Induction of Apoptotic Cell Death by the Dietary Flavonol, Quercetin, in MCF-7 and MDA-MB-231 Breast Adenocarcinoma Cell Lines

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Abstract

Numerous studies have provided considerable evidence on quercetin's potential as a chemotherapeutic agent. Quercetin is a highly bioavailable dietary flavonoid that induces apoptosis in different cancer cell lines. Currently, the reported effects of this compound have been observed at concentration ranges of 40-250 µM. The purpose of this study is to investigate the apoptotic effects of quercetin in a dose- and time-dependent manner, at concentration ranges of 300-600 µM. At higher concentrations, there was no clear and observable relationship between the dose- and time-dependent treatment of quercetin and the number of viable MDA-MB-231 and MCF7 breast cancer cells as well as the percentage of apoptotic bodies. These unexpected results could be either attributed to the limitations of the experimental design, or could prove to be a novel finding. However, as with the latter, such inferences could not be drawn due to constraints in time and resources. For future experiments, a more comprehensive and elaborate experimental design is warranted.

Introduction

Breast cancer is the second leading cause of cancer-related deaths and a significant problem in terms of both morbidity and mortality in Canada (Canadian Cancer Society 2013). It is a complex, heterogeneous disease caused by an interplay of both non-genetic and genetic factors, and comprises different entities with distinct clinical, morphological, and molecular characteristics; each representing unique sets of risk factors, pathology, clinical manifestation, responses to therapy, and outcomes (Mavaddat et al 2010, Weigelt et al 2010).

One of the major hallmarks of cancer, as described by Hanahan and Weinberg (2011), is the ability to resist cell death. Apoptosis is a form of cell death in which the cell is disassembled progressively and is taken up by nearby cells or phagocytes (Okada and Mak 2004).Apoptosis signalling pathways are divided into the death receptor pathway, or extrinsic pathway, that is induced by cytokines of the tumor necrosis factor (TNF) family secreted by cytotoxic T cells or natural killer (NK) cells, which includes TNF-α and TNF-related apoptosis-inducing ligand (TRAIL); and the mitochondrial pathway, or intrinsic pathway, which integrates various intracellular signals in response to genotoxic stress (Strohecker 2008, Russo et al 2012). Commitment to apoptosis in the extrinsic and intrinsic pathways is caused by the activation of initiator caspases-8 and -9, respectively, resulting in a proteolytic cascade and the eventual activation of effector caspases such as caspase-3; the main effector caspase that cleaves most cellular components in apoptotic cells (Figure 3; Kumar 2007, Hanahan and Weinberg 2011). Ultimately, these effector caspases execute apoptosis via proteolytic cleavage of ICAD (inhibitor of CAD) in the DNA fragmentation factor (DFF) complex, resulting in the release and activation

of the endonuclease CAD (caspase-activated DNase) and subsequent cleavage of chromosomal DNA (Degen et al 2000).

The study of naturally-occurring compounds has recently garnered attention because of their presumed low toxicity and potential to be chemotherapeutic agents. An example of such compounds include flavonoids, a large group of biologically active, polyphenolic compounds present in plants. Quercetin (Qu; 3,3',4',5,7-pentahydroxylflavone) is a highly bioavailable dietary flavonoid commonly present in plant-derived foods such as apples, tomatoes, and onions (Murakami et al 2008, Gibellini 2011, Russo et al 2012). Quercetin has gained much attention as a potential chemotherapeutic agent, with its effects targeting various mechanisms attributed to the growth, survival, and development of cancer. Numerous studies have consistently provided evidence of the pro-apoptotic effects of quercetin in various cancer cell lines and tumors, such as cervical, glioma, colorectal, and leukemia cancers (Dajas 2012, Russo et al 2012). From these studies, quercetin was observed to act on both intrinsic and extrinsic pathways of apoptosis through interactions with the receptors and various proteins. In studies involving MCF-7 and MDA-MB-231 cell lines, quercetin has been shown to induce apoptosis within concentrations ranging from 12.5-300 μM. Results from these studies show that the apoptotic property of quercetin increases with concentration (Chien et al 2009, Duo et al 2012, Pham et al 2012, Deng et al 2013, Huang et al 2013); however, effects of quercetin at higher concentrations have not been reported. Therefore, in this study, the apoptotic effects of quercetin in human breast cancer cell lines MDA-MB-231 and MCF7 will be examined.

Materials and methods

Chemicals and reagents

Quercetin (product no: Q4951) was obtained as a solid from Sigma-Aldrich Canada Co. (Oakville, ON, Canada) and was dissolved to its stock concentration of 0.0992M with dimethyl sulfoxide (DMSO). RPMI-1640 medium (SKU # 22409-015) and fetal bovine serum (FBS; SKU # 12483-020) by Gibco were obtained from Life Technologies (Burlington, ON, Canada). XTT Cell Viability Assay Kit (Cat. #: 30007) was purchased from Biotium (Hayward, CA, USA).

Cell culture

MDA-MB-231 and MCF-7 cell lines were obtained from Concordia University College of Alberta (Edmonton, AB). Cell lines were plated in 75 cm^2 tissue culture flasks and grown in 12mL RPMI supplemented with 10% FBS at 37^0 C and 5% CO_2, under humidified conditions. Medium was replaced every two to three days. Confluent cells were split through digestion with 0.25% Trypsin-EDTA solution and resuspended to a cell concentration of ~$1x10^6$ cells/mL.

XTT assay

An XTT cell proliferation assay was used to determine the percent viability of the samples in the 24-hour and 48-hour treatments. Procedures were followed as recommended in the Cell Proliferation Kit by Biotium. Because of the extreme sensitivity of the test, a low concentration of ~$5x10^3$ cells per well of MDA-MB-231 and MCF7 cells would be used. Cells were initially trypsinized and 100 µL of the solution were transferred to 96-well plates and incubated for 24-hours. After incubation, media was removed from the wells and replaced with

100 µL of either DMSO alone or 0, 300, 450, and 600 µM quercetin in RPMI with 10% FBS media. After 24 and 48 hours incubation, 50 µL of XTT (2,3-Bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide salt) solution was added to each well, and the plate was incubated at 37^0 C between 5 to 24 hours. Absorbance of cells were measured at a wavelength of 450 nm at a background of 655 nm through a spectrophotometer plate reader.

DAPI Staining

A DAPI staining protocol was done to determine apoptotic cell death in the cell samples in the 24-hour and 48-hour treatments. Cells grown in 75 cm^2 tissue culture flasks were trypsinized and 2ml were transferred in 6-well plates containing 70% EtOH-sterilized microscope coverslips, and was incubated for 24 hours at 37^0 C. Each well contained ~100,000 cells. After incubation, media was removed from the wells and replaced with 2ml of either DMSO alone or 0, 300, 450, and 600 µM quercetin in RPMI with 10% FBS media. After 24 and 48 hours incubation, cells were stained by 4',6-diamidino-2-phenylindole (DAPI) fluorescent stain, with procedures followed as recommended by Chazotte (2011), and visualized and photographed under a fluorescent microscope.

Statistical analysis

For XTT analysis, a student's t-test via Microsoft Excel was used to analyze the differences between the control and the quercetin-treated groups. From nine replicates in each experiment, data was expressed as mean ±SE.

Results

Cytotoxic effects of quercetin on human breast cancer cell lines MDA-MB-231 and MCF7

The potential cytotoxic effects of quercetin on human breast cell lines MDA-MB-231 and MCF7 were investigated via XTT assay. Cells were treated with different concentrations of quercetin for 24 and 48 hours. In MDA-MB-231 cell lines, quercetin slightly decreased the percentage of viable cells compared to control cells in the 24-hour, but no significant effect was found in the 48-hour (Figures 1A and 1B). In MCF7 cell lines, no significant effects were observed in the 24-hours, but there is an increase in viable cells compared to control cells in 48-hour treatment (Figures 2A and 2B).

Effects of quercetin on apoptosis in MDA-MB-231 and MCF7 cells

MDA-MB-231 and MCF7 cells were treated with different concentrations of quercetin for 24 and 48 hours. In both cell lines, apoptotis was observed in all treatments (Figures 3 and 4). As shown in Figure 3, compared to the control, the percent of apoptotic MDA-MB-231 cells slightly increased with concentration in both 24 and 48-hour treatments, whereas no observable effects were observed in both 24 and 48-hour treatments in MCF7 cells (Figure 4).

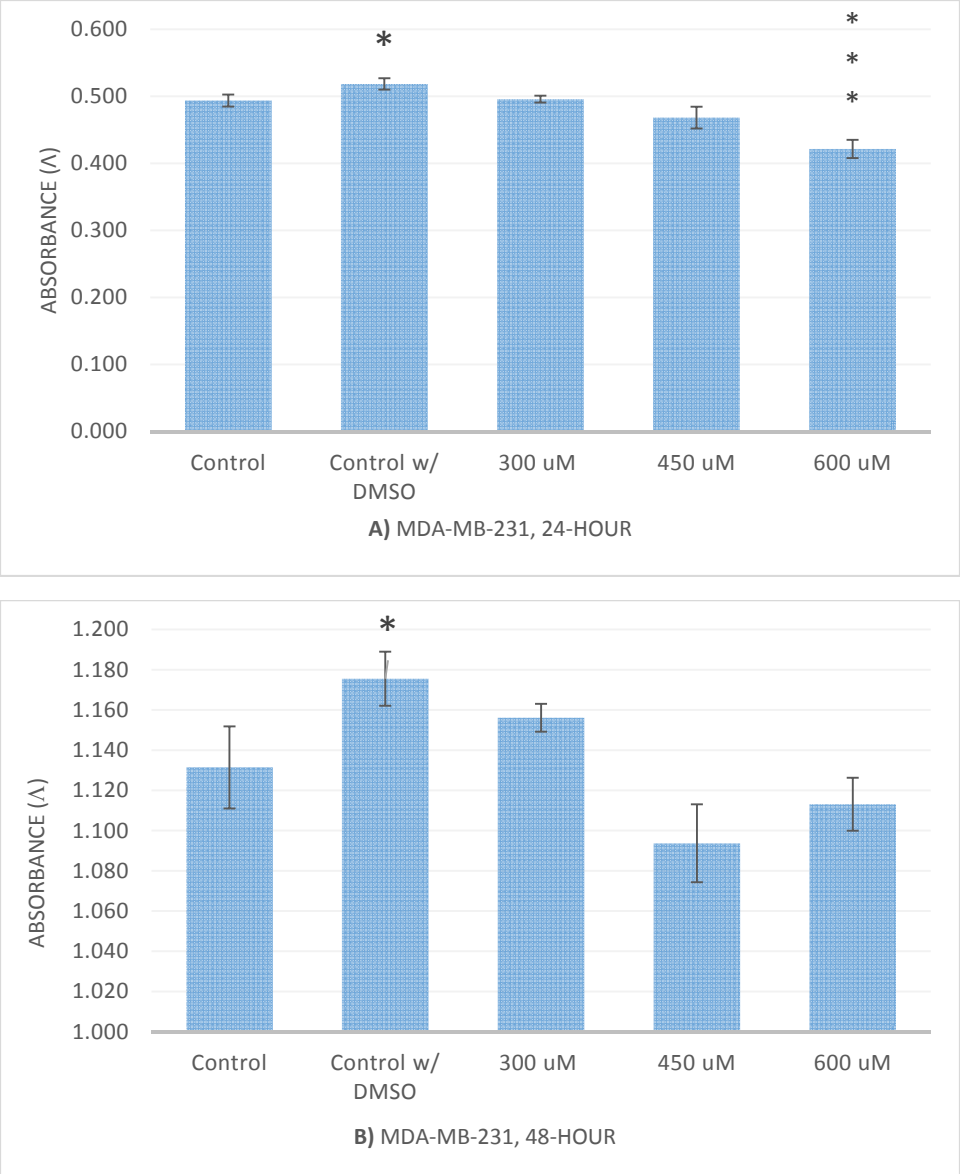
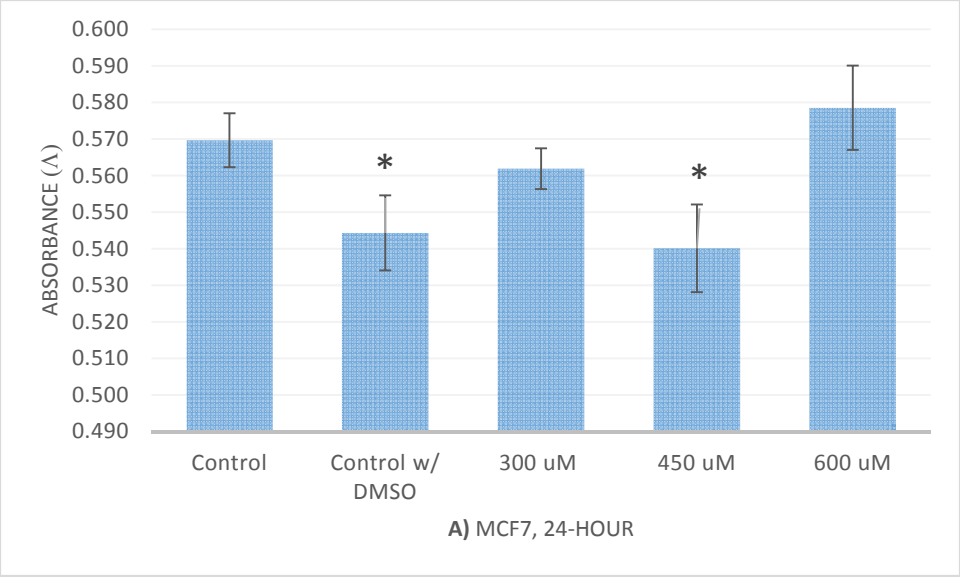


*Figure 1. Average absorbance values of viable human breast adenocarcinoma MDA-MB-231 cells after treatment with quercetin. Cells (5.0 x 10^3 cells/well; 96-well plates) were treated with different concentrations of quercetin for **A)** 24-hours and **B)** 48-hours. Viable cells were determined by XTT assay as described by Biotium (Hayward, CA). Each point is mean ±SE of nine replicates. *Significantly different from the control at $p < 0.05$. *** Significantly different from the control at $p < 0.001$.*



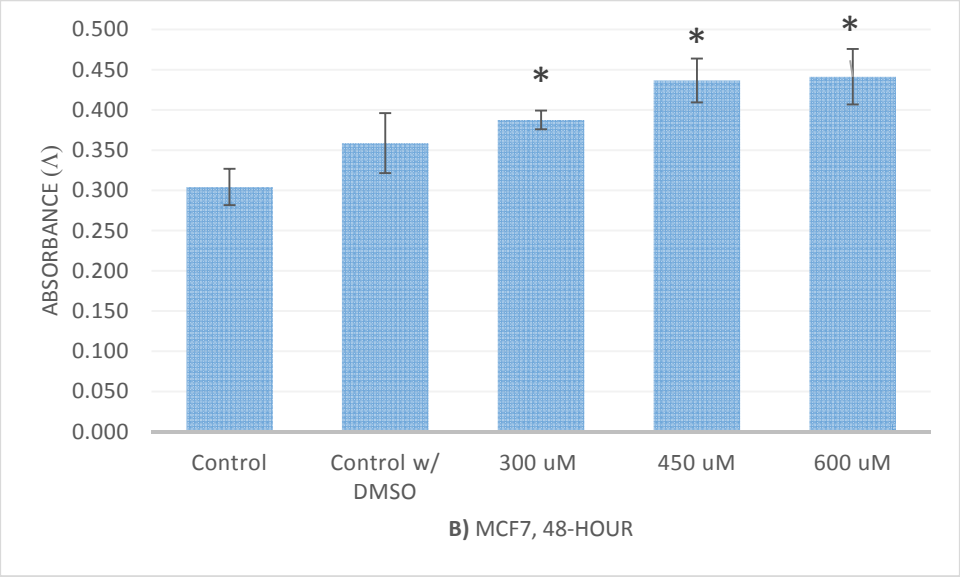


*Figure 2. Average absorbance values of viable human breast adenocarcinoma MCF& cells after treatment with quercetin. Cells (5.0 x 10^3 cells/well; 96-well plates) were treated with different concentrations of quercetin for **A)** 24-hours and **B)** 48-hours. Viable cells were determined by XTT assay as described by Biotium (Hayward, CA). Each point is mean ±SE of nine replicates. *Significantly different from the control at $p < 0.05$.*

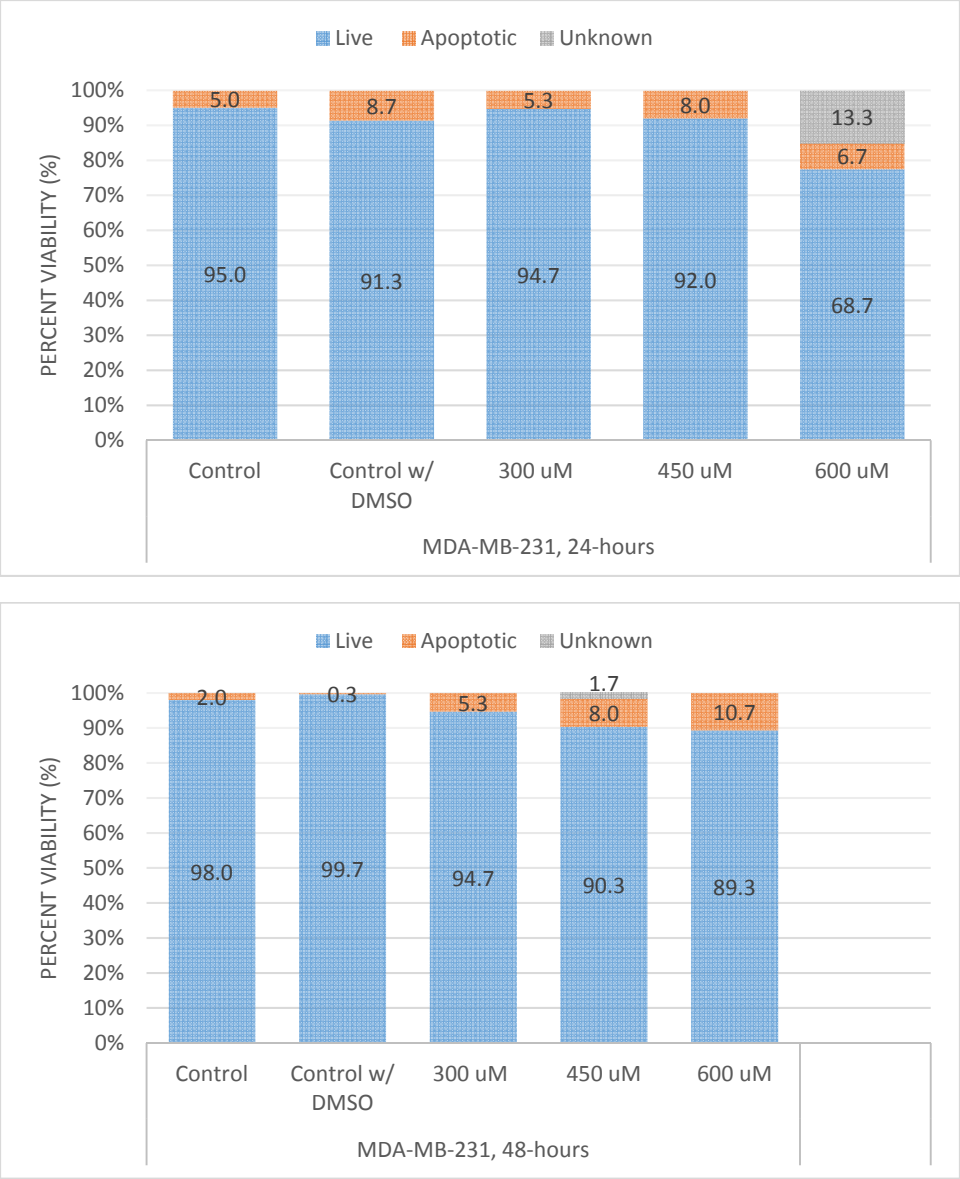


Figure 3. Percentage of viable human adenocarcinoma MDA-MB-231 cells after treatment with quercetin. Cells (1.0 x 10^5 cells/well; 6-well plates) were treated with different concentrations of quercetin for 24 (above) and 48 (below) hours. Cells were stained with DAPI fluorescent stain as described by Chazotte (2011).. Each point is an average of three replicates.

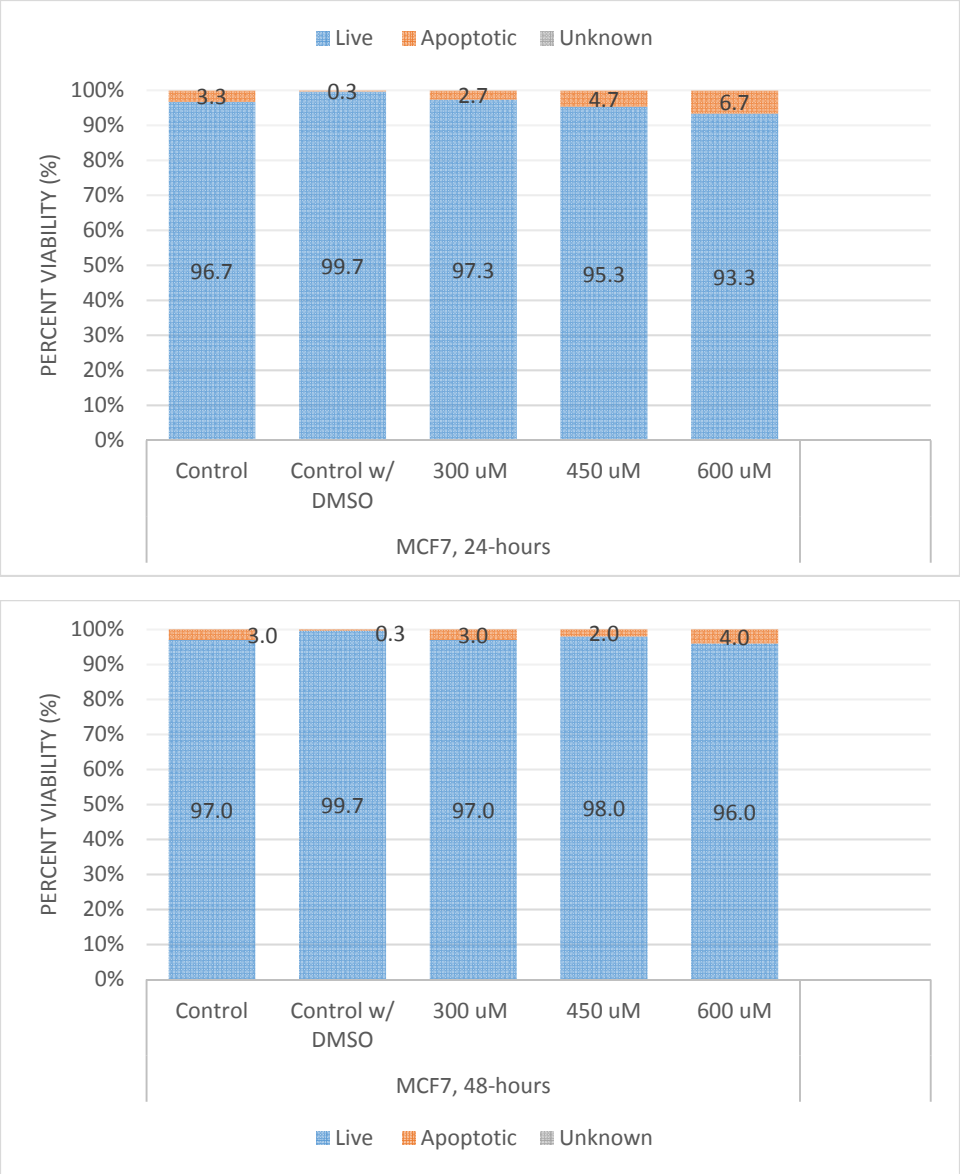


Figure 4. Percentage of viable human adenocarcinoma MCF7 cells after treatment with quercetin. Cells (1.0 x 10^5 cells/well; 6-well plates) were treated with different concentrations of quercetin for 24 (above) and 48 (below) hours. Cells were stained with DAPI fluorescent stain as described by Chazotte (2011).. Each point is an average of three replicates.

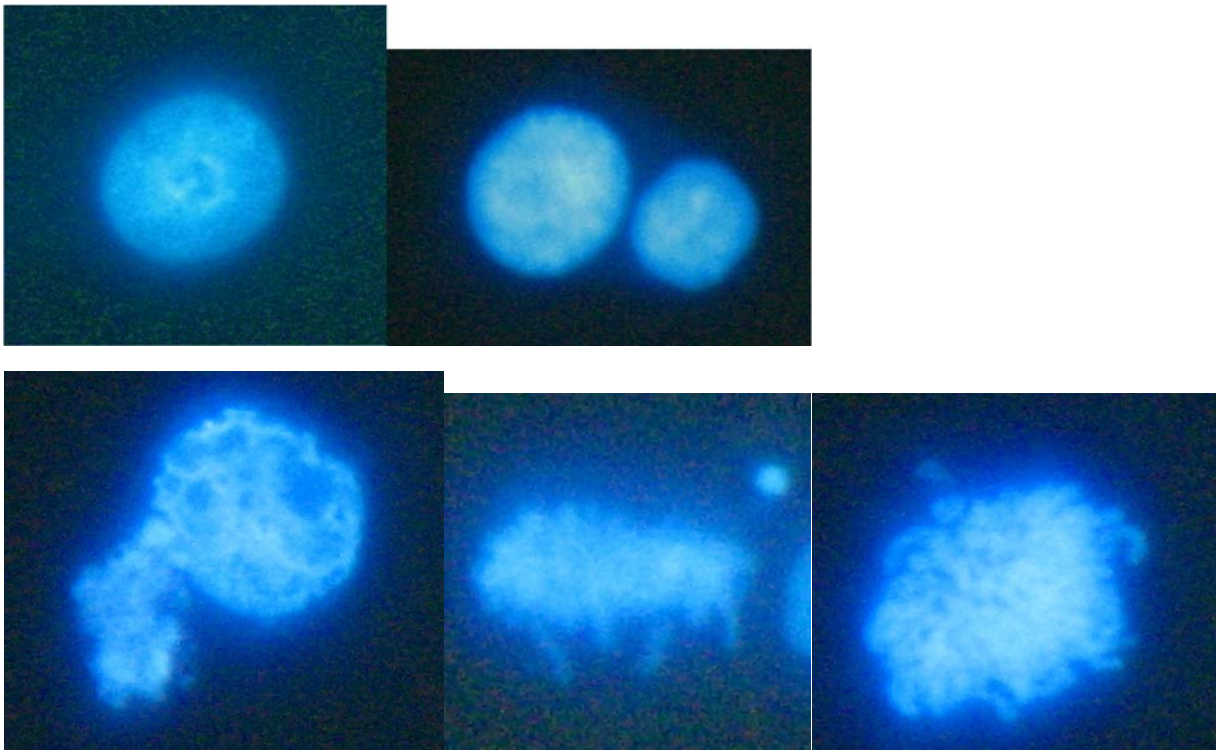


Figure 5. Apoptosis examinations by 4',6-diamidino-2-phenylindole (DAPI) staining for human breast cancer cell lines MDA-MB-231 and MCF7 after treatment with quercetin. Cells were incubated with various concentrations of quercetin for 24 and 48 hours. Cells were visualized at 40X magnification under fluorescent microscope to determine live (top) and apoptotic (bottom) cells.

Discussion

Research on dietary compounds have been done to examine their ability to induce apoptosis in cancer cells. Flavonoids have been shown to induce cytotoxic activities in numerous cancer cell types (Russo et al 2012), although the apoptotic effects of quercetin remain unclear. Since it is well-documented that quercetin's apoptotic effects increases in a concentration- and time-dependent manner (Chen et al 2010, Dajas 2012, Russo et al 2012), and the concentrations of quercetin used in the literature were within 40-250 μM (Russo et al 2007, Chein et al 2009, Tan et al 2009), this study examined the flavonoid's concentration- and

time-dependent apoptotic effects at higher concentrations in MDA-MB-231 and MCF7 breast cancer cell lines.

Quercetin exhibits hormesis, a biphasic dose-dependent response in which the flavonoid is an antioxidant in low concentrations (<40 μM) while becoming a pro-oxidant at concentrations greater than 40 μM or for longer incubation time at that concentration (Chen et al 2010, Vargas and Burd 2010). Although cytotoxicity is not an attractive outcome in normal cells, quercetin's hormetic property would be highly beneficial against cancer cells. To determine the cytotoxic effects of the flavonoid on the human breast cancer cell lines, an XTT assay was done and contrary to the expected outcome, results show that there is no clear relationship between the dose- and time-dependent treatment of quercetin and the number of viable cells in a population (Figures 1A and 1B; 2A and 2B). These results are possibly pervaded by the limitations of the experimental design. For example, in order to draw strong inferences from comparing samples from two or more groups, at least three independent experiments (with replicates) should be done. However, due to time constraints and with limited resources available (especially the XTT assay), only one experiment with nine replicates (samples were taken from the same flask) was done for each cell line at different time frames in order to finish the experiment on time as well as to conserve resources for later experiments. Although many replicates were done for each experiment, the absorbance values taken (including its errors) only reflects the accuracy of measurement, but not its precision; that is, the reproducibility and repeatability of the differences between the treated population and the control. Finally, based on the results, it is possible to acknowledge measurement errors attributed to the lack of stringency in the XTT protocol. For example, after trypsinizing and counting the cells, 100 μl of

the cell suspension was immediately transferred to the 96-well plates which possibly caused the cell suspension in each well to not reflect the appropriate or ideal concentration of ~$5x10^3$ cells/well. Thus, in future experiments, centrifugation of the cells before transferring them to the plates could ensure uniformity of cell concentration in each well.

Numerous studies have confirmed quercetin's ability to induce apoptosis through its interactions in various cellular pathways in various cancer cell lines, especially MDA-MB-231 and MCF7 breast cancer cells (Dajas 2012, Russo et al 2012). At concentration ranges of 40-250 μM, quercetin was found to act on both the intrinsic and extrinsic pathways of apoptosis. In the intrinsic pathway, quercetin upregulates the activities of pro-apoptotic proteins including caspase-3 and -9, Bax, and p53 while downregulating anti-apoptotic protein activity including survivin and Bcl-2 (Chein et al 2009, Tan et al 2009, Duo et al 2012, Deng et al 2013). Furthermore, quercetin could induce apoptosis in the extrinsic pathway through the upregulation of the Fas ligand, as well working synergistically with death receptors such as CD95 and tumor necrosis factor α (TNF-α)-related apoptosis-inducing ligand (TRAIL) to induce apoptosis (Russo et al 2007, Siegelin et al 2008). To confirm apoptotic cell death in the treated samples, DAPI (4',6-diamidino-2-phenylindole) staining was done. Unlike the expected outcome, results show that apoptosis was observed in all treatments, including controls, in both MDA-MB-231 and MCF7 cell lines, and no clear relationship was observed between the dose- and time-dependent treatment of quercetin and the number of apoptotic bodies in a population (Figures 3 and 4). Additionally, the small percentage of apoptotic bodies and the presence of irregularly-shaped cells opens in some treatments in Figures 3 and 4 suggests the possibility of other forms of cell death, such as autophagy or necrosis, triggered by quercetin.

Furthermore, when looking at the number of viable cells from the XTT assay, particularly in the MCF7 48-hour treatment (Figures 1A and 1B; 2A and 2B), there is a possibility that other cellular mechanisms, possibly those involved in cell survival, might come into play at quercetin concentrations higher than 250 µM. However, such conclusions could not be drawn since no experimental protocol was done to confirm these results due to constraints in time and especially in resources. For example, Western Blot is a technique to detect the presence of protein biomarkers involved or associated with apoptosis, such as the caspases or the pro-apoptotic Bax. However, this procedure was not done because of the amount of time needed to carry out the protocol, as well as the cost of the primary antibodies required for this protocol.

Conclusion

In this study, quercetin at high concentrations does not cause a significant effect in inducing apoptosis in MDA-MB-231 and MCF7 breast cancer cell lines. The results are reflective of the limitations of experimental design, and thus a definitive conclusion could not be made regarding the dose- and time-dependent apoptotic effects of quercetin. Since the effects of the flavonoid at higher concentrations (300 µM>) have not yet been done, it is sensible to continue in this direction for future studies under a comprehensive, elaborate experimental design.

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