Int J Clin Exp Pathol 2017;10(3):2554-2567
www.ijcep.com /ISSN:1936-2625/IJCEP0046619

Original Article

Oxidative stress pathways of flavonoid toxicity in human breast tumor cells

Maryam Taabodi[1], Eric B May[1], Kelly M Mack[3], Katherine S Squibb[2], Ali B Ishaque[1]

[1]Department of Natural Sciences, University of Maryland Eastern Shore, MD, USA; [2]Department of Medicine, University of Maryland School of Medicine, MD, USA; [3]Association of American Colleges and Universities, 1818 R Street, NW, Washington DC, USA

Received December 4, 2016; Accepted January 15, 2017; Epub March 1, 2017; Published March 15, 2017

Abstract: African-American women have a higher breast cancer mortality rate than Caucasian women. Harmful reactive oxygen species (ROS) exert oxidative stress in cells of the human body and lead to several types of DNA damage. Recent studies have shown that flavonoids may protect against cancer through inhibition of oxidative damage. Naringenin, a universal flavonoid, can also inhibit the proliferation of cancer cells. This project studied the oxidative stress (cell death) pathways of naringenin in ER-positive (MCF-7), ER-negative (MDA-MB-468), and non-tumorigenic human breast cell (MCF-10A) lines. Cellular levels of ROS, superoxide (O_2^-), glutathione (GSH), and mitochondrial membrane potential (MMP) were assayed using the FACS Calibur flow cytometer and cell quest software for data collection. The results showed that naringenin induced oxidative stress that resulted in cell death in all cell lines. The highest production of ROS and O_2^- was observed in ER-negative cells. Naringenin did not have any effect on GSH levels in ER-negative and ER-positive but a partial increase occurred in non-tumorigenic human breast cells. The results for MMP showed that naringenin significantly increased the loss of MMP in all breast cells. In conclusion, the oxidative stress that resulted in cell death from exposure to naringenin may contribute to the cancer-preventive effects associated with an increased dietary intake of fruits containing flavonoids. We have presented evidence that naringenin is able to induce cell death in both ER-positive and ER-negative breast cancer cells; however the magnitude of the effect of naringenin is quite different. This finding suggests naringenin may have potential as a treatment for ER-negative breast cancer.

Keywords: Breast cancer, estrogen receptor, flavonoids, naringenin, oxidative stress

Introduction

African American women have lower incidence rates but higher mortality rates for breast cancer than Caucasian women [1] and are more likely to be diagnosed at the later stages of breast cancer [2-4]. The reason for mortality differences between racial groups may be cultural but also due to a different hormone receptor status [5-9]. It is increasingly recognized that breast cancer is a disease with distinct clinical behavioral and molecular properties. For example, estrogen receptor (ER) positive and negative cancers are the two most distinct breast cancer subtypes and predict the likelihood of benefits from antiestrogen therapy [10, 11]. Breast cancer tumors that are ER-positive and human epidermal growth factor receptor 2 positive (ER+/HER2+) are much more likely to respond to endocrine therapy that blocks estrogen's effects in the body, than ER-negative (ER-/HER2+) tumors [5, 12]. According to the Surveillance, Epidemiology, and End Results [13], women with ER-positive tumors have better prognosis and need less aggressive treatment than women with ER-negative tumors.

Cancer cells have higher levels of oxidative stress than normal cells [14, 15]. Increased levels of oxidative stress result from an imbalance between the production of reactive oxygen species (ROS) and reduction of glutathione (GSH) level in the cell and high levels of ROS are known to kill cancer cells [16-22]. GSH plays a vital role in maintaining redox homeostasis and it is the main non-enzymatic component of intracellular antioxidant defense mechanisms, acting as a small scavenger molecule in living

organisms [22-24]. GSH is able to scavenge single oxygen and hydroxyl radicals directly, detoxify lipid hydroperoxides and hydrogen peroxides by the activity of glutathione peroxidase, and regenerate other antioxidant molecules [25, 26]. An early stage of oxidative stress starts with disruption of mitochondria including changes in membrane and redox potential. DNA fragmentation is preceded by disruption of mitochondria, which results in a decrease in mitochondrial membrane potential (MMP) [27-30]. This reduction in MMP is accompanied by the production of ROS contributing to cell death [17, 31-34]. There is growing awareness that oxidative stress plays an important role in various health problems and that consumption of antioxidants can prevent and even reverse these effects [24, 35-37].

Numerous studies suggest that bioflavonoids have potential beneficial effects and exert preventive effects in carcinogenesis essentially due to their antioxidant, anti-inflammatory, and anti-proliferative activities [38-40]. Naringenin, a flavonoid found in grapefruit, can alter ROS metabolism by directly lowering the intracellular pool of GSH [14, 41-44]. It was the aim of this study was to better understand the cytotoxicity of naringenin and its oxidative stress pathway(s) in human breast cancer cells and investigate the possible use of naringenin as an alternative treatment for estrogen receptor negative breast cancer.

Material and methods

Cell culture

MCF-7 (ER-positive), MDA-MB-468 (ER-negative) and MCF-10A (non-tumorigenic, control) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). For routine maintenance, Eagle's Minimal Essential Medium (EMEM) with 2.0 mM L-glutamine, supplemented with 0.01 mg/ml bovine insulin, 10% fetal bovine serum, and 0.5% penicillin and streptomycin was used for ER-positive cells; Leibovitz's L-15 medium with 2.0 mM L-glutamine supplemented with 10% fetal bovine serum, 0.5% penicillin and streptomycin was used for ER-negative cells; and Mammary Epithelial Growth Medium (MEGM) supplemented with 100 ng/ml cholera toxin was used for non-tumorigenic cells. All cells were cultured in 175 cm^2 flasks at 37°C in a humidified 95% air and 5% carbon dioxide (CO_2) incubator. Cells were allowed to grow and form a monolayer in the flasks. Cells were grown to 90-95% confluence, washed with phosphate buffered saline (PBS) and harvested by trypsinization (0.05% (w/v) trypsin) once a week following manufacture's protocol (ATCC cell culture). All media were purchased from Invitrogen (Grand Island, NY) and fetal bovine serum, penicillin, and streptomycin purchased from ATCC.

Cell toxicity assay (cell death) by measuring of intracellular ROS, O_2^-, GSH, and MMP

All breast cells were grown in 6-well plates with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin, at 37°C in a 5% CO_2 incubator. Cells were allowed to grow to 90% confluence. Naringenin (Sigma Aldrich, St. Louis, MO) at different concentrations (0.0, 2.5, 10.0, or 50.0 μM) was added to the wells with 5.0 mL MEM without phenol red followed by incubation for 24 h. After 24 h of exposure to naringenin, 4.0 μL of the appropriate fluorescent indicator was added to the cells. 2',-7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA), dihydroethidium (DHE), 5-chloromethylfluorescein diacetate (CMFDA) and rhodamine 123 (RHO) for detection of cell toxicity (cell death) using ROS, O_2^-, GSH and MMP, respectively using a FACS Calibur flow cytometer instrument (Becton Dickenson, San Jose, CA).

Intracellular ROS were monitored by H_2DCFDA dye, which is a cell permeable indicator for ROS [33, 45]. The superoxide radical (O_2^-) was monitored by DHE dye, which is cell permeable and reacts with O_2^- to form ethidium that in turn intercalates in DNA and exhibits a red fluorescence [46]. The intracellular reduction of GSH level was monitored by CMFDA dye, which is a fluorescent chloromethyl derivative that freely spreads through the membranes of live cells and exhibits bright green fluorescence in the cytoplasm [47]. The mitochondrial membrane potential (MMP) was monitored by RHO dye, which is a cell permeable cationic dye that exhibits green fluorescence in mitochondria of living cells [48].

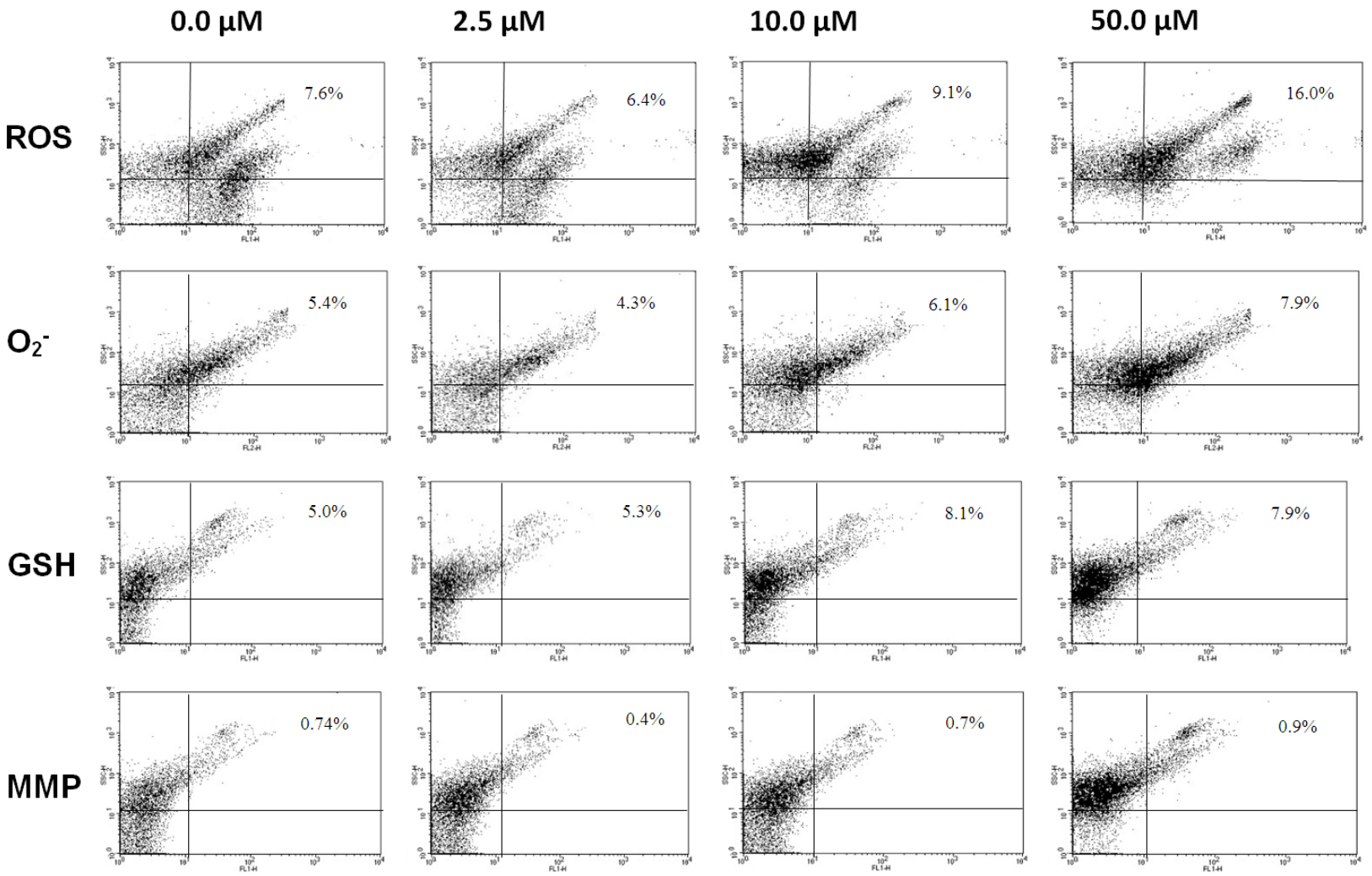

Figure 1. Flow cytometric analysis of NT (MCF-10A Cells) naringenin concentration (µM). Flow cytometric analysis of NT (MCF-10A) following exposure to fluorescent dye for reactive oxygen species (ROS), superoxide radical (O_2^-), glutathione (GSH) and determining of a change in mitochondrial membrane potential (MMP). Percents indicate portion of dead cells reacting positive for each dye at 0.0 µM, 2.5 µM, 10.0 µM and 50.0 µM naringenin.

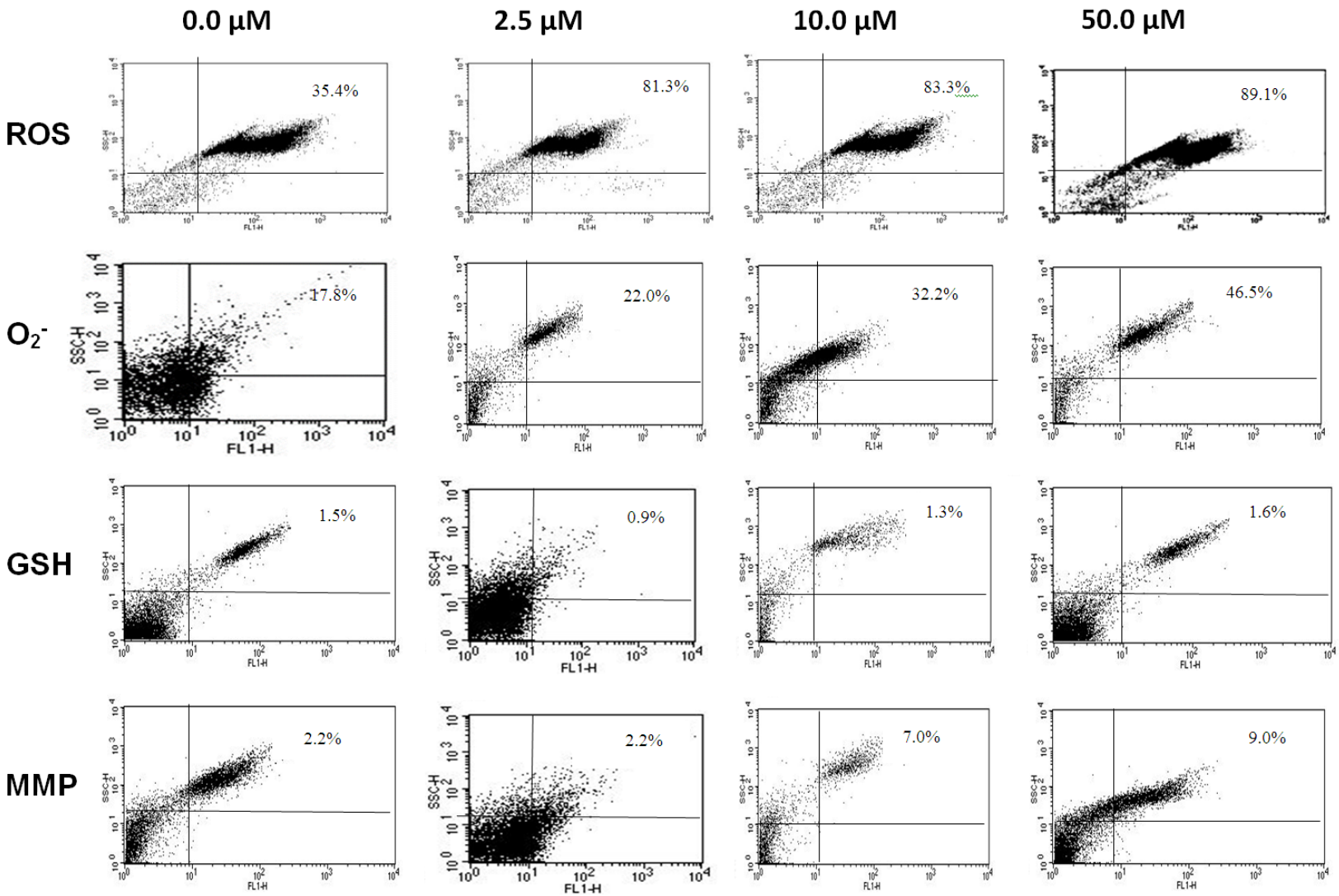

Figure 2. Flow cytometric analysis of ER- (MDA-MB-468 Cells) naringenin concentration (µM). Flow cytometric analysis of ER- (MDA-MB-468) following exposure to fluorescent dye for reactive oxygen species (ROS), superoxide radical (O_2^-), glutathione (GSH) and determining of a change in mitochondrial membrane potential (MMP). Percents indicate portion of dead cells reacting positive for each dye at 0.0 µM, 2.5 µM, 10.0 µM and 50.0 µM naringenin.

FACS calibur flow cytometer

After the breast cells were exposed to H_2DCFDA, DHE, CMFDA and RHO dyes for four hours for detection of ROS, O_2^-, GSH and MMP, respectively; the cells were centrifuged, the supernatant fluid with the dyes was decanted, and the cells were suspended in 1 ml PBS. The fluorescence intensity for 10,000 events for each sample was determined using a FACS Calibur flow cytometer to sort and analyze the breast cells. Results for percent dead cells showing positive for the dye were calculated with Cell Quest software (Becton Dickenson, San Jose, CA).

Statistical analysis

The effects of naringenin (0.0, 2.5, 10.0, and 50.0 μM) and cell type (ER+, ER-, NT) and their interactions resulting in cell death due to oxidative stress (ROS, O_2^-, GSH, and MMP) was assessed by two-way ANOVA using a 4 by 3 factorial design (version 6.0, Prism, GraphPad Software, San Diego, CA). Regardless of which oxidative stressor was assessed, cell death was always affected (P<0.05) by a naringenin by cell type interaction. To better understand these interactions, two means comparison tests were done. First, within a cell type, mean percent of dead cells reacting positive for given dye for 0.0 μM naringenin was compared to mean cell death for 2.5 μM, 10.0 μM, and 50.0 μM naringenin using Dunnet's multiple comparison test at P<0.05. Second, within a dose of naringenin, mean cell death among cell types was compared using Dunnet's multiple comparison test at P<0.05.

Results

Reactive oxygen species (ROS)

The effect of naringenin on percent of dead cells exhibiting a positive reaction with the H_2DCFDA dye (ROS) was significantly (P<0.05) correlated with cell type (NT, ER-, or ER+) and concentration of naringenin in the medium (**Figures 1**-**4**; **Table 1**). With respect to cell type ER- (MDA-MB-468) exhibited the strongest response (**Figure 4**; **Table 1**) with percent mortality of 35.4±1.28%, 81.3±1.0%, 83.3±0.74%, and 89.1±1.34% for 0.0 μM, 2.5 μM, 10.0 μM, 50.0 μM naringenin, respectively. While NT (MCF-10A) and ER+ (MCF-7) showed significant differences (P<0.05) **Figures 1**-**4**; **Table 1**). The percent differences as compared to ER- were not high (NT = 7.6±0.63%, ER+ = 1.9±0.62%, at 0.0 μM naringenin; NT = 6.4±0.24%, ER+ = 12.6±0.79% at 2.5 μM naringenin; NT = 9.1± 0.54%, ER+ = 18.3±0.55% at 10.0 μM naringenin and NT = 16±0.45%, ER+ = 22.2±2.23% at 50 μM naringenin). Between concentrations for ER- there was a very significant (P<0.05) difference between 0.0 μM and 2.5 μM naringenin with percents of 35.4±1.28% and 81.3±1.0% respectively. However, the differences in ROS for ER-, while significant (P<0.05) between 2.5 μM, 10.0 μM and 50.0 μM naringenin, were not as great as between 0.0 μM and 2.5 μM with percents of 81.3±1.0%, 83.3±0.74%, and 89.1±1.34%, respectively. For NT and ER+ there was no significant difference (P<0.05) between naringenin concentrations of 0.0 μM and 2.5 μM. However, between 2.5 μM and 10.0 μM; and 10.0 μM and 50.0 μM naringenin significant (P<0.05) differences were seen with NT showing a change of 6.4±0.24% to 9.1±0.54% between 2.5 μM and 10.0 μM and 9.1±0.54% to 16±0.45% between 10.0 μM and 50.0 μM naringenin. For ER+ a similar response occurred with a change in percent of 12.6±0.79% to 18.3±0.55% between 2.5 μM and 10.0 μM and 18.3±0.55% to 22.2±2.28% between 10.0 μM and 50.0 μM naringenin.

Superoxide (O_2^-)

For superoxide radicals (O_2^-) the effect of naringenin on percent of dead cells exhibiting a positive reaction to DHE dye was again significantly (P<0.05) correlated with cell type (**Figures 1**-**3** and **5**; **Table 1**). For ER- cells a strong and significant (P<0.05) response has seen at all concentrations of naringenin (0.0 μM, 2.5 μM, 10.0 μM and 50.0 μM) with percent dead cells reacting positive at 17.8± 1.06%, 22.0±0.73%, 32.2±0.76% and 46.5± 1.07%, respectively. For NT and ER+ cells differences were significant (P<0.05) but to a lesser in degree than ER- with NT at 5.4±0.70% and ER+ at 2.3±0.44%, NT at 4.3±0.35% and ER+ 6.3±0.41%, NT at 6.1±0.49% and ER+ at 9.6±0.65%, and NT at 7.9±0.59% and ER+ at 22.2±2.23% at 0.0 μM, 2.5 μM, 10.0 μM and 50.0 μM nareningenin, respectively. Between concentrations there was a significant (P< 0.05) difference with ER- cells between 0.0 μM

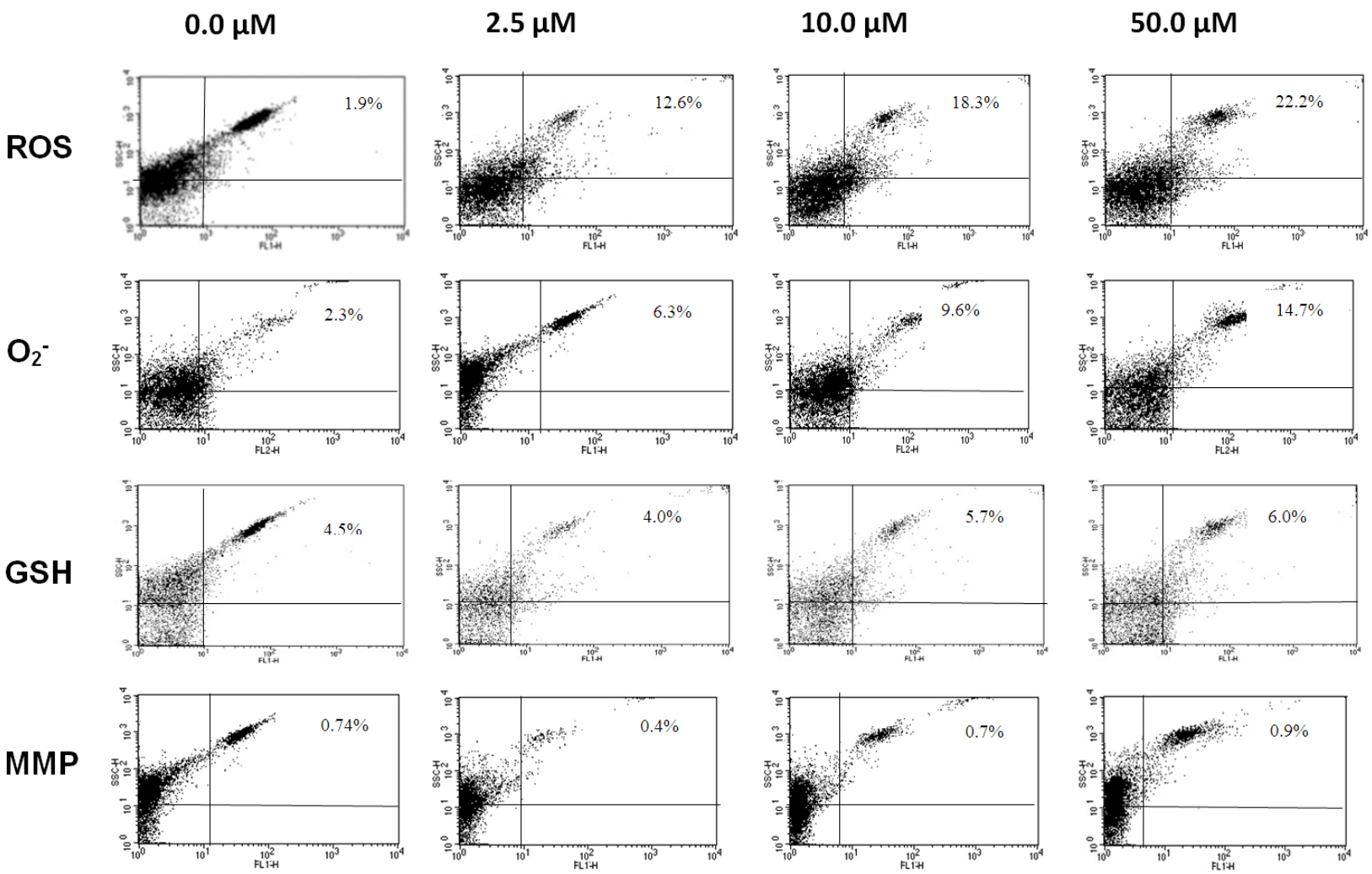

Figure 3. Flow cytometric analysis of ER+ (MCF-7 Cells) naringenin concentration (µM).

Table 1. Percent positive dead NT, ER- and ER+ cells for reactive oxygen species (ROS), superoxide radical (O_2^-), glutathione (GSH), and change in mitochondrial membrane potential (MMP) at 0.0 μM, 2.5 μM, 10.0 μM and 50 μM naringenin

	0.0 μM				2.5 μM				10.0 μM				50.0 μM			
	ROS	O_2^-	GSH	MMP	ROS	O_2^-	GSH	MMP	ROS	O_2^-	GSH	MMP	ROS	O_2^-	GSH	MMP
NT (MCF-10A)	7.6± 0.63%	5.4± 0.70%	5.0± 0.53%	0.74± 0.14%	6.4± 0.24%	4.3± 0.35%	5.3± 0.50%	0.4± 0.12%	9.1± 0.54%	6.1± 0.49%	8.1± 0.39%	0.7± 0.09%	16.0± 0.45%	7.9± 0.82%	7.9± 0.59%	0.9± 0.06%
ER+ (MCF-7)	1.9± 0.62%	2.3± 0.44%	4.5± 0.61%	0.74± 0.19%	12.6± 0.79%	6.3± 0.41%	4.0± 0.50%	0.4± 0.14%	18.3± 0.55%	9.6± 0.65%	5.7± 0.42%	0.7± 0.19%	22.2± 2.23%	14.7± 3.6%	6.0± 0.78%	0.9± 0.09%
ER- (MDA-MB-468)	35.4± 1.28%	17.8± 1.06%	1.5± 0.51%	2.2± 0.52%	81.3± 1.0%	22.0± 0.73%	0.9± 0.52%	2.2± 0.52%	83.3± 0.74%	32.2± 0.76%	1.3± 0.36%	7.0± 0.45%	89.1± 1.34%	46.5± 1.07%	1.6± 0.58%	9.0± 0.62%

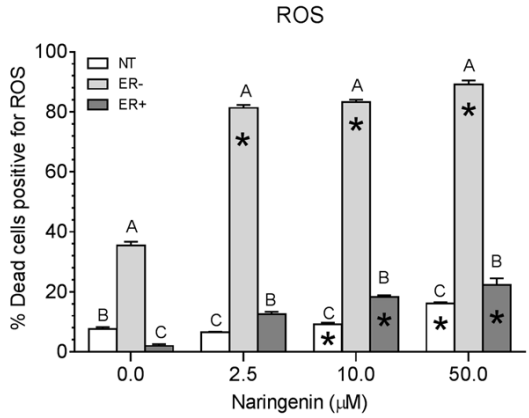

Figure 4. Effect of naringenin on generation of reactive oxygen species (ROS) and cell death in non-tumorigenic (NT), estrogen receptor negative (ER-), and estrogen receptor positive (ER+) breast cancer cell lines. ROS reported as percent dead cells exhibiting a positive reaction with the H_2DCFDA dye. Bars are means ±95% confidence intervals for six samples. Bars within a cell type with an asterisk differ (*P*<0.05) from the corresponding bar at 0 μM naringenin. Bars within a dose of naringenin with different superscripts differ at *P*<0.05.

and 2.5 μM, 2.5 μM and 10.0 μM, and 10.0 μM and 50.0 μM naringenin with percent differences of 17.8±1.06% and 22.0±0.73%, 22.0±0.73% and 32.2±0.76%, 32.2±0.76% and 46.5±1.07%, respectively. Lesser differences (*P*<0.05) were seen with NT and ER+ cells with no significant differences between 0.0 μM and 2.5 μM naringenin (NT = 5.4±0.70% and 4.3±0.35%, ER+ = 2.3±0.44% and 6.3±0.41%). Between 2.5 μM, 10.0 μM and 50.0 μM significant (P<0.05) differences were seen but to a lesser degree than with ER- cells with NT cells exhibiting percents of 4.3±0.35%, 6.1±0.49%, 7.9±0.28% and ER+ cells exhibiting percents of 6.3±0.41%, 9.6±0.65%, and 22.2±2.23% at 2.5 μM, 10.0 μM and 50.0 μM naringenin, respectively.

Glutathione (GSH)

With respect to GSH response following addition of RHO to the cell medium the result was less clear. NT and ER+ cells were not significantly (*P*<0.05) different at 0.0 μM naringenin (5.0±0.53% and 4.5±0.61%, respectively, but ER- was significantly (*P*<0.05) than both at 0.74±0.19% (**Figures 1**-**3** and **6**; **Table 1**). At 2.5 μM naringenin all 3 cell types were significantly (P<0.05) different with NT = 5.3±0.50%, ER+ = 2.2±0.52% and ER- = 0.4±0.14%. The differences at 10.0 μM and 50 μM were again significant (P<0.05) with NT = 8.1±0.39% and 7.9±0.59%, respectively; ER+ = 5.7±0.42% and 6.0±0.78%, respectively; ER- = 1.3±0.36% and 1.6±0.58%, respectively. Between concentrations GSH for NT cells was not significantly (P>0.05) different between 0.0 μM and 2.5 μM naringenin with GSH positive reactions at 5.0±0.53% and 5.3±0.50%, respectively. A significant (P<0.05) difference was seen between 2.5 μM and 10.0 μM naringenin with percents positive of 5.3±0.50% and 8.1±0.39%, respectively. Between 10.0 μM and 50.0 μM naringenin no significant (P<0.05) difference was observed with percents positive of 8.1±0.39% and 7.9±0.59%, respectively. ER- cells did not show any significant (P>0.05) between concentrations with percent positives of 1.5±0.51% at 0.0 μM; 0.9±0.52% at 2.5 μM; 1.3±0.36% at 10.0 μM; and 1.6±0.58% at 50.0 μM naringenin. ER+ cells showed no significant (P<0.05) differences between 0.0 μM and 2.5 μM naringenin with percent positives of 2.2±0.52% and 4.0±0.50%, respectively; but did show significant (P<0.05) differences between 2.5 μM and 10.0 μM naringenin with percent positives of 4.0±0.50% and 5.7±0.42%, respectively. As with NT cells between 10.0 μM and 50 μM naringenin no significant (P<0.05) differences were seen with values at 8.1±0.39% and 7.9±0.59%, respectively.

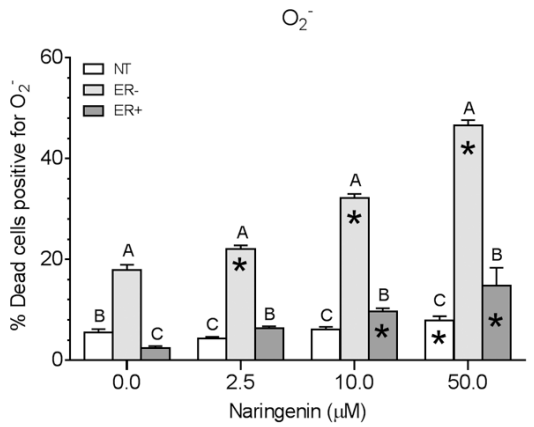

Figure 5. Effect of naringenin on generation of superoxide radicals (O_2^-) and cell death in non-tumorigenic (NT), estrogen receptor negative (ER-), and estrogen receptor positive (ER+) breast cancer cell lines. O_2^- reported as percent dead cells exhibiting a positive reaction with the DHE dye. Bars are means ±95% confidence intervals for six samples. Bars within a cell type with an asterisk differ (P<0.05) from the corresponding bar at 0 μM naringenin. Bars within a dose of naringenin with different superscripts differ at P<0.05.

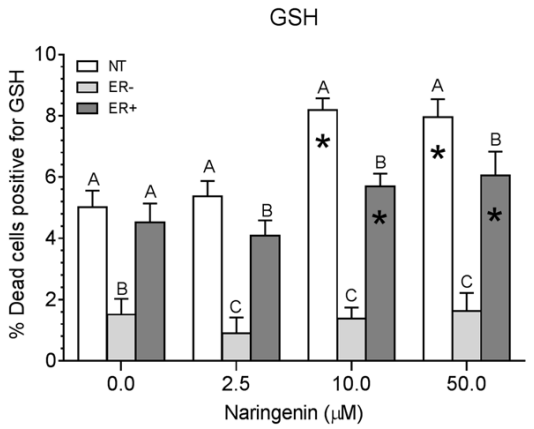

Figure 6. Effect of naringenin on generation of glutathione (GSH) and cell death in non-tumorigenic (NT), estrogen receptor negative (ER-), and estrogen receptor positive (ER+) breast cancer cell lines. GSH reported as percent dead cells exhibiting a positive reaction with the CMFDA dye. Bars are means ±95% confidence intervals for six samples. Bars within a cell type with an asterisk differ (P<0.05) from the corresponding bar at 0 μM naringenin. Bars within a dose of naringenin with different superscripts differ at P<0.05.

Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) was clearly related to cell type for ER-, but not NT or ER+ (**Figures 1**-**3** and **7**; **Table 1**). At all concentrations of naringenin (0.0 μM, 2.5 μM, 10.0 μM and 50.0 μM) NT and ER+ cells did not exhibit any significant (P<0.05) differences with NT cells showing percent positive dead cells of 0.74±0.14% at 0.0 μM, 0.4±0.12% at 2.5 μM, 0.7±0.09% at 10.0 μM and 0.9±0.06% at 50.0 μM; and ER+ cells percents of 0.74±0.19% at 0.0 μM, 0.4±0.14% at 2.5 μM, 0.7±0.19% at 10.0 μM and 0.9±0.09% at 50.0 μM. ER- cells exhibited significant (P<0.05) differences at 0.0 μM and 2.5 μM naringenin with percents of 2.2±0.52% and 2.2±0.52%; and a much more pronounced response at 10.0 μM and 50.0 μM with percents of 7.0±0.45% and 9.0±0.62%. Between concentrations of naringenin no significant (P<0.05) differences were seen in NT and ER+ cells with percents of 0.74±0.14% and 0.74±0.19% for NT and ER+ at 0.0 μM, 0.4±0.12% and 0.4±0.14% at 2.5 μM, 0.7±0.09% and 0.7±0.19% at 10.0 μM and 0.9±0.06% and 0.9±0.09% at 50.0 μM. The ER- cells did show significant (P<0.05) differences between all concentrations of naringenin with percents of 2.2±0.52% at 0.0 μM, 2.2±0.52% at 2.5 μM, 7.0±0.45% at 10.0 μM and 9.0±0.62% at 50.0 μM.

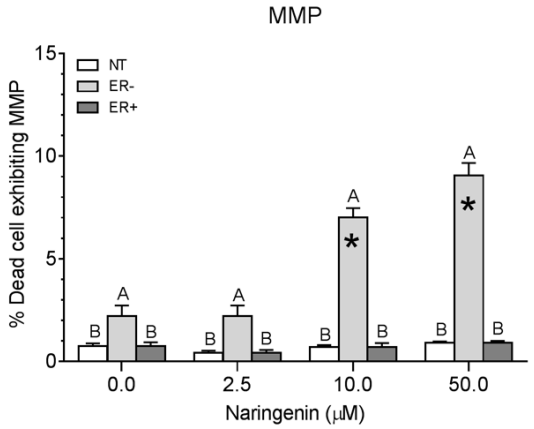

Figure 7. Effect of naringenin on mitochondrial membrane potential (MMP) and cell death in non-tumorigenic (NT), estrogen receptor negative (ER-), and estrogen receptor positive (ER+) breast cancer cell lines. MMP reported as percent dead cells exhibiting a positive reaction with the RHO dye. Bars are means ±95% confidence intervals for six samples. Bars within a cell type with an asterisk differ (P<0.05) from the corresponding bar at 0 μM naringenin. Bars within a dose of naringenin with different superscripts differ at P<0.05.

Discussion

Much interest has been directed toward the use of natural products as preventive and

remedial treatments for a variety of diseases, specifically as anticancer treatment [44, 49-51]. Thousands of flavonoids are known to occur in nature and are one of the largest groups of natural products [52]. As potential anticancer drugs flavonoids have been studied on a variety of cancer cell lines [44, 50, 53-56]. Examining different pathways to determine mode of action [41, 42, 44, 57-61]. The potential beneficial effects of flavonoids on carcinogenesis use in their antioxidative, anti-inflammative, and antiproliferation activities [36, 62, 63]. Naringenin, a flavonoid found in high concentrations in grapefruit and other citrus fruits [63, 64-66], has been shown to exhibit antiproliferative effects and was able to cause death in various cancer cell lines [14, 49, 56, 67]. The study reported here applied flow cytometry and specific fluorescent dyes (H_2DCFDA, DHE, CMFDA and RHO) to evaluate intracellular ROS, O_2^-, GSH and MMP responses in NT, ER- and ER+ human breast cell lines. It is known that oxidative damage to mitochondrial membranes from ROS and O_2^- is involved in many diseases such as Alzheimer's disease [56] and cancer [68, 69]. In order to better understand the intracellular process involved in inducing cell death due to naringenin we determined the ROS and O_2^- responses to varying concentrations of naringenin and corresponding changes in GSH and MMP.

In examining the intracellular production of ROS and O_2^- at varying concentrations of naringenin in the media ROS showed a significant increase in ER- cells between 0.0 μM and 2.5 μM naringenin and while not significant between 2.5 μM and 10.0 μM, and 10.0 μM and 50.0 μM ROS response remained high at all three naringenin concentrations (**Figure 4**). This corresponds to O_2^- values (**Figure 5**) where there was a direct coordination with recent dead cells showing positive increasing as naringenin concentrations increased. This suggests that both ROS and O_2^- in ER- cells are profoundly affected by naringenin. However, this was not the case with NT and ER+ cells where ROS percents (**Figure 4**) remained the same between 0.0 μM and 2.5 μM naringenin and between 2.5 μM and 10.0 μM, and 10.0 μM and 50.0 μM naringenin showing significance for both NT and ER+ cells between 2.5 μM and 10.0 μM naringenin but not significant between 10.0 μM and 50.0 μM naringenin. These results are similar to O_2^- percents with significant differences between 10.0 μM and 50.0 μM naringenin for NT and ER+ cells. These results support the assumption that bioflavonoid compounds can induce growth inhibition in breast tumor cells through increasing rates of apoptosis [69-71] and support the finding of McLean [72] where amino-flavones induce ROS formation and oxidative DNA damage in ER- breast cell lines. In a review by Sac [53] there is substantial evidence that flavonoids through induction of oxidative stress inhibit tumor growth in ER- cells. The O_2^- radical can rapidly change into other reactive oxygen species such as hydrogen peroxide and hydroxyl radicals [21, 73-76] thus it is clear that naringenin has significant effect in increase ROS and O_2^- in estrogen negative breast tumor cell lines which is supported by other studies [70, 71, 73, 77].

Protective mechanisms such as GSH serve to break down free radicals and minimize oxidative damage [19, 23]. In this study GSH response appeared to be minimal (**Figure 6**) with the highest percent of cells positive for GSH at 8.1±0.39% in NT cells. Significant increases in GSH did not occur until 10.0 μM naringenin was added to the medium for NT and ER+ cells. ER- cells did not respond to increasing naringenin; which when compared with ROS and O_2^- responses showing similarly low percents confirming studies [26] that ROS, O_2^- and GSH production are much lower in non-malignant cell line [26, 78-80]. In general with GSH levels remaining low in spite of increase in ROS and O_2^- as naringenin concentration were increased, it would appear that naringenin may, as suggested by others [12, 41, 78, 81, 82] that flavonoids can cause GSH to be depleted and increase ROS. Whether this is due to GSH being converted to GSSH (Glutathione disulfide) at a rate that far exceeds regeneration or if some other mechanism is involved cannot be answered by this study. None the less, GSH is important in preventing cell death [20, 22, 23, 83, 84].

Cell death is usually proceeded by mitochondrial degeneration [28] which was determined in this study by the uptake of the RHO dye by mitochondria in dead cells indicating a change in the MMP [28, 30, 31, 37, 85-87]. In this study MMP did not show any significant change in percent cell positive in ER- cells between 0.0

μM and 2.5 μM naringenin, but did exhibit significant differences between 2.5 μM and 10.0 μM, and 10.0 μM and 50.0 μM naringenin showing a near linear response (**Figure 7**). This is consistent with finding by Suski [88] who found that during apoptosis MMP and activity of the respiratory chain decreased coincident with an increase in ROS production. However, for NT and ER+ no significant changes in MMP were detected (**Figure 7**). Overall the percent of dead cells positive for RHO dye was low with the highest percent at 9.0±0.62% for ER- cells. This suggests that other mechanisms may be at play affecting mitochondrial integrity including deficiency in oxidizable substances for mitochondria, blockage of respiration, and withdrawal of growth factors and lack of extra cellular glucose supply [27, 89-92]. In addition, pathways leading to cell death do not necessarily involve mitochondria but rather can directly affect nuclear DNA leading to cell death.

Take in it is entirety there was a clear difference in cell susceptibility to naringenin between NT, ER- and ER+ cells with respect to oxidative stress. Of significant ROS and O_2^- percents (**Figures 4** and **5**) were highest in ER- cells which is in agreement with other studies [26, 79] were flavonoids exhibited low cytotoxicity in normal human cells. GSH while not showing high percent positive cells was more significant in NT and ER+ cells, and very low in ER- cells (**Figure 6**). As noted previously this may be due to high ROS and O_2^- which overwhelms the rate at which GSH is produced or regenerated from GSSH [23, 35, 93]. The change in MMP was clear in ER- cells, but low; which suggests that naringenin did increase oxidative stress on mitochondria as shown by several researches [85-87, 91]. It is probably not the major pathway leading to cell death as discussed by several authors [59]. Since anti-cancer therapeutic strategies are focusing on oxidative stress [20, 94-97] early studies have suggested that cancer cells have high metabolic activities and therefore produce high levels of ROS as compared with normal cells which in this study only ER- showed high ROS and O_2^- activity and is consistent with previous studies [68, 70-72, 74].

Acknowledgements

This research was supported by a U56 grant from National Institute of Health (NIH). Thanks to the Toxicology Laboratory at the Department of Natural Sciences at the University of Maryland Eastern Shore for equipment and facility support. Special thanks go to Dr. Jeanine Harter-Dennis for her invaluable assistance in statistical analysis of the data and to Dr. Thomas P. Oscar for his review and helpful suggestions during preparation of this work for publication.

Disclosure of conflict of interest

None.

Address correspondence to: Maryam Taabodi, Department of Natural Sciences, University of Maryland Eastern Shore, MD, USA. Tel: 410-651-7527; Fax: 410-651-7739; E-mail: mtaabodi@umes.edu

References

[1] United States Cancer Statistics. 2007. www.cdc.gov/cancer.

[2] Joslyn SA and West MM. Racial differences in breast carcinoma survival. Cancer 2000; 88: 114-123.

[3] Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S, Deming SL, Geradts J, Cheang MC, Nielsen TO, Moorman PG, Earp HS, Millikan RC. Race, breast cancer subtypes, and survival in the Carolina breast cancer study. JAMA 2006; 295: 2492-2502.

[4] Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T and Thun MJ. Cancer statistics. CA Cancer J Clin 2008; 58: 71-96.

[5] Joslyn SA. Hormone receptors in breast cancer: racial differences in distribution and survival. Breast Cancer Res Treat 2002; 73: 45-59.

[6] Bauer KE, Brown M, Cress RD, Parise CA and Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype. Cancer 2007; 109: 1721-1728.

[7] Dunnwald LK, Rossing MA and Li CI. Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. Breast cancer Res 2007; 9: R6.

[8] Sineshaw HM, Gaudet M, Ward EM, Flanders WD, Desantis C, Lin CC and Jemal A. Association of race/ethnicity, socioeconomic status, and breast cancer subtypes in the national cancer data base (2010-2011). Breast Cancer Res Treat 2014; 145: 753-763.

[9] Li CI, Malone KE and Daling JR. Differences in breast cancer stage, treatment, and survival

by race and ethnicity. Arch Intern Med 2003; 163: 49-56.
[10] Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, Hess KR, Stec J, Ayers M, Wagner P, Morandi P, Fan C, Rabiul I, Ross JS, Hortobagyi GN, Pusztai L. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res 2005; 11: 5678-5685.
[11] Gibellini L, Pinti M, Nasi M, De Biasi S, Roat E, Bertoncelli L and Cossarizza A. Interfering with ROS metabolism in cancer cells: the potential role of quercetin. Cancer (Basel) 2010; 2: 1288-1311.
[12] Surveillance, epidemiology, and end results (SEER). 2011.
[13] Kanno S, Tomizawa A, Hiura T, Osanai Y, Shouji A, Ujibe M, Ohtake T, Kimura K and Ishikawa M. Inhibitory effects of naringenin on tumor growth in human cancer cell lines and sarcoma S-180-implanted mice. Biol Pharm Bull 2005; 28: 527-530.
[14] Khandrika L, Kumar B, Koul S, Maroni P and Koul KH. Oxidative stress in prostate cancer. Cancer Lett 2009; 282: 125-136.
[15] Deneke SM, Baxter DF, Phelps DT, Fanburg BL. Regulation of cellular glutathione. Am J Physiol 1989; 257: L163-173.
[16] Tan S, Sagara Y, Liu Y, Maher P and Schubert D. The regulation of reactive oxygen species production during programmed cell death. J Cell Biol 1998; 141: 1423-1432.
[17] Filomeni G, Rotilio G and Ciriolo MR. Disulfide relays and phosphorylative cascades: partners in redox-mediated signaling pathways. Cell Death Differ 2005; 12: 1555-1563.
[18] Valko M, Rhodes CJ, Moncol J, Izakovic M and Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006; 160: 1-40.
[19] Du ZX, Zhang HY, Meng X, Guan Y and Wang HQ. Role of oxidative stress and intracellular glutathione in the sensitivity to apoptosis induced by proteasome inhibitor in thyroid cancer cells. BMC Cancer 2010; 9: 56.
[20] Liou GY and Storz P. Reactive oxygen species in cancer. Free Radic Res 2010; 44: 497-496.
[21] Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, Marinari UM and Domenicotti C. Role of glutathione in cancer progression and chemoresistance. Oxid Med Cell Longev 2013; 2013: 972913.
[22] Schafer FQ and Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 2001; 30: 1191-212.
[23] Santos RX, Correia SC, Zhu X, Smith MA, Moreira PI, Castellani RJ, Nunomura A and Perry G. Mitochondrial DNA oxidative damage and repair in aging and Alzheimer's disease. Antioxid Redox Signal 2013; 18: 2444-2457.
[24] Cooke MS, Evans MD, Dizdaroglu M and Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J 2003; 17: 1195-1214.
[25] Syng-Ai C, Kumari AL and Khar A. Effect of curcumin on normal and tumor cells: role of glutathione and bcl-2. Mole Cancer Ther 2004; 3: 1101-1108.
[26] Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH and Thompson CB. Growth factors can influence cell growth and survival through effects on glucose metabolism. Mol Cell Biol 2001; 21: 5899-5912.
[27] Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007; 35: 495-516.
[28] Perry SW, Norman JP, Barbieri J, Brown EB and Gelbard HA. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. Biotechniques 2011; 50: 98-115.
[29] Ouyang L, Zhao S, Wang FT, Zhou TT, Liu B and Bao JK. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell Prolif 2012; 45: 487-498.
[30] Kroemer G, Zamzami N and Susin SA. Mitochondrial control of apoptosis. Immunol Today 1997; 18: 44-51.
[31] de Groot H and Rauen U. Tissue injury by reactive oxygen species and the protective effects of flavonoids. Fund Clin Pharmacol 1998; 12: 249-255.
[32] Chompoosor A, Saha K, Ghosh PS, Macarthy DJ, Miranda OR, Zhu ZJ, Arcaro KF and Rotello VM. The role of surface functionality on acute cytotoxicity, ROS generation and DNA damage by cationic gold nanoparticles. Small 2010; 6: 2246-2249.
[33] Maharjan S, Oku M, Tsuda M, Hoseki J and Sakai Y. Mitochondrial impairment triggers cytosolic oxidative stress and cell death following proteasome inhibition. Sci Rep 2014; 4: 5896-5907.
[34] Circu M and Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med 2010; 48: 749-762.
[35] Hidalgo M, Sánchez-Moreno C and Pascual-Teresa S. Flavonoid-flavonoid interaction and its effect on their antioxidant activity. Food Chem 2010; 121: 691-696.
[36] Mikhed Y, Daiber A and Steven S. Mitochondrial oxidative stress, mitochondrial DNA damage and their role in age-related vascular dysfunction. Int J Mol Sci 2015; 16: 15918-15953.
[37] Stoner GD and Mukhtar H. Polyphenols as cancer chemopreventive agents. J Cell Biochem Suppl 1995; 22: 169-180.

[38] Brownson DM, Azios NG, Fuqua BK, Dharmawardhane SF and Mabry TJ. Flavonoid effects relevant to cancer. J Nutr 2002; 132: 3482S-3489S.
[39] Chae SC, Lee JH and Sang SU. Recent studies on flavonoids and their antioxidant activities. EXCLI J 2013; 12: 226-230.
[40] Galati G, Moridani MY, Chan TS and O'Brien PJ. Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: glutathione oxidation and conjugation. Free Radic Biol Med 2001; 30: 370-382.
[41] Galati G, Sabzevari O, Wilson JX and O'Brien PJ. Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. Toxicology 2002; 177: 91-104.
[42] Kanno S, Shouji A, Hirata R, Asou K and Ishikawa M. Effects of naringin on cytosine arabinoside (Ara-C)-induced cytotoxicity and apoptosis in P388 cells. Life Sci 2004; 75: 353-365.
[43] Kumar S and Pandey AK. Chemistry and biological activities of flavonoids: an overview. Sci World J 2013; 11-12: 162750.
[44] Leshem Y, Seri L and Levine A. Induction of phosphatidylinositol 3-kinase-mediated endocytosis by salt stress leads to intracellular production of reactive oxygen species and salt tolerance. Plant J 2007; 51: 185-197.
[45] Li GX, Hu H, Jiang C, Schuster T and Lü J. Differential involvement of reactive oxygen species in apoptosis induced by two classes of selenium compounds in human prostate cancer cells. Int J Cancer 2007; 120: 2034-2043.
[46] Markovic J, Borrás C, Ortega Á, Sastre J, Viña J and Pallardó FV. Glutathione is recruited into the nucleus in early phases of cell proliferation. J Biol Chem 2007; 282: 20416-20424.
[47] Scaduto RC Jr and Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J 1999; 76: 469-477.
[48] Virgili F, Acconcia F, Ambra R, Rinna A, Totta P and Marino M. Nutritional flavonoids modulate estrogen receptor alpha signaling. IUBMB Life 2004; 56: 145-151.
[49] Li RF, Feng YQ, Chen JH, Ge LT, Xiao SY and Zuo XL. Naringenin suppresses K562 human leukemia cell proliferation and ameliorates Adriamycin-induced oxidative damage in polymorphonuclear leukocytes. Exp Ther Med 2015; 9: 697-706.
[50] Luo Y, Li B, Liu D, Zhang L, Chen Y, Jia B, Zeng B, Zhao H and Yuan Y. Engineered biosynthesis of natural products in heterologous hosts. Chem Soc Rev 2015; 44: 5265-5290.
[51] Keinan-Boker L, van Der Schouw YT, Grobbee DE and Peeters PH. Dietary phytoestrogens and breast cancer risk. Am J Clin Nutr 2004; 79: 282-288.
[52] Liu HL, Jiang WB and Xie MX. Flavonoids: recent advances as anticancer drugs. Recent Pat Anticancer Drug Discov 2010; 5: 152-164.
[53] Sak K. Cytotoxicity of dietary flavonoids on different human cancer types. Phcog Rev 2014; 8: 122-146.
[54] Mohamed SA, Orabi KY, Al-Quattan E and Al-Attiyah RJ. Growth inhibitory and chemo-sensitization effects of naringenin, a natural flavanone purified from Thymus vulgaris, on human breast and colorectal cancer. Cancer Cell Int 2015; 15: 46.
[55] Kocyigit A, Koyuncu I, Dikilitas M, Bahadori F and Turkkon B. Cytotoxic, genotoxic and apoptotic effects of naringenin-oxime relative to naringenin on normal and cancer cell lines. APJTB 2016; 6: 872-880.
[56] Wardman P. The fate of glutathione thiyl radicals in biological systems. Free Radic Biol Med 1990; 9: 62.
[57] Sadik CD, Seis H and Schewe T. Inhibition of 15-lipoxigenases by flavonoids: structure-activity relations and mode of action. Biochem Pharmacol 2003; 65: 773-781.
[58] Duprez L, Wirawan E, Berghe TV and Vandenabeele P. Major cell death pathway at a glance. Microbial Infec 2009; 11: 1050-1062.
[59] Rietjens IM, Sotoca AM, Vervoor J and Louisse J. Mechanisms underlying the dualistic mode of action of major soy isoflavones in relation to cell proliferation and cancer risks. Mol Nutr Food Res 2013; 57: 100-113.
[60] Kuete V, Nkuete AH, Mbaveng AT, Wiench B, Wabo HK, Tane P and Effertha T. Cytotoxicity and modes of action of 4-hydroxy-2, 6-dimethoxychalcone and other flavonoids toward drug-sensitive and multidrug-resistant cancer cell lines. Phytomed 2014; 21: 1651-1657.
[61] Middleton E, Kandaswami C and Theoharides TC. The effects of plant flavonoids in mammalian cells: implication for inflammation, heart disease and cancer. Pharmacol Rev 2000; 52: 673-751.
[62] Meiyanto E, Hermawan A and Anindyajati I. Natural products for cancer-targeted therapy: citrus flavonoids as potent chemopreventive agents. Asian Pac J Cancer Prev 2012; 13: 427-36.
[63] Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S and Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997; 138: 863-870.
[64] Mayer IA. Treatment of HER2-positive metastatic breast cancer following initial progression. Clin Breast Cancer 2009; 9: S50-7.

[65] Thilakarathna SH and Rupasinghe HP. Flavonoid bioavailability and attempts for bioavailability enhancement. Nutrients 2013; 5: 3367-3387.
[66] Song HM, Park GH, Eo HJ, Lee JW, Kim MK, Lee JR, Lee MH, Koo JS and Jeong JB. Anti-proliferative effect of naringenin through p38-dependent downregulation of cyclin D1 in human colorectal cancer cells. Biomol Ther (Seoul) 2015; 23: 339-344.
[67] Chen YC, Shen SC and Lin HY. Rutinoside at C7 attenuates the apoptosis-inducing activity of flavonoids. Biochem Pharmacol 2003; 66: 1139-1150.
[68] Masgras I, Carrera S, de Verdier PJ, Brennan P, Majid A, Makhtar W, Tulchinsky E, Jones GD, Roninson IB and Macip S. Reactive oxygen species and mitochondrial sensitivity to oxidative stress determine induction of cancer cell death by p21. J Biol Chem 2012; 287: 9845-9854.
[69] Murray TJ, Yang X and Sherr DH. Growth of a human mammary tumor cell line is blocked by galangin, a naturally occurring bioflavonoid, and is accompanied by down-regulation of cyclins D3, E, and A. Breast Cancer Res 2006; 8: R17.
[70] Pedro M, Lourenço CF, Cidade H, Kijjoa A, Pinto M and Nascimento MS. Effects of natural prenylated flavones in the phenotypical ER (+) MCF-7 and ER (-) MDA-MB-231 human breast cancer cells. Toxicol Lett 2006; 164: 24-36.
[71] McLean L, Soto U, Agama K, Francis J, Jimenez R, Pommier Y and Brantley E. Aminoflavone induces oxidative DNA damage and reactive oxidative species-mediated apoptosis in breast cancer cells. Int J Cancer 2008; 122: 1665-1674.
[72] Szatrowski TP and Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 1991; 51: 794-798.
[73] Fridovich I. Superoxide anion radical (O_2^-), superoxide dismutases, and related matters. J Biol Chem 1997; 272: 18515-18517.
[74] Perales-Clemente E, Folmes CD and Terzic A. Metabolic regulation of redox status in stem cells. Antioxid Redox Signal 2014; 21:1648-1659.
[75] Schieber M and Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol 2014; 24: R453-R462.
[76] Wang L and Di LJ. BRCA1 and estrogen/estrogen receptor in breast cancer: where they interact? Int J Biol Sci 2014; 10: 566-575.
[77] Matsuo M, Sasaki N, Saga K and Kaneko T. Cytotoxicity of flavonoids toward cultured normal human cells. Biol Pharm Bull 2005; 28: 253-259.
[78] Robert SM, Ogunrinu-Babarinde T, Holt KT and Sontheimer H. Role of glutamate transporters in redox homeostasis of the brain. Neurochem Int 2014; 73: 181-191.
[79] Stanley JA, Neelamohan R, Suthagar E, Vengatesh G, Jayakumar J, Chandrasekaran M, Banu SK and Aruldhas MM. Lipid peroxidation and antioxidants status in human malignant and non-malignant thyroid tumors. Hum Exp Toxicol 2016; 35: 585-597.
[80] Alam MA, Subhan N, Rah MM, Uddin SJ, Reza HM, Sarker SD. Effect of citrus flavonoids, naringin and naringenin, on metabolic syndrome and their mechanisms of action. Adv Nutr 2014; 5: 404-417.
[81] Scherbakov AM and Andreeva OE. Apigenin inhibits growth of breast cancer cells: the role of ERα and HER2/neu. Acta Naturae 2015; 7: 133-139.
[82] Day RM and Suzuki YJ. Cell proliferation, reactive oxygen and cellular glutathione. Dose Response 2005; 3: 425-442.
[83] Tessoulin B, Descamps G, Moreau P, Maïga S, Lodé L, Godon C, Marionneau-Lambot S, Oullier T, Le Gouill S, Amiot M and Pellat-Deceunynck C. PRIMA-1 Met induces myeloma cell death independent of p53 by impairing the GSH/ROS balance. Blood 2014; 124: 1626-1616.
[84] Szeto HH. Cell-permeable, mitochondrial-targeted, peptide antioxidants. AAPS J 2006; 8: E277-E283.
[85] Zhang DX and Gutterman DD. Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. Am J Physiol Heart Circ Physiol 2006; 292: H2023-31.
[86] Blasiak J, Glowacki S, Kauppinen A and Kaarniranta K. Mitochondrial and nuclear DNA damage and repair in age-related macular degeneration. Int. J Mol Sci 2013; 14: 2996-3010.
[87] Suski JM, Lebiedzinska M, Bonora M, Pinton P, Duszynski J and Wieckowski MR. Relation between mitochondrial membrane potential and ROS formation. Methods Mol Biol 2012; 810: 183-205.
[88] Halestrap AP. The regulation of the matrix volume of mammalian mitochondria in vivo and in vitro and its role in the control of mitochondrial metabolism. Biochim Biophys Acta 1989; 973: 355-382.
[89] Kan O, Baldwin SA and Whetton AD. Apoptosis is regulated by the rate of glucose transport in an interleukin 3 dependent cell line. J Exp Med 1994; 180: 917-923.
[90] Kroemer G and Reed JC. Mitochondrial control of cell death. Nature Med 2000; 6: 513-519.
[91] Bernardi P, Petronilli V, Di Lisa F and Forte M. A mitochondrial perspective on cell death. Trends Biochem Sci 2001; 26: 112-117.

[92] Anderson CP, Tsai JM, Meek WE, Liu RM, Tang Y, Forman HJ, Reynolds CP. Depletion of glutathione by buthionine sulfoxine is cytotoxic for human neuroblastoma cells lines via apoptosis. Exp Cell Res 1999; 246: 183-192.

[93] Lecane PS, Karaman MW, Sirisawad M, Naumovski L, Miller RA, Hacia JG and Magda D. Motexafin gadolinium and zinc induce oxidative stress responses and apoptosis in B-cell lymphoma lines. Cancer Res 2005; 65: 11676-11688.

[94] Trachootham D, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H and Huang P. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by β-phenylethyl isothiocyanate. Cancer Cell 2006; 10: 241-252.

[95] Allen BG, Bhatia SK, Buatti JM, Brandt KE, Lindholm KE, Button AM, Szweda LI, Smith BJ, Spitz DR and Fath MA. Ketogenic diets enhance oxidative stress and radio-chemo-therapy responses in lung cancer xenografts. Clin Cancer Res 2013; 19: 3905-3913.

[96] Gorrini C, Harris IS and Mak TW. Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discov 2013; 12: 931-947.

[97] Hecht F, Pessoa CF, Gentile LB, Rosenthal D, Carvalho DP and Fortunato RS. The role of oxidative stress on breast cancer development and therapy. Tumour Biol 2016; 37: 4281-4291.