flow cytometer with Annexin V-FITC (fluorescein isothiocyanate) and PI (Propidium iodide) staining kit (BD Biosciences) to distinguish early apoptotic from necrotic cells as previously described.

Statistical analysis

SPSS (v. 20.0 Chicago, IL) was used for statistical analyses. All the data were expressed as Mean ± SD. First of all, the data were tested for homogeneity of variances. Significant time/dose interactions were found for variables and subgroups were analyzed further by testing the effect of concentration within each group using for repeated-measures ANOVA. Accordingly time/dose interactions was found significantly ($P<0.05$). Therefore, differences between the groups were evaluated with one-way analysis of variance followed by the Duncan test in each time groups and the difference between times was analyzed separately by paired t test in each groups.

RESULTS

Effect of quercetin and hesperetin on cell proliferation and apoptosis

As a result of the analysis, time/dose interaction was found to be significant and therefore the comparisons between dose groups were made for each period of time. Cell growth rate was monitored for 135 hours with various concentration of hesperetin. The cells were inhibited by different concentration (10-175 µM) of hesperetin compared to control [Figure 1]. Statistical results were given in the Table 1 for effect of hesperetin on cells proliferation. A statistically difference was not found significantly ($P>0.05$) between dose groups after the treatment for 24 h and 48 h. Differences of between dose groups significantly were observed statistically after the treatment for 72 h [Table 1]. The treatment of MCF-7 cells with hesperetin for 48 h did not affect cell proliferation [Figure 1]. Hesperetin significantly decreased cell proliferation at 96 h of treatment in a dose- and time-dependent manner ($p=0.026$). Inhibition of cell proliferation was seen hesperetin treatment at concentrations of 150 and 175 µM (decreased up to 50.6% and 56.9% at 96 h and decreased up to 52.8% and 60.6% at 120 h compared to the control, respectively ($P<0.05$) [Table 2]. IC_{50} dose of hesperetin ($IC_{50:}$ 115 µM) calculated at 96 hours (r^2:0.99) and

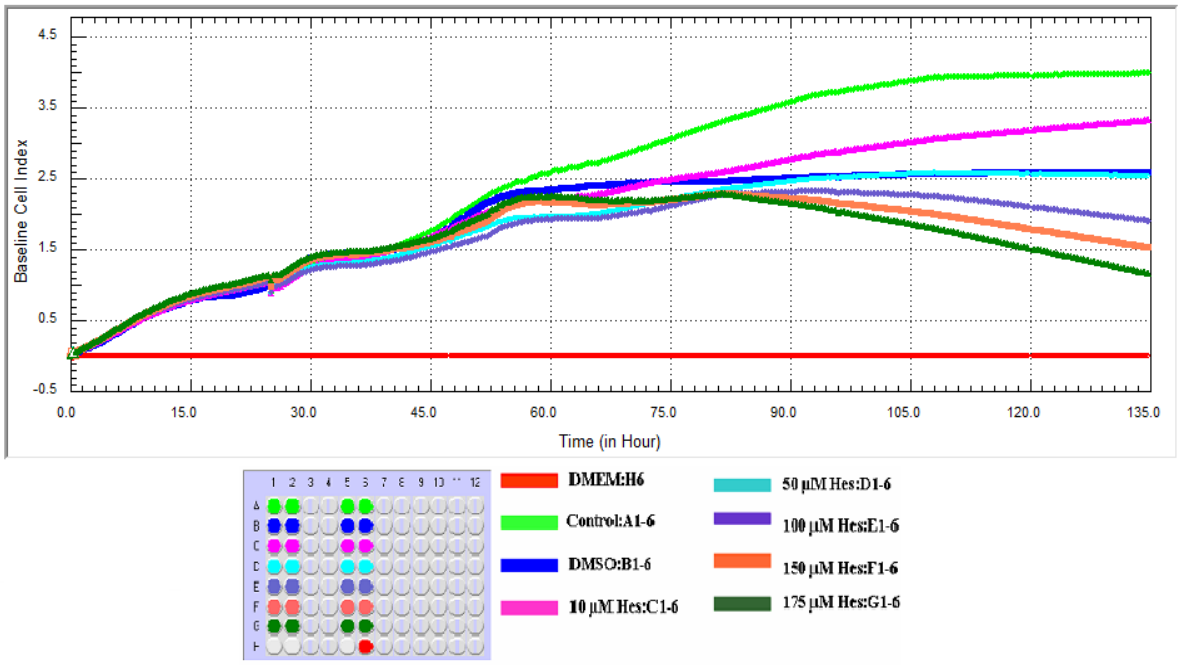

Figure 1: Real-time monitoring of cell adhesion and cell proliferation. Effect of hesperetin on cell proliferation and viability of MCF-7 cells. Cells were exposed to either vehicle (0.1% dimethyl sulfoxide in medium) or hesperetin (10-175 µM). Cells were examined for 135 h using a real-time cell analyzer

Table 1: Cell index as mean ± standard deviation (M ± SD) on MCF-7 cells

	96 h		120 h		135 h	
CONTROL	3.62 ± 0.52	a	3.76 ± 0.55	a	3.83 ± 0.30	a
DMSO	2.21 ± 0.04	b	2.28 ± 0.11	bc	2.24 ± 0.14	c
10 µM Hes	2.43 ± 0.58	b	2.76 ± 0.67	b	2.84 ± 0.42	b
50 µM Hes	2.19 ± 0.52	b	2.24 ± 0.47	bc	2.19 ± 0.21	c
100 µM Hes	1.88 ± 0.44	bc	1.75 ± 0.37	c	1.68 ± 0.13	d
150 µM Hes	1.79 ± 0.42	c	1.62 ± 0.35	cd	1.52 ± 0.30	d
175 µM Hes	1.71 ± 0.42	c	1.48 ± 0.26	d	0.76 ± 0.14	e
P	0.026		0.001		0.000	

MCF 7 cells were treated with the indicated concentrations of hesperetin (Hes). Cell proliferation and viability were measured using a real time cell analyzer. Data are presented as mean ± SD. Groups with different superscript letters in each column represent significant difference between groups ($P < 0.05$).

Table 2: Cell index as mean ± standard deviation (M ± SD) and percentages of cell viability

	96 h		120 h	
	Cell index	Cell via.(%)	Cell index	Cell via.(%)
CONTROL	3.62 ± 0.52	100	3.76 ± 0.55	100
10 µM Hes	2.43 ± 0.58	67.1	2.76 ± 0.67	73.4
50 µM Hes	2.19 ± 0.52	60.5	2.24 ± 0.47	59.6
100 µM Hes	1.88 ± 0.44	51.9	1.75 ± 0.37	46.5
150 µM Hes	1.79 ± 0.42	49.4	1.62 ± 0.35	43.1
175 µM Hes	1.71 ± 0.42	47.2	1.48 ± 0.26	39.4

All data are reported as the percentage change in comparison with the vehicle-only group. which were arbitrarily assigned 100% viability.

apoptosis analyze was set up by using calculated dose of hesperetin.

Cell proliferation rates were observed various concentrations of quercetin at 135 h. The cells were inhibited by different concentration (125-250) µM of quercetin compared to control [Figure 2]. Statistical results were given in the Table 3 for effect of quercetin on cells. The dose groups were found different compared to control, except 225 and 250 µM after the treatment for 24 h. It was seen a similar situation in terms of statistical significance at next times, but the degree of differences between dose groups were changed from time to time due to interaction time/dose [Table 3]. The treatment of MCF-7 cells with quercetin after the treatment for 48 h did not affect cell proliferation [Figure 2]. Quercetin significantly decreased cell proliferation after the treatment for 72 h of treatment in a dose-and time-dependent manner ($P<0.05$). Inhibition of cell proliferation was seen quercetin treatment at concentrations of 200 and 250 µM (decreased up to 57.4% and 68.5% at 96 h and decreased up to 70.4% and 81.1% at 120 h compared to the control, respectively $P<0.05$) [Table 4]. IC_{50} dose of quercetin ($IC_{50:}$ 200 µM) calculated at 96 hours (r^2:0.99) and apoptosis analyze was set up by using calculated dose of quercetin. As a result of the analysis, time/dose interaction was found to be significant and therefore the comparisons between dose groups were made separately for each period of time. It is observed that flavonoids induce apoptosis in MCF-7 cells at 96 hours as well as cell viability [Figure 3]. Apoptosis was in an increasing trend: it was 9-fold for hesperetin and 13.7-fold for Quercetin (Annexin V positive, PI negative) at 96 hours [Table 5].

DISCUSSION AND CONCLUSION

Flavonoids are generally known as inhibitors of cell proliferation. Several recent studies have shown anti-proliferative effects of flavonoid compounds on cancer cells. Studies have been emphasized to reduce risk of cancer if major food sources of these compounds were consumed frequently.[3,8,9,11,20,27-30] Anti-proliferative effects of flavonoids on cancer cells were described by several mechanisms. These compounds were interested that may inhibit cell cycle or induce apoptosis.[3,8] Braganhol *et al.* analyzed effects of quercetin on human glioma cells (U138MG). The cells were treated with 10-100 µM of quercetin and were analyzed at 24, 48 and 72 h after treatment by MTT analyze. The cells proliferation reduced with 30 µM of quercetin at these times for 22, 58, 74%, respectively, but these rates changed with 100 µM as 31, 70, 76%. Quercetin caused G2/M phase arrest by increasing the caspase 3 and caspase 7 in this study.[31] Chou *et al.* Studied effect of quercetin on MCF-7 cells and were treated with different dose (10-175 µM) of quercetin. Effect of quercetin was analyzed at 24 and 48 h and inhibition of cells was observed in a dose-time dependent manner by MTT analyze.[27] Also the cell cycle was analyzed and numbers of cells were found to decrease significantly in G0/G1 phase. Quercetin caused S phase arrest by decreasing the protein expression of CDK2, cyclins A and B.[24] Alshatwi *et al.* investigated effects of hesperetin on cell viability in human cervical cancer cells (SiHa). SiHa cells were exposed to various concentrations of hesperetin (0-1000 µM) for 24 and 48 h and were measured by MTT assay. According to this study, hesperetin did not inhibit the cells at lower concentrations, but cell viability reduced at a hesperetin concentration of 500 µM after 24 and 48 h of the treatment. Hesperetin significantly inhibited the cells in a time-and dose-dependent manner at the high concentration (1000 µM).[32] In another study, Choi *et al.* searched anticancer effect of hesperetin on MCF-7 cells. Cell proliferation was inhibited by hesperetin induced G1-phase cell cycle arrest on MCF-7 cells. Also cyclin dependent kinases was examined in this context. Effect 10, 50 and 100 µM of hesperetin was analyzed on MCF-7 at 24, 48 and 72 h by MTT assay. They have been observed to inhibit the cell proliferation a dose-time dependent manner. Accordingly, maximum inhibition was seen at 72 h with 100 µM hesperetin.[8] In our study, proliferation of cells was inhibited in a time- and dose-dependent manner by flavonoids. MCF-7 cells were treated with different doses (125-250 µM) of quercetin and (10-175 µM) hesperetin. This effect was performed by a real-time cell analyzer. Inhibitory effect of these flavonoids was observed by MTT after the treatment for 24 h by according to the mentioned studies, but we significantly observed effect of these flavonoids after the treatment for 72 h and subsequent times with a real-time cell analyzer. Hesperetin and Quercetin shows specific inhibitory activity for cancer cell growth,[15,16] but the mechanisms underlying the effects of hesperetin and quercetin in the induction of cell cycle arrest in human breast cancer cells are still unknown.

According to our results, 115 µM for hesperetin and 200 µM for quercetin may useful for treatment of breast cancer in later times. Furthermore the flavonoids induced apoptosis of cells. Flavonoids inhibit the cell proliferation via induce cell apoptosis. Flavonoids may have shown inhibitor effects on cell proliferation by increasing Bax or decreasing Bcl-2 or effecting caspase activation, which are effective in apoptosis process. Moreover Quercetin was effective at earlier times and at higher concentrations than hesperetin on inhibition of MCF-7 proliferation. It may be a reason that quercetin have two more hydroxyl groups, unlike hesperetin. Because hydroxyl groups which are found flavonoids provide antoxidant or antiproliferative characteristic property of them.

Our results are generally consistent with this literature in terms of to inhibit the cell proliferation a dose-time dependent manner. The difference between the results could be attributable to methodology and different application concentrations. Classical viability assays are both time-consuming and non-sensitive methods. Furthermore the specifity of methods is low and quite obvious to the mistakes of manipulation. In the present work, we conducted experiments with a new real-time system that investigated the effects of the hesperetin and quercetin on MCF-7 cell line by real-time and continuous monitoring of cell proliferation and viability. Furthermore, the real-time cell analysis system allowed for calculation of time-dependent different concentrations.[33,34] Compared with conventional end point cell-based

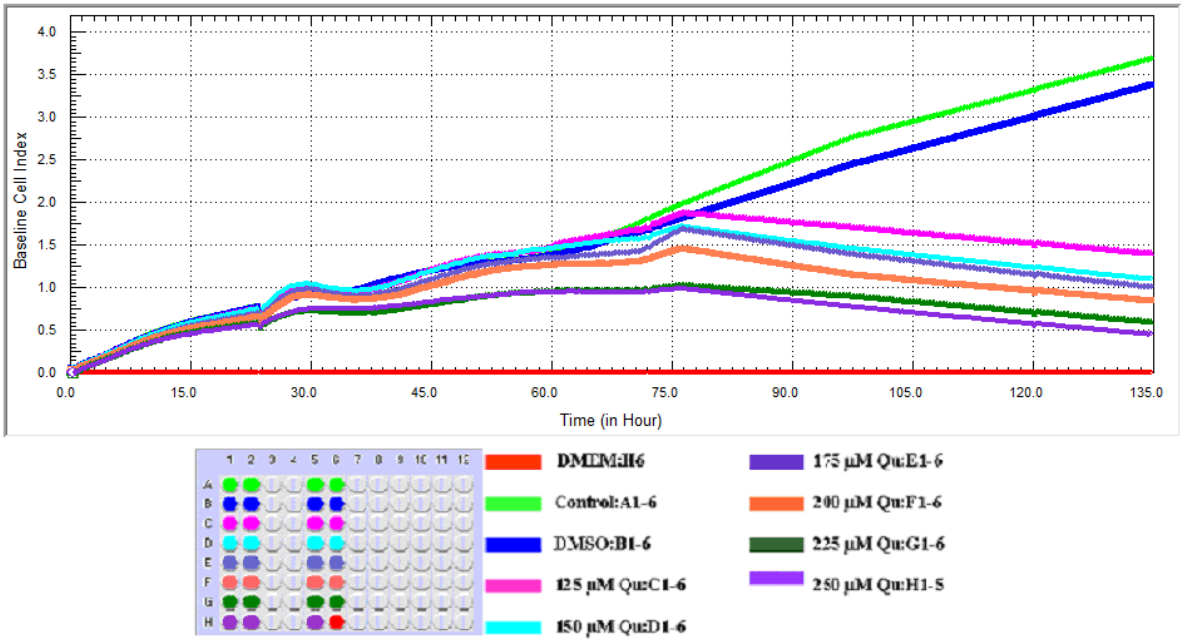

Figure 2: Real-time monitoring of cell adhesion and cell proliferation. Effect of quercetin on cell proliferation and viability of MCF-7 cells. Cells were exposed to either vehicle (0.1% dimethyl sulfoxide in medium) or quercetin (125-250) µM. Cells were examined for 135 h using a real-time cell analyzer

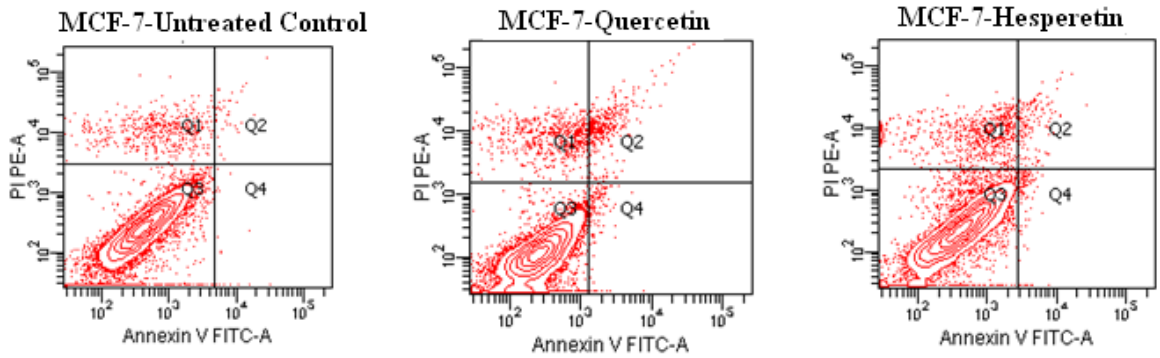

Figure 3: Effects of flavonoids on MCF-7 cell apoptosis analysed at 96 h by flow cytometry with Annexin V-FITC/PI staining, Annexin V-FITC in conjunction with PI staining was used to distinguish early apoptotic (Annexin V-FITC positive, PI negative; Q4) from late apoptotic or necrotic cells (Annexin V-FITC positive, PI positive; Q2)

Table 3: Cell index as mean ± standard deviation (M ± SD) on the MCF-7 cells

	72 h		96 h		120 h		135 h	
CONTROL	1.71 ± 0.44	a	2.70 ± 0.64	a	3.27 ± 0.76	a	3.70 ± 0.64	a
DMSO	1.71 ± 0.01	a	2.53 ± 0.22	a	3.01 ± 0.28	a	3.48 ± 0.22	a
125 µM Qu	1.81 ± 0.28	a	1.64 ± 0.24	b	1.52 ± 0.46	b	1.42 ± 0.17	b
150 µM Qu	1.80 ± 0.21	a	1.45 ± 0.11	bc	1.32 ± 0.14	bc	1.20 ± 0.03	bc
175 µM Qu	1.79 ± 0.03	a	1.42 ± 0.05	bc	1.30 ± 0.09	cd	1.17 ± 0.10	bc
200 µM Qu	1.52 ± 0.06	ab	1.15 ± 0.08	bcd	1.03 ± 0.19	cd	0.92 ± 0.08	bcd
225 µM Qu	1.05 ± 0.22	b	0.95 ± 0.16	cd	0.79 ± 0.18	de	0.63 ± 0.09	cd
250 µM Qu	1.01 ± 0.22	b	0.80 ± 0.17	d	0.62 ± 0.21	e	0.43 ± 0.12	d
P	0.002		0.001		0.000		0.000	

MCF 7 cells were treated with the indicated concentrations of quercetin (Qu). Cell proliferation and viability were measured using a real time cell analyzer. Data are presented as mean ± SD. Groups with different superscript letters in each column represent significant difference between groups ($P < 0.05$).

Table 4: Cell index as mean ± standard deviation (M ± SD) and percentages of cell viability

	72 h		96 h		120 h	
	Cell index	Cell via.(%)	Cell index	Cell via.(%)	Cell index	Cell via.(%)
CONTROL	1.71 ± 0.44	100	2.70 ± 0.64	100	3.27 ± 0.76	**100**
125 µM Qu	1.81 ± 0.28	105.8	1.64 ± 0.24	60.7	1.52 ± 0.46	**46.5**
150 µM Qu	1.80 ± 0.21	105.3	1.45 ± 0.11	53.7	1.32 ± 0.14	**40.4**
175 µM Qu	1.79 ± 0.03	104.7	1.42 ± 0.05	52.6	1.30 ± 0.09	**39.8**
200 µM Qu	1.52 ± 0.06	88.9	1.15 ± 0.08	42.6	1.03 ± 0.19	**31.5**
225 µM Qu	1.05 ± 0.22	61.4	0.95 ± 0.16	35.2	0.79 ± 0.18	**24.2**
250 µM Qu	1.01 ± 0.22	59.0	0.80 ± 0.17	29.6	0.62 ± 0.21	**18.9**

All data are reported as the percentage change in comparison with the vehicle-only group. which were arbitrarily assigned 100% viability.

Table 5: Apoptosis values of MCF-7 after flavonoids treatment

	Annexin V(-) PI(-)(%)	Annexin V(-) PI(+)(%)	Annexin V(-) PI(+)(%)
Control (No treatment)	92.3	0.3	0.5
Quercetin (200 µM)	86.1	4.1	2.1
Hesperetin (115 µM)	81.1	2.7	2

Apoptosis analyzes of MCF-7 after flavonoids treatment at 96 h. Early apoptotic (Annexin V(-)/PI(+)); late apoptotic Annexin V(-)/PI(+); live cells Annexin V(-)/PI(-)

assays, dynamic monitoring of cell response, such as cell adhesion, spreading, proliferation, and cell death, is an advantage of the real-time system to optimize the cell concentration for *in-vitro* assays; it also allows both cell and assay conditions to be constantly obtained before and during the experiments.[35]

Conflict of interest

The authors declare no conflict of interest.

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