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Structure-Function Relationship Between Quercetin, Its Methylated Derivatives and Cytotoxicity in Triple Negative Breast Cancer Cells

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STRUCTURE-FUNCTION RELATIONSHIP BETWEEN QUERCETIN, ITS METHYLATED DERIVATIVES AND CYTOTOXICITY IN TRIPLE NEGATIVE BREAST CANCER CELLS

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ABSTRACT

STRUCTURE-FUNCTION RELATIONSHIP BETWEEN QUERCETIN, ITS METHYLATED DERIVATIVES AND CYTOTOXICITY IN TRIPLE NEGATIVE BREAST CANCER CELLS

by Jyoti Phatak

Quercetin is a member of the flavonoid family, found in fruits and vegetables, and has shown cytotoxic effects on many cancers, including breast cancer. Quercetin's methylated derivatives, 3-*O*-methyl quercetin, pentamethyl quercetin, isorhamnetin, and tamarixetin, differ in their substitution patterns of the methyl capping at the 5-hydroxyl groups. Cell death assays performed on MDA-MB-231 cells showed increased cytotoxicity with quercetin and all its methylated derivatives, except pentamethyl quercetin, after 72 hours of treatment. Loss of the mitochondrial outer membrane potential (MOMP) was seen using a JC-1 assay after 15 min incubation with compounds, except for pentamethyl quercetin. We saw no change in MOMP after 24 hours of treatment with all quercetin derivatives. Treatment with compounds showed no change in intracellular reactive oxygen species (ROS). However, the generation of ROS by TBHP, positive control, was decreased after treatment, indicating that these compounds can function as an anti-oxidant by decreasing cellular ROS. The quercetin derivatives showed cell cycle arrest at various phases. Our data from the cell viability assay suggest that a structure-function relationship exists between methylation of the hydroxyl groups available on the quercetin molecule and its cytotoxicity in breast cancer cells, where the presence of at least one hydroxyl group is necessary for the compound to exert a cytotoxic effect.

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LIST OF ABBREVIATIONS

ADP: adenoside diphosphate

ATP: adenoside triphosphate

AIF: apoptosis-inducing factor

Apaf-1: apoptosis protease activating factor-1

ATCC: American type culture collection

Bcl-2: B cell lymphoma

Bax: Bcl-2-associated X protein

Bak: Bcl-2-homologous antagonist

Bid: BH3 interacting-domain death agonist

Bad: Bcl-2-associated death promoter

BH3: Bcl-2 homology domain 3

CDK: cyclin-dependent kinase

CCCP: carbonyl cyanide m-chlorophenyl hydrazone

DMEM: Dulbecco's modification of eagle's medium

DISC: death-inducing signaling complex

DMSO: dimethyl sulfoxide

DR: death receptor

Endo G: endonuclease G

ER: estrogen receptor

FBS: fetal bovine serum

FADD: Fas-associated protein with death domain

HER2: human epidermal growth factor receptor-2

IC: inhibitory concentration

IAP: inhibitors of apoptosis

IFN-γ: interferon-gamma

IL-1β: interleukin-2-1-beta

LLC: Lewis lung cancer

MOMP: mitochondrial outer membrane permeabilization

mTOR: mammalian target of rapamycin

MMP: matrix metalloproteinase

NADH: nicotinamide adenine dinucleotide

NFκB: nuclear factor kappa B

NOD-like receptor

PBS: phosphate buffered saline

Puma: p53 upregulated modulator of apoptosis

PARP: poly-ADP-ribose polymerase

PR: progesterone receptor

ROS: reactive oxygen species

RNS: reactive nitrogen species

RIP1: receptor-interacting protein1 kinase

RIP3: receptor-interacting protein3 kinase

tBid: truncated Bid

Introduction

Cancer is the second leading cause of death in the United States and many other nations of the world (R. Siegel, Miller, & Jemal, 2015). It is characterized by uncontrolled cell growth and invasion of bodily tissues such as the lung, colon, and breast. The American Cancer Society estimates that in 2016, there will be 1,685,210 new cancer cases diagnosed and 595,690 cancer-related deaths in the United States alone (R. L. Siegel, Miller, & Jemal, 2016).

Breast cancer is a diverse disease in reference to its origin, disease progression, and response to treatment (Ossovskaya et al., 2011). With nearly 250,000 new cases in the U.S. each year, breast cancer accounts for 12% of all cancer diagnoses and is the leading cause of cancer-related death in women, claiming the lives of more than 40,000 annually in the United States (R. L. Siegel et al., 2016). Micro-array profiling of invasive breast carcinoma has identified five expression pattern subtypes, distinguished by the presence or absence of estrogen (ER), progesterone (PR) hormone receptors, and the human epidermal growth factor receptor (HER2). These subtypes include luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), normal breast-like, HER2 over-expressing (ER-, PR- and HER2 +), and basal-like or triple negative breast cancer (ER-, PR-, HER2-) (Bauer, Brown, Cress, Parise, & Caggiano, 2007). Current treatment options for breast cancer largely depend on cancer stage and subtype and include local treatments such as surgery, radiation therapy, and systemic treatment such as chemotherapy. For cancers overexpressing hormone receptors, the treatment plan may

also include hormone therapy, and HER2+ cancers may be additionally treated with targeted therapies (American Cancer Society, 2016).

The triple negative breast cancer (TNBC) subtype accounts for approximately 20% of all breast cancers (Hurvitz & Mead, 2015), and presents with an aggressive clinical course and a poor prognosis (Gluz et al., 2009). Due to the absence of all three targetable surface receptors, TNBC presents a significant therapeutic challenge, as patients do not respond to the targeted receptor treatments available for other breast cancer subtypes (De Laurentiis et al., 2016). Currently, the treatment choices for triple negative breast cancer are limited to chemotherapy, radiation, and combination therapies (Hurvitz & Mead, 2015). In addition to causing unwanted side-effects such as nausea and the loss of hair follicles, these therapies are expensive and subject the patient to additional health risks such as drug toxicity and healthy cell damage (Botchkarev, 2003; Du, Osborne, & Goodwin, 2002; Early Breast Cancer Trialists' Collaborative Group, 2005). Due to the challenges of treating TNBC and the harsh side effects of current treatment options, there is a pressing need to identify naturally available anti-cancer agents with therapeutic potential.

Flavonoids are naturally available bioactive compounds that belong to the family of phytochemicals, pigment molecules found in fruits, vegetables, soy products, tea, and red wine (Yan-Hwa, Chang, & Hsu, 2000). These compounds have been associated with positive health outcomes and more recently have been investigated for their potential use as anti-cancer agents. Flavonoids exhibit many functional characteristics lending to their promise in cancer therapeutics, including the ability to induce apoptosis, anti-angiogenic

activity, and inhibition of cell invasion, as well as antiproliferative properties and growth suppressive effects (N. Sharma, Dobhal, Joshi, & Chahar, 2011). Based on their chemical structure, flavonoid compounds can be subdivided into different classes. These classes include flavonols, flavones, isoflavones, flavanols, anthocyanidins, and flavanones (Table 1) (Heim, Tagliaferro, & Bobilya, 2002).

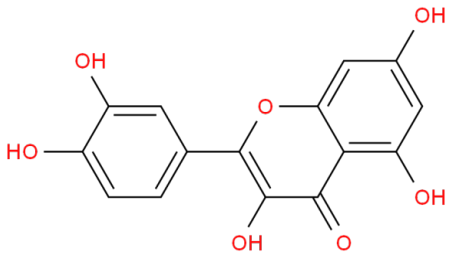
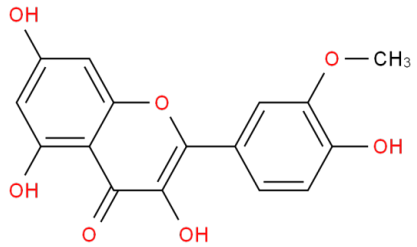
Table 1
Flavonoid sub-types and their examples. The table depicts different types of flavonoids and examples of the respective categories.

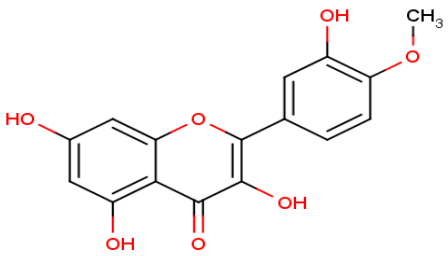
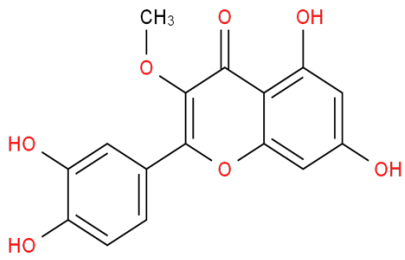
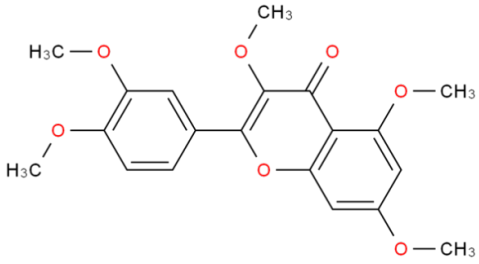
Type of flavonoid	Examples
Flavonols	Kaempferol, Quercetin, Myricetin
Flavones	Apigenin, Luteolin
Isoflavones	Daidzein, Genistein
Flavanols	Catechin, GalloCatechin
Anthocyanidins	Cyanidin, Delphinidin, Malvidin
Flavanones	Naringenin, Hesperetin

Quercetin, a flavonol, is the most abundant of all the flavonoids (A. Sharma & Gupta, 2010). Quercetin displays a spectrum of biological activities, functioning in anti-viral, anti-bacterial, anti-carcinogenic, and anti-inflammatory capacities (A. Sharma & Gupta, 2010). Studies have shown that upon ingestion, quercetin may undergo different metabolic conversions such as sulfation, methylation, and glucuronidation (Murota & Terao, 2003). The methylated derivatives isorhamnetin, tamarixetin, 3-*O*-methyl

quercetin, and pentamethyl quercetin are naturally available and found in several fruits, vegetables, and herbs (Table 2).

Table 2
Natural sources of quercetin and its methylated derivatives.

Name	Natural source	Reference
Quercetin 	Berries, apples, onions, tea, nuts, and red wine	(Crozier, Lean, McDonald, & Black, 1997; Hertog, Hollman, & van de Putte, 1993; Wach, Pyrzyńska, & Biesaga, 2007)
Isorhamnetin 	Gingko Biloba, Onions, sea buckthorn	(Park & Lee, 1996; Y. Wang, Cao, & Zeng, 2005; Zu, Li, Fu, & Zhao, 2006)

Tamarixetin 	Indian head ginger, gingko biloba	(Pandurangan, Bose, & Banerji, 2011; Peng, Li, Zhu, Han, & Yu, 2005)
3-*O*-Methylquercetin 	Laurel leaf cistus, macela	(Bettega, Teixeira, Bassani, Barardi, & Simões, 2004; Ustun, Ozcelik, Akyon, Abbasoglu, & Yesilada, 2006)
Pentamethyl Quercetin 	Green tea, sea buckthorn, cranberries	("FIavonoI Glycosides," 1967; Tsujimura & September, 1930)

Previous studies have demonstrated the ability of quercetin and the methylated derivatives isorhamnetin, and tamarixetin to induce cell death in cancer cells (Z. Li & Gao, 2013; Nicolini et al., 2013; Sun et al., 2012). Although the compounds are

suggested to cause cell death through the intrinsic pathway of apoptosis, the exact mechanism remains unclear.

Knowledge of quercetin's bioavailability, including its intestinal absorption and metabolic conversion is crucial for understanding its anti-carcinogenic properties and for determining its therapeutic potential (Murakami, Ashida, & Terao, 2008). Previous studies have demonstrated that unmodified quercetin exhibits a limited bioavailability. Hence, it has become necessary to identify a more bioavailable form of quercetin that can perform similar functions in vivo and can act as an effective anti-cancer agent. Additional studies have found that methylated flavonoids are more freely bioavailable and exhibit higher cytotoxic potential in comparison to their parent forms (Wen & Walle, 2006).

Our research compares the cytotoxicity between quercetin and its methylated derivatives isorhamnetin, tamarixetin, 3-*O*-methyl quercetin, and pentamethyl quercetin. We suggest that the methyl capping of the free hydroxyl groups on the parent quercetin molecule may make these derivatives of quercetin structurally more favorable as anti-cancer agents. Therefore, we conducted a study to determine the structure-function relationship between quercetin, its methylated derivatives, and cytotoxicity induced by these flavonols in triple negative breast cancer cells. Understanding the structural characteristics of these flavonol compounds and the relationship between the structure and cytotoxic effects on cancer cells is critical for improving their efficiency as a chemopreventive or a chemotherapeutic drug candidate.

Literature Review

Cell Death

Cell death is a critical biological process required for normal functioning of multicellular organisms. The mechanism of death depends largely on morphological characteristics of a cell, the biochemical indicators present, and the circumstances that trigger cell death. Based on these factors, cell death can be classified into the following categories: autophagy, apoptosis, necrosis, and necroptosis (Kroemer et al., 2009).

Autophagic cell death. Autophagic cell death or type II cell death is characterized by a unique cellular morphology, in which cell death takes place in the absence of chromatin condensation and instead is associated with large autophagic vacuolization of the cytoplasm (Kroemer et al., 2009). It can be triggered by deprivation of growth factors, resulting in a decrease of intracellular nutrients and subsequent activation of the nutrient sensing signals that stimulate the autophagic pathway (Levine & Yuan, 2005). The pathway for autophagic cell death involves sequestration of cytoplasmic material in double-membrane vesicles known as autophagosomes (Fink, Cookson, Fink, & Cookson, 2005). The degradation of the sequestrated cytoplasmic material within these vesicles releases nucleotides, amino acids, and free fatty acids, which are then reused for biosynthesis and production of ATP. Autophagic cell death is further characterized by a lack of tissue inflammatory response (Levine & Yuan, 2005).

Autophagy has also been shown to play a role in controlling unregulated cell growth associated with tumor development. An example of this is the study of a tumor suppressor gene, *beclin* 1, a human homolog of the yeast autophagy gene APG6 (Liang et

al., 1999). *Beclin* 1 interacts with the anti-apoptotic protein Bcl-1, which further prevents the Bax-dependent release of mitochondrial cytochrome c (Koneri, Goi, Hirono, Katayama, & Yamaguchi, 2007). The role of autophagy in cancer can be cancer preventing or cancer promoting. It provides cancer prevention by sequestering damaged organelles, permitting cellular differentiation, increasing protein catabolism, and stimulating autophagic cell death. Autophagy promotes cancer by helping with the survival of starving cells, which may facilitate tumor initiation and progression (Levine & Yuan, 2005). When a tumor grows beyond its supply of blood, it undergoes nutrient starvation. Previous studies suggest that these transformed cells may utilize autophagy for cell survival under nutrient deprivation conditions (Lum, DeBerardinis, & Thompson, 2005).

Apoptosis. Apoptosis is morphologically characterized by rapid chromatin condensation and budding of the cellular membrane, along with the formation of apoptotic bodies containing well-preserved organelles, which are later phagocytosed and digested by nearby resident cells (Kroemer et al., 2009). In preparation for cell death, apoptotic cells undergo double-stranded cleavage of nuclear DNA at the linker region between nucleosomes, resulting in oligonucleosomal DNA fragments (Kerr, Winterford, & Harmon, 1994). Several distinct subtypes of apoptosis exist, which are morphologically similar. Various stimuli such as irradiation, growth factor deprivation, and chemotherapeutic drugs trigger these subtypes of apoptosis, all of which ultimately lead to DNA damage. There are two main pathways through which apoptosis may be

initiated in a cell: the extrinsic pathway (receptor-mediated) and the intrinsic pathway (mitochondrial-mediated) (Kroemer et al., 2009).

Extrinsic pathway. The extrinsic pathway of apoptosis, also known as the cytoplasmic pathway, is activated through Fas death receptor, a member of the tumor necrosis factor (TNF) receptor family (S. Wang & El-Deiry, 2003). This pathway is initiated by ligation of cell surface death receptors (DRs) such as Fas, CD95, and TNF-related apoptosis-inducing ligand (TRAIL) with their extracellular ligand. The ligation of CD95 or the TRAIL agonistic receptors R1 and R2 by their ligands results in the clustering of receptor death domains, which then recruits adaptor molecules such as Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD) (Kischkel et al., 2000). The complex formed by the ligand-receptor and adaptor protein is referred to as the death-inducing signaling complex (DISC). Formation of this complex leads to the assembly of initiator caspase-8 and caspase-10, which self-processes to activate effector caspases -3, 6, and 7 through release of activated caspase enzymes (O'Brien & Kirby, 2008). The release of activated caspase enzymes results in DNA cleavage and fragmentation, which are characteristic of apoptosis (Martin, 2014).

Intrinsic pathway. The intrinsic, or mitochondrial pathway of apoptosis is initiated within a cell in response to triggers such as cellular stress, DNA damage, radical oxygen species (ROS), radiation, hormone or growth factor deprivation, chemotherapeutic agents, and cytokines, or glucocorticoids (Grunnet et al., 2009; Pelicano et al., 2003; Schmidt et al., 2004). The mitochondria integrate the pro-apoptotic signaling environment via the B-cell lymphoma 2 (Bcl-2) family of proteins to control

cell death (Elkholi, Renault, Serasinghe, & Chipuk, 2014). The Bcl -2 family proteins are critical regulators that reside immediately upstream of the mitochondria and consist of both anti- and pro-apoptotic members. These proteins have conserved α-helices with sequence conservation clustered in the Bcl-2 homology (BH) domain (Kutzki et al., 2002). The anti-apoptotic members exhibit homology in all BH1 to BH4 segments. The pro-apoptotic members are further subdivided into multidomain members Bax and Bak and the BH3-only members including Bid, Bim, Bad, and Puma (Scorrano & Korsmeyer, 2003).

For pro-apoptotic activity, the Bcl-2 family members must undergo heterodimerization of the BH3 domain (Adams & Cory, 1998). During apoptosis, Bax, a 21 KDa protein, translocates to the mitochondria, in which it undergoes a conformational change before integrating itself into the mitochondrial membrane (Walensky & Gavathiotis, 2011). The translocation of Bax can be triggered by different stimuli such as alterations in mitochondrial bioenergetics or Cathepsin D (lysosomal aspartyl protease) (Bidère et al., 2003; Smaili, Hsu, Sanders, Russell, & Youle, 2001). Upon localization to the mitochondria, Bax undergoes a conformational change from its monomer form to a pore-forming oligomer, generating a proteolipid pore in the outer mitochondrial membrane (Epand, Martinou, Montessuit, Epand, & Yip, 2002). Formation of this pore causes permeability of the membrane and leads to the release of mitochondrial proteins such as cytochrome c and mitochondria-derived activator of caspase (i.e., IAP-binding protein). Cytochrome c, once released into the cytosol, interacts with cellular adaptor proteins (i.e., Apaf-1) and procaspase-9. The binding of these three components forms

the apoptosome, which then activates procaspase-3. The activated caspase-3 further initiates the caspase cascade that leads to apoptosis (*Figure 1*) (Scorrano & Korsmeyer, 2003).

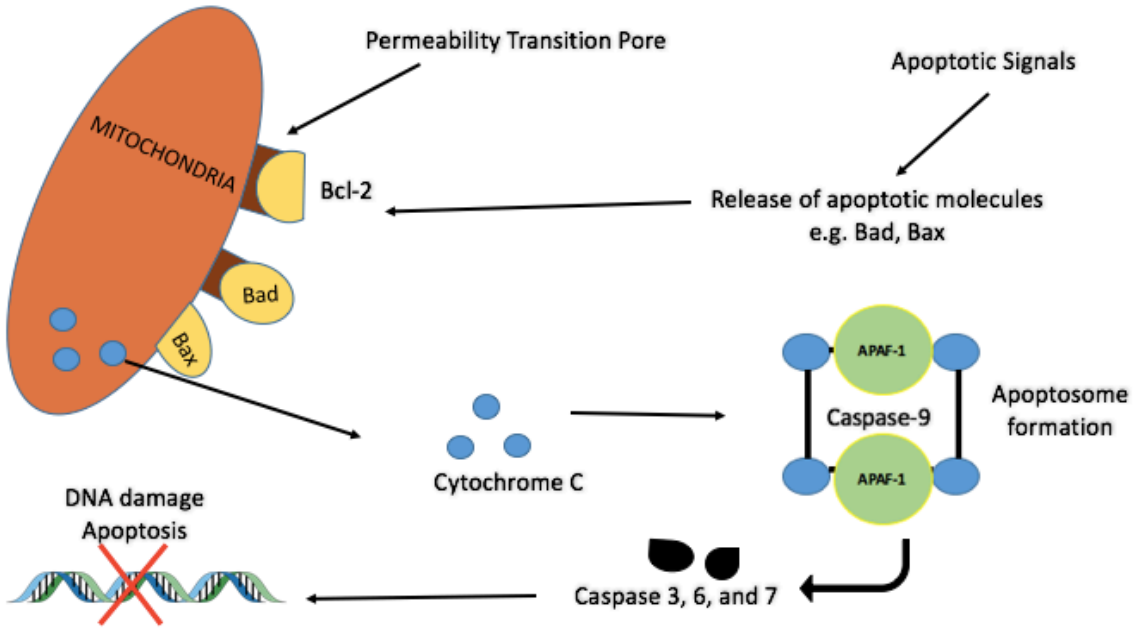


Figure 1. Schematic representation of intrinsic pathway of apoptosis. Intrinsic apoptosis is triggered by apoptotic signals which activate the BH3 only proteins, causing the release of apoptotic molecules Bax and Bak from the outer mitochondrial membrane and the subsequent loss of mitochondrial outer membrane potential by the formation of the permeability transition pore. Cytochrome c is released into the cytosol, complexing with the APAF-1 and caspase-9 to form the apoptosome. This complex activates caspase-3, triggering the caspase cascade leading to apoptosis.

Caspases play a crucial role in apoptosis. They exist as inactive pro-enzymes which are then activated by cleavage at specific aspartate sites (Martin, 2014). The caspase family consists of 15 mammalian members, divided into three subclasses based on the structure and function of their prodomains (Chowdhury, Tharakan, & Bhat, 2008). Caspases with larger prodomains (caspase-1, 4, 5, 12, 13, and 14) play a role in cytokine

maturation and inflammatory responses and are grouped as inflammatory caspases. The second subclass includes caspases-8 and 10, which possess long prodomains (>90 amino acids) and constitute the initiators of apoptosis caspases. The last group of caspases, those with shorter prodomains (caspase-3, 6, and 7), are called effector or executioner caspases (Chowdhury et al., 2008). Procaspases are distinct caspases that are present in the cytoplasm in an inactive form. They can be activated by (1) exposure to another activated caspase, (2) autocatalysis, or (3) binding with an activator protein such as Apaf-1, procaspase-9 and cytochrome c (O'Brien & Kirby, 2008).

BH3-only members (Bim, Puma, Bid, Bcl-x_L/Bcl-2-associated death promoter (Bad), Bcl-2-interacting killer (Bik), and Noxa) display sequence conservation only in the amphipathic α-helical BH3 region and control anti-apoptotic Bcl-2 proteins (Adams & Cory, 1998). These proteins stimulate the apoptotic pathway by binding to a groove on the surface of pro-survival proteins via their BH3 region. Different apoptotic stimuli require different BH3-only proteins for killing cells. For example, Puma is shown to be necessary for p53-mediated apoptosis, which is induced by DNA damage or anoxia (Michalak, Villunger, Adams, & Strasser, 2008). The BH3-only protein Bim is critical for inducing cell death in response to growth factor deprivation, ER stress, and deregulated calcium flux in diverse cell types (Puthalakath et al., 2007). The Bax and Bak proteins also interact with BH3-only proteins. Upon activation by caspase-8 cleavage, cytosolic p22 Bid engages with Fas or TNFR1 receptors on the cell surface, resulting in the formation of truncated Bid (tBid). The tBid further interacts with Bax to induce conformational change and oligomerization of Bax (Wei et al., 2000).

Studies have shown initiation of apoptosis through direct binding of Bim and tBid to Bax or Bak (Kuwana et al., 2005). Furthermore, it was recently demonstrated that genetic deletions of Bid, Bim, and Puma prevented the homo-oligomerization of Bax and Bak, and thereby the cytochrome c mediated activation of caspases in response to death signals in neurons and T-lymphocytes, even in the presence of other BH3 molecules (D. Ren et al., 2010).

In addition to the release of cytochrome c, the oligomerization of Bax causes the release of several other pro-death substances from the mitochondria such as apoptosis inducing factor, Smac/DIABLO, HtrA2/Omi, and several procaspases. Smac and its murine homolog, DIABLO, are nuclear- encoded mitochondrial proteins that contain a localization signal from the mitochondria. This signal is proteolytically removed upon mitochondrial import, generating a mature 23kDa protein. This mature protein exposes the IAP-binding motif (IBM) at the N-terminus of Smac/DIABLO (Fulda & Debatin, 2006). Although the mitochondrial function of Smac/Diablo is not well-understood, *in vitro* studies conducted in Smac -/- cells have demonstrated that in the absence of Smac, cleavage of procaspase-3 is inhibited despite the addition of cytochrome c (Fulda & Debatin, 2006).

Smac/DIABLO also promotes caspase activation through neutralization of inhibitor of apoptosis proteins (IAPs). Omi/HtrA2 is a nuclear-encoded, 49 kDa protein with an N-terminal mitochondrial localization signal that mediates its translocation into the mitochondrial intermembrane space (Saelens et al., 2004). Similar to

Smac/DIABLO, Omi/HtrA2 blocks the IAPs through its N-terminal IAP-binding motif, presented in a trimeric configuration (Yang, Church-Hajduk, Ren, Newton, & Du, 2003).

Emerging evidence suggests an additional apoptotic pathway which proceeds in the absence of caspases, a mechanism known as caspase-independent cell death (Lockshin & Zakeri, 2004). The caspase-independent pathway of cell death has been associated with several factors, including apoptosis-inducing factor (AIF) and endonuclease G. The mitochondrial-nuclear translocation of AIF is one example of caspase-independent apoptosis. Apoptosis-inducing factor (AIF) resides primarily in the mitochondrial intermembrane space in healthy cells and is thought to promote cell death by binding to DNA and inducing large-scale fragmentation (Norberg, Orrenius, & Zhivotovsky, 2010). The release of AIF is mediated by direct proteolysis of the protein, calpain. Calpains are a family of calcium-dependent, non-lysosomal cysteine proteases, which upon activation are shown to cause cleavage of AIF. Studies have shown that the deletion of calpain-1 alone is sufficient to affect AIF truncation (Polster, Basanez, Etxebarria, Hardwick, & Nicholls, 2005). Redistribution of AIF to the nucleus and large-scale DNA fragmentation are both caspase- and ATP-independent processes (Daugas et al., 2000). Endonuclease G (Endo G) may function similarly to AIF in promoting caspase-independent cell death. While it was initially identified as a protein involved in mitochondrial DNA replication, recent studies have shown Endo G to be released from the mitochondria during apoptosis, with subsequent nuclear localization and apparent involvement in nuclear DNA breakdown (L. Y. Li, Luo, & Wang, 2001; van Loo et al., 2001).

Necrosis. Necrotic cell death is characterized by an irreversible loss of plasma membrane integrity, gain in cytosolic volume, and marked swelling of organelles (Golstein & Kroemer, 2007). It is a consequence of crosstalk between several biochemical and molecular pathways at different cellular levels (Festjens, Vanden Berghe, & Vandenabeele, 2006). Several molecular signals have been identified as necessary for necrotic cell death such as members of TNF family, RIP1, PARP, and Cyclophilin D (CypD) (Golstein & Kroemer, 2007).

Members of the TNF receptor family are shown to initiate necrotic cell death. When treated with TNF-α, L929 mouse fibrosarcoma cells initiate a complex multi-step signal transduction pathway in which FAS-associated death domain (FADD) is recruited to the TNF receptor-1, and necrosis is induced presumably through the death domain (Boone et al., 2000). Necrosis in L929 cells is dependent on the death domain of TNFR-55 (Boone et al., 2000). When L929 cells were transfected with the FAS gene, apoptotic cell death was seen after anti-FAS addition, yet the cell line maintained sensitivity to the necrotic effect of TNF, indicating that apoptotic and necrotic events can coexist in the same cell (Vercammen et al., 1998).

Receptor inducing protein 1 (RIP1) is a specific kinase which is recruited to the death-inducing signaling complex after the occupation of TNF-R1, and when activated it can moderate several effects including necrosis (Golstein & Kroemer, 2007). Jurkat cells deficient in RIP1 resist necrotic cell death induced by the Fas/TNF-R/TRAIL-R pathway, implicating that RIP1 plays a critical role as a molecular signal for necrosis. There is also evidence of RIP1 involvement in other models of necrotic signaling (Chan et al., 2003).

Knockdown of RIP1 expression has been shown to interrupt the lethal signal transduction cascade in necrosis induced by alkylation of DNA and activation of (PARP) poly(ADP-ribose) polymerase (Xu, Huang, Liu, & Han, 2006).

PARP (ADP-ribose) polymerase, is a nuclear enzyme containing a Zn-binding domain. Activation of PARP can result in necrotic cell death through depletion of NAD^+ (Galluzzi et al., 2011), and subsequent necrosis is initiated by over-activation of the DNA-repair enzyme PARP-1. PARP inhibitors block necrotic cell death in Burkitt's lymphoma (Ha & Snyder, 2016).

Cyclophilin D (CypD) is a mitochondrial matrix protein that can interact with inner membrane proteins. Knockdown of the gene encoding CypD induces resistance to necrosis caused by ROS or Ca^{2+} overload in hepatocytes and fibroblasts (Nakagawa et al., 2005). CypD is also a mitochondrial target of cyclosporin A, which has been previously shown to decrease cell loss caused by necrotic cell death, for example, TNF-α treated hepatocytes (Pastorino et al., 1996). CypD interacts with the inner membrane proteins including adenine nucleotide translocator (ANT), which participates in the opening of non-specific channels causing dissipation of the inner mitochondrial transmembrane potential (Nakagawa et al., 2005).

Necroptosis. In contrast to the unregulated and uncontrollable cell death process associated with cellular necrosis, a programmed form of necrotic death may also occur through a process known as 'necroptosis'. The necroptotic mechanism of cell death is observed in specific cellular conditions such as those arising from neurodegeneration,

ischemia-perfusion injuries, and infection. It is known to be initiated by activation of several receptors such as CD95 (FAS) binding to CD95L (FASL), TNF receptor 1 (TNFR1), TNFR2, TNF-related apoptosis-inducing ligand receptor 1 (TRAIL1), and TRAIL2. The TNF receptors stimulate TRADD to recruit RIP1K. The RIP1K and RIP3K receptor-interacting proteins, in the absence of caspase-8, subsequently phosphorylate, leading to the formation of the necrosome. The formation of the necrosome by these receptor-interacting proteins such as RIP1 and RIP3 is considered one of the most critical characteristics of necroptosis. (Wu et al., 2014).

Flavonoids

Flavonoids are potent natural compounds which have long been associated with positive health outcomes. A member of the polyphenol family, flavonoids are widely distributed in a variety of plants, vegetables, and fruits, with more than 4000 naturally occurring forms already identified (Galati & O'Brien, 2004). Because of their many health-promoting attributes, flavonoids have been a research target for some years. More recent insight regarding the biochemical mechanisms producing flavonoid-associated health benefits have led investigators to explore a potential therapeutic application for these compounds. Perhaps most notably, some flavonoids have been shown to impede the initiation, promotion, and progression of cancer by regulating enzymes and signaling pathways that participate in vital cell processes such as cell differentiation, apoptosis, angiogenesis, metastasis, and inflammation (W. Ren, Qiao, Wang, Zhu, & Zhang, 2003).

Structure of flavonoids. Flavonoids have a generic structure similar to the diphenyl propanes, C_6-C_3-C_6, with two benzene rings joined by a three-carbon chain

with an oxygen bridge, resulting in the formation of two phenolic rings (A and B) and a pyrane ring (C). Based on variations in the number and the positioning of the hydroxyl groups, flavonoids are further classified into six major groups: flavonols, flavones, flavanones, anthocyanidins, isoflavones, and flavanols. Some flavonoids have hydroxyl groups on their structure at positions 3, 5, 7, 3',4', and/or 5', with the exception of the isoflavone group, where the B-ring is attached to 3-carbon on pyran.

Flavonoids also vary in their hydroxyl group attachments. When present without sugar molecules attached, they are referred to as aglycones, whereas when one or more sugar molecules have replaced the hydroxyl groups, they are called flavonoid glycosides. The presence of glycosides on flavonoid molecules plays a crucial role in the bioavailability of these molecules within the human body. The hydroxyl groups on flavonoids may also be replaced with methyl groups (methyl capping), resulting in *O*-methylated flavonoids. The presence of the methyl capping is necessary for flavonoid cell permeability and anti-cancer properties (Walle, 2009).

Flavonoid as anti-oxidants. Flavonoids are most commonly recognized for their anti-oxidant activity (Pietta, 2000). The mechanism of anti-oxidant action in the body may proceed through several channels such as by inhibition of enzymes responsible for reactive oxygen species-formation, by chelation of trace elements responsible for free radical production, by direct scavenging of reactive oxygen species, and by up-regulation or protection of antioxidant defenses (Pietta, 2000). In flavonoids, the anti-oxidant activity can be directly attributed to the availability of phenolic free hydroxyl groups. These phenolic hydroxyl groups provide the flavonoid molecules with their electron-

donating ability to detoxify free-radicals (Murakami et al., 2008). Although studies have suggested that flavonoids possess both excellent iron chelating and radical scavenging properties, there are inconsistencies regarding the structure-function relationship and influence of structure on anti-oxidant potential. The B-ring hydroxyl configuration is thought to be the most important determinant of the compound's ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Haenen, Paquay, Korthouwer, & Bast, 1997). The hydroxyl groups on the B-ring donate hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, while A-ring substitution of the hydroxyl group has no known significance in relation to the anti-oxidant activity (van Acker et al., 1996). Planarity allows conjugation, electron dislocation, and increase in flavonoid radical stability. The absence of the 3-OH revokes the coplanarity and conjugation, thereby undermining the anti-oxidant potential of flavonoids (van Acker et al., 1996).

Role of flavonoids. Flavonoids have demonstrated several biological properties that may play a significant role in cancer chemoprevention. In addition to serving as anti-oxidants, flavonoids may interfere with cancer-associated cellular functions by inhibiting cell division and proliferation, reducing oxidative stress levels, and inducing apoptosis, as well as by aiding in cellular detoxification and helping to stimulate the immune system (W. Ren et al., 2003). A large body of data has been generated to study the relationship between the consumption of flavonoid-rich foods and cancer prevention (N. Sharma et al., 2011).

Quercetin

The flavonol quercetin is the most abundant of all flavonoids (A. Sharma & Gupta, 2010). The chemical formula for quercetin is $C_{15}H_{10}O_7$ with a molecular weight of 302.24 g/mole. Quercetin is found in various food products such as berries, apples, onions, tea, nuts, and red wine (Crozier et al., 1997; Hertog et al., 1993; Wach et al., 2007). The five hydroxyl groups on the structure of quercetin make it the most potent anti-oxidant among all known flavonoids (Letan, 1966). Average dietary intake of quercetin has been estimated as 5-40 mg/day, although these levels may vary with the consumption of a flavonol-rich diet. Still, dietary consumption remains far below the suggested upper limit, as quercetin is recognized as safe in oral dosages of 1,000 mg/day or intravenous dosages of 765 mg/day (Harwood et al., 2007).

When ingested, the quercetin glycoside is cleaved by β-glycosidase, resulting in the formation of quercetin aglycone (Spencer, Kuhnle, Williams, & Rice-Evans, 2003). The bioavailability of quercetin aglycone and its glycosides plays a significant role in the efficacy of their biochemical properties. Quercetin is commonly digested in its glycoside form, where one or more sugar groups are bound to the phenolic group by glycosidic linkage (Murota & Terao, 2003). Quercetin glycosides from the diet pass through small intestine, and enter the cecum and colon, in which they are hydrolyzed to aglycone (where the sugar moiety is removed from the glycosidic form of quercetin) by enterobacteria (Noteborn, Jansen, Benito, & Mengelers, 1997). The aglycone form of quercetin is lipophilic, and thus passes through the phospholipid bilayer of cellular membranes to enter the circulation, in which it is subjected to *O*-methylation,

glucuronidation, and/or sulfation in the liver (Noteborn et al., 1997). A human study conducted to evaluate the absorption of quercetin supplementation showed that oral quercetin effectively increased blood plasma levels after a 2-week regimen. In the study, 18 men and 18 women were given daily oral quercetin aglycone supplementation for two weeks, at a dose of (Q50) 50 mg/day, (Q100) 100mg/day, and (Q150) 150mg/day. Though researchers observed varying levels of individual blood plasma concentrations based on the participant's fat mass or gender, study participants, on average, were able to absorb ~150 mg/day (Egert et al., 2008). Compared with the baseline, there was a significant increase in the plasma concentrations of quercetin by 178% (Q50), 359% (Q100), and 570% (Q150) (Egert et al., 2008).

Quercetin as an anti-oxidant. Quercetin possesses a range of favorable biochemical attributes, including anti-inflammatory, anti-histaminic, anti-oxidant, anti-carcinogenic, and anti-viral properties. (Russo, Spagnuolo, Tedesco, Bilotto, & Russo, 2012). Quercetin is considered a strong anti-oxidant due to its ability to scavenge free radicals and bind transition metal ions. These properties allow quercetin to inhibit lipid peroxidation, which is a process that involves the conversion of unsaturated fatty acids to free radicals (Hollman, van Trijp, Mengelers, de Vries, & Katan, 1997). The radical-scavenging activity of quercetin is largely dependent on the *O*-dihydroxyl structure (catechol) at the 3' and 4' –positions on the B ring of the quercetin molecule (Letan, 1966). The electron donating ability of quercetin to ROS prevents damage to cellular DNA. Additionally, at high intracellular concentrations, quercetin acts as a pro-oxidant. Quercetin at concentrations greater than 40 μM has shown to induce apoptosis in

transformed cells, presumably by becoming an ROS itself (Metodiewa, Jaiswal, Cenas, Dickancaite, & Segura-Aguilar, 1999).

Quercetin's effects on cancer. Apoptosis induced by quercetin occurs through the mitochondrial-mediated pathway (Chien et al., 2009; Duo, Ying, Wang, & Zhang, 2012; Kim, Moon, Ahn, & Cho, 2013). The mitochondrial pathway of apoptosis has been confirmed by a measured loss of mitochondrial outer membrane potential (MOMP) upon treatment with quercetin in various cancer cell lines. Exposure to quercetin causes loss of MOMP in colorectal cancer (HT-29) cells, data were supported by a decrease in the cell viability, an increase in apoptosis rate, and activation of caspase-3, in correlation with activation of caspase-9 (Z. Li & Gao, 2013). In a study of triple negative breast cancer cells treated with quercetin at a concentration range of 200-250 μM, results have shown an increase in the production of p53, increased activation of caspases-9 and 3, and increased levels of cytochrome c (Chien et al., 2009), consistent with activation of mitochondrial apoptosis.

Quercetin also induces cell cycle arrest at G1 and/or G2/M phase in several cancer cell lines such as colon, leukemia, breast, lung, hepatoma, and oral cancer. An investigation of the molecular signaling pathway of quercetin showed the induction of apoptosis in human breast MCF-7 cancer cells and cell cycle arrest at S phase. These changes were accompanied by alterations in the levels of cell cycle regulated proteins such as CDK2, and cyclins A and E, suggesting that alterations in cell division may play an important role in apoptosis induced by quercetin. (Chou et al., 2010).

Quercetin is also able to cause mitochondrial-mediated cell death in the absence of p53 (Zhang, Zhao, & Wang, 2009). Additionally, quercetin may work with death-domain stimulators such as tumor necrosis factor α (TNF-α)- related apoptosis-inducing ligand (TRAIL) to induce cell death in cancer cells through the extrinsic apoptosis pathway (Siegelin, Reuss, Habel, Rami, & von Deimling, 2009).

Methylated derivatives of quercetin. Methylated derivatives of quercetin act as potent chemopreventive agents with increased hepatic metabolic stability (Walle et al., 2007). Blocking of the free hydroxyl groups by methylation eliminates the influence of highly efficient conjugation pathways, thereby limiting the metabolic clearance from the intestinal epithelial cells as well as in the liver (Wen & Walle, 2006).

Isorhamnetin, a 3′-*O*-methylated metabolite of quercetin, is a flavonol, commonly found in plants such as *Ginkgo biloba* Linne and *Persicaria thunbergii* H.Gross (Lee et al., 2008). Isorhamnetin exhibits anti-tumor and anti-oxidant activity in several cell lines, including human hepatocellular and human esophageal squamous cancer cells. The biological effects of quercetin may be largely attributed to the activity of its derivatives such as the 3-*O* methylated metabolite isorhamnetin. Isorhamnetin can induce both dose- and time-dependent apoptosis and necrosis in HCT-116 colorectal cancer cells, which are also shown to arrest at G2/M phase of the cell cycle upon treatment (Jaramillo et al., 2010). In Lewis lung cancer cells (LLC), isorhamnetin-induced apoptosis occurs through activation of the mitochondrial pathway, demonstrated by loss of MOMP, increased mitochondrial Bax, release of cytochrome c into the cytosolic fraction, decreased production procaspase-9, and increased caspase-3 levels, along with increases

in PARP cleavage, DNA fragmentation, and sub-G1 apoptotic bodies. In C57BL/6 mice bearing LLC, treatment with isorhamnetin at dosage levels of up to 0.5 mg/Kg resulted in a significant reduction in the size and weight of excised tumors (Lee et al., 2008). Isorhamnetin has also been shown to have potential inhibitory effects on adhesion, migration, and invasion of MDA-MB 231 human breast carcinoma cells. These inhibitory effects of isorhamnetin are achieved by downregulating the activity of (Matrix Metalloproteinase) MMP-2 and MMP-9, a family of endopeptidases that have the ability to degrade all ECM components and therefore play a significant role in tumor invasion and tissue remodeling (C. Li et al., 2015).

Less-studied methylated derivatives of quercetin may also have therapeutic potential in cancer treatment. Flavonoid aglycone, 3-*O*-methylquercetin, is naturally found in *Nicotiana tabacum L. trichomes.* It has been shown to participate in the inhibition of prostaglandin and phosphodiesterase, the inactivation of free radicals and in inhibition of viral RNA synthesis (Schwingel, Schwingel, Storch, Barreto, & Bassani, 2014). The metabolite 3-*O* methyl quercetin has also been studied for its anti-viral activity, where it inhibits viral replication at an early stage, reducing the viral RNA and protein synthesis (Schwingel et al., 2014). Though its anti-cancer activity has not yet been extensively studied, the potential for 3-*O* methyl quercetin in cancer treatment is notable due to its cytotoxic activity in various human cancer cell lines such as MCF-7 breast cancer cells, Hep2 hepatoma cells, HL60-leukamia cells, A431-epidermoid carcinoma, HeLa-cervical cancer cells, and HOS-hosteosarcoma (Carini, Klamt, & Linck, 2014). Similarly, pentamethyl quercetin induces weak cytotoxicity in MDA-MB 231

cells, along with cell cycle arrest at G1 phase (Yadegarynia et al., 2012). This compound has also been found to have low toxicity with the ability to reverse drug resistance for SN-38 (an active metabolite of irinotecan, a drug used for cancer treatment and a substrate of ABCG2) in breast cancer resistance protein ABCG2 overexpressing cells (Gallus, Juvale, & Wiese, 2014).

Tamarixetin, a naturally occurring flavonoid, is structurally very similar to quercetin, and very little research has been conducted to explore its potential application in anti-tumor therapy. Tamarixetin contains a methoxyl group at position 4′ of the phenylbenzo-γ-pyrone core (Spencer et al., 2003). It has been reported to be more cytotoxic and a more potent inducer of apoptosis than quercetin in human myeloid HL-60 cells (Nicolini et al., 2013). The methylated derivative of tamarixetin is also capable of passing through biological membranes more easily than quercetin due to its high lipophilic nature and its ability to reach a higher intracellular concentration (Nicolini et al., 2013). This review exemplifies the need for continued research investigating the anticancer potential of methylated flavonoid compounds. Our objective was to compare the quercetin compound to its methylated derivatives, in their ability to induce cell death in triple negative breast cancer cells.

Methods

Materials

Flavonoids were purchased from Indofine Chemicals and prepared by dissolving in 100% dimethyl sulfoxide (DMSO) to produce a 50 mM stock solution.

Cell Culture

MDA-MB 231 cells (human breast adenocarcinoma cells), HeLa cells (cervical cancer cells), and MCF-10A cells (immortalized non-transformed primary breast epithelial cells) were purchased from the American Type Culture Collection (ATCC). MDA-MB 231 and HeLa cells were maintained using Dulbecco's Modification of Eagle's medium (DMEM) with high glucose, L-glutamine, and sodium pyruvate (Corning), and supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic as recommended by ATCC. The MCF-10A cells were grown in mammary epithelial cell growth media (MEGM) (Lonza) with 25.0 mL horse serum (Invitrogen), 0.5 ml human epithelial growth factor (hEGF), 0.5 mL hydrocortisone, 2.0 mL bovine pituitary extract (BPE), 0.5 mL of gentamycin sulfate (GA-1000), and 0.5 mL insulin (Lonza). All cell lines were grown at 37°C under humidified 5% CO_2.

Cell Viability Assay

Cell viability was measured using a trypan blue exclusion assay (TBE). For this assay, 0.5 x 10^6 cells per well were seeded in a 6-well culture dish. After 24 h of incubation, the medium was pipetted out and the cells were treated with 12.5, 25, 50 and 100 μM of flavonoids or with the vehicle control, dimethyl sulfoxide (DMSO). Cells were harvested by trypsinization at 72 h post treatment and re-suspended in 1 mL of 1X PBS. The cell suspension was mixed with trypan blue at a 1:1 ratio. Cells lacking trypan

blue were counted using a TC10 automated cell counter (Bio-Rad) and the percentage of live cells was calculated in comparison to the DMSO control.

Measuring Mitochondrial Outer Membrane Potential

Loss of mitochondrial outer membrane potential (MOMP) was determined using flow cytometry. When the mitochondrial membrane is intact, JC-1 dye (Invitrogen) forms red aggregates, but a loss of membrane permeability leads to a decrease in the proton gradient across the membrane, which resulted in the release of green JC-1 monomers into the cytosol. The ratio of red/green fluorescence is an indicator of MOMP change, with a higher ratio indicating intact mitochondrial membranes and a lower ratio indicating loss of MOMP (Facts, 2011). The protocol was followed per manufacturer recommendation (Invitrogen). Briefly, 1 x 10^6 MDA-MB 231 cells were treated for 15 min with 100 μM of the flavonoid compounds or CCCP as a positive control and DMSO as a negative control treatment, along with 15 μL of the 200 μM JC-1 dye in 1X PBS at 37°C. Change in the MOMP was then measured on a FACSCalibur cell analyzer (BD Biosciences) by collecting and analyzing 25,000 events using a CellQuest Pro flow cytometry analysis software. The parameters for the flow cytometer were set to FSC: E-1, SSC: 326, FL1: 320, FL2: 300, and compensation of FL2: 22% FL1. The assay was repeated with 0.3 x 10^6 cells per well seeded in a 6-well dish. After 24 h, the media was replaced with the treatment and incubated for another 24 h. A washout experiment was also performed as described above, with the exception that cells were washed with 1X PBS three times following the harvesting of cells and before the cells were being

analyzed. The samples were analyzed on the flow cytometer using Cell Quest Pro, as they were for the 15 min treatment.

Detection of Reactive Oxygen Species

The generation of reactive oxygen species upon treatment with flavonoids was measured using CM-H_2DCFDA dye. The dye (5-(and-6)-chloromehtyl-2', 7'-dichlorodihydrofluorescin diacetate) is an acetyl ester that passively diffuses into the cells, in which its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation leads to the formation of a fluorescent adduct that is trapped inside the cell (Eruslanov & Kusmartsev, 2010). The CM-H_2DCFDA dye was dissolved in DMSO to prepare a 10 mM stock. For this experiment, 1.5 x 10^5 cells per well were seeded in a 6-well dish. After 24 h, the cells were harvested and resuspended in 1 mL 1X PBS. The cells were treated and stained together for 15 min using 10 μM CM-H_2DCFDA dye at 37^0C. Cells were also treated with DMSO as a negative control and 5 μM *tert*-Butyl-hydroperoxide (TBHP) as a positive control and further analyzed using a FACSCalibur cell analyzer (BD Bioscience). The flow cytometer instrument settings were set to FSC: E-1, SSC: 358, FL1: 245, and FL2: 300 and were kept constant for all samples.

Cell Cycle Analysis

Cells were seeded at 2.5 x 10^5 cells in a 10 mm culture dish (Corning). After 24 h, cells were treated with flavonoid compounds. Then 24 h and 48 h after treatment, cells were harvested via trypsinization and counted on an automated cell counter. Cells at 1 x 10^6 confluency were resuspended in 1ml of cold 1X PBS. Briefly, the cells were fixed in

absolute ethanol and stored at -20ºC. The fixed cells were centrifuged at 1000 rpm, washed three times in cold 1X PBS and then resuspended in 1 ml of 50 µg/mL PI-staining solution and 150 µl of 100 µg/ml of RNase A. After incubation for 40 min at 37ºC, cells were analyzed on FACSCalibur (Pozarowski & Darzynkiewicz, 2004).

Statistical Analysis

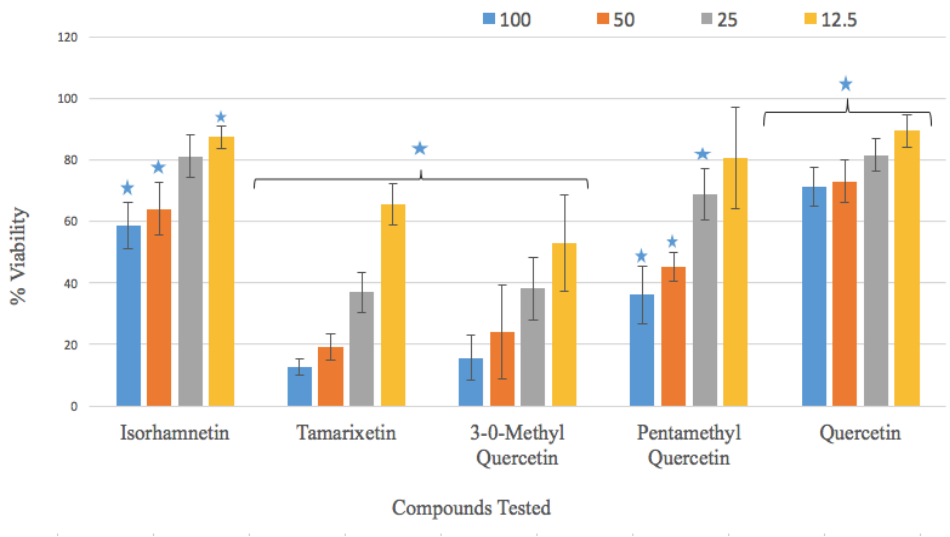
The IC50 values for cytotoxicity assays were calculated using GraphPad prism software version 6.0. For measuring differences between two groups, data were analyzed using two-tailed Unpaired Student t-tests with a $p \leq 0.05$.

Results

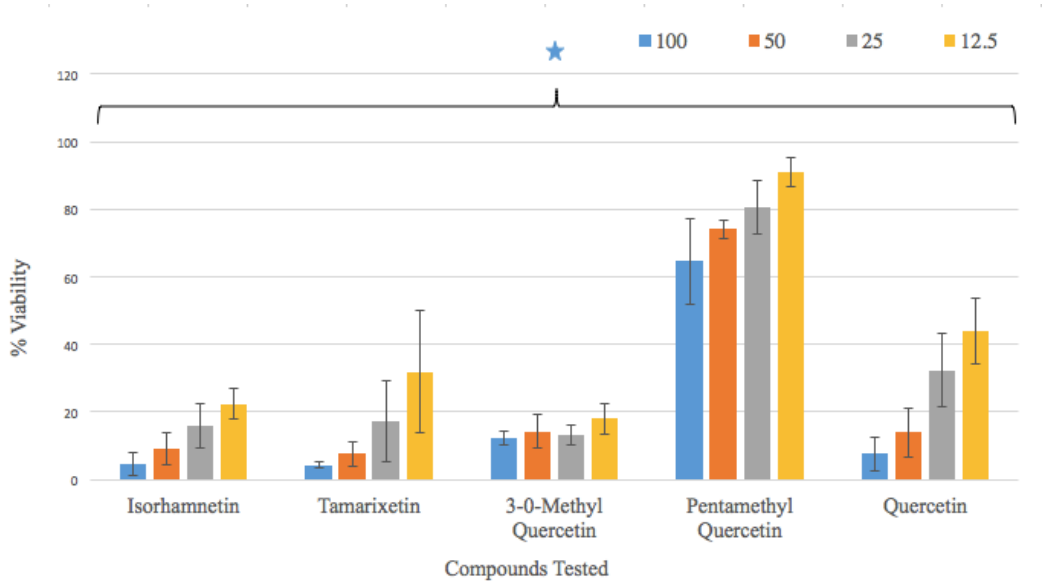
Cytotoxic Effect of Quercetin and Its Methylated Derivatives on MDA-MB 231 Cells

Cell viability was evaluated using classical trypan blue exclusion (TBE) assay. MDA-MB 231 triple negative breast cancer cells were treated with quercetin, isorhamnetin, tamarixetin, 3-*O*-methylquercetin, and pentamethyl quercetin at concentrations of 12.5, 25, 50, and 100 µM and harvested 72 h post-treatment (*Figure 2A*). The assay was repeated using HeLa cells (*Figure 2B*) to determine if the observed effects were specific to breast cancer cell, and on MCF-10A cells (*Figure 2C*), a non-transformed breast cancer cell line, to determine if the cytotoxic effects of these compounds were specific to cancer cells.

A.



B.



C.

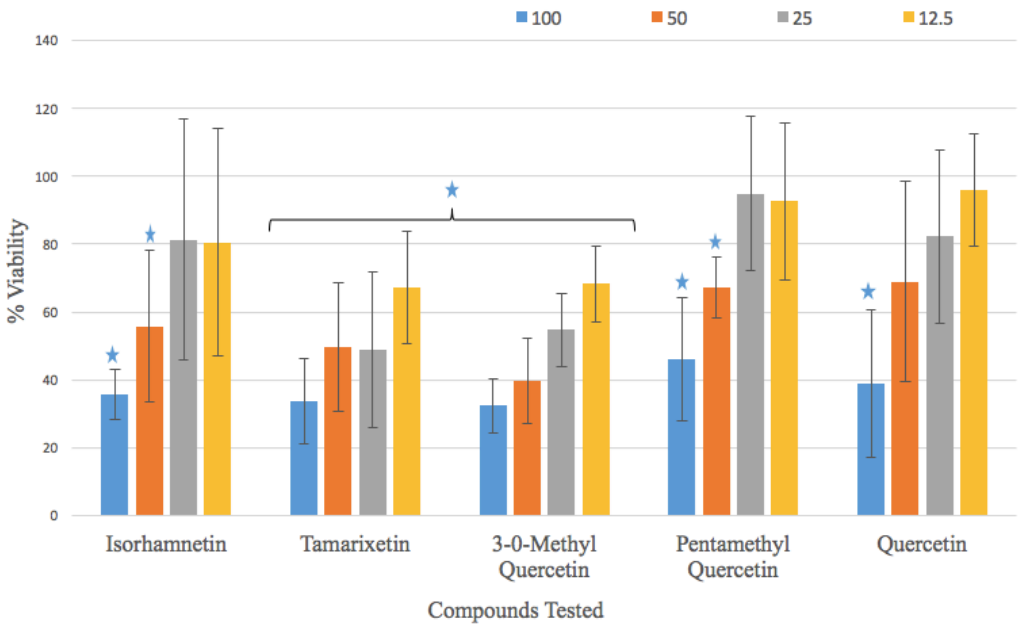


Figure 2. Trypan blue exclusion assays performed on A) MDA-MB 231 cells, B) HeLa cells, and C) MCF -0A cells after treatment with 12.5, 25, 50, and 100 µM of quercetin and its methylated derivatives. Percent viability was calculated in comparison to the negative DMSO control. Error bars represent standard deviation. The star indicates statistical significance, using $p \leq 0.05$ in a student t-test, n=3.

The cells were prepared for the TBE assay as described in methods. DMSO treated cells were used as a negative control. The trypan blue dye is used to stain dead cells that do not have an intact membrane while live cells appear clear and are counted using an automated cell counter. The data were analyzed by calculating the IC50 curves for all treatments (Table 3).

Table 3
IC50 calculation for cell death induced by quercetin and its methylated derivatives after 72 h. IC50 values are reported in μM. Data in parenthesis indicate range for the 95% confidence interval.

Compounds	MDA-MB 231	MCF-10A	HeLa
Isorhamnetin	112.1 (75.24 to 167.10)	78.86 (34.85 to 178.40)	3.721 (3.116 to 4.44)
Tamarixetin	16.72 (10.37 to 26.98)	35.46 (24.63 to 51.05)	5.453 (4.610 to 6.45)
3-O-Methyl Quercetin	15.17 (13.07 to 17.61)	28.35 (20.27 to 39.64)	3.683 (1.313 to 10.33)
Pentamethyl Quercetin	50.05 (38.99 to 64.25)	89.62 (61.70 to 130.20)	170.3 (92.53 to 313.50)
Quercetin	175.9 (90.20 to 342.90)	90.42 (62.04 to 131.8)	9.983 (8.765 to 11.37)

The IC50 data and the TBE assay data were used together to evaluate the cytotoxic abilities of the compounds. The compounds 3-*O*-methylquercetin and tamarixetin were the most efficient in inducing cell death, but their effects were not specific to cancer cells. Isorhamnetin was more effective in HeLa cells and MCF-10A cells but did not show the same efficacy in MDA-MB 231 cells. Quercetin was also highly effective in HeLa cells, but not in the other two cell lines. Pentamethyl quercetin was ineffective in all three cell lines. In summary, our findings indicated that the

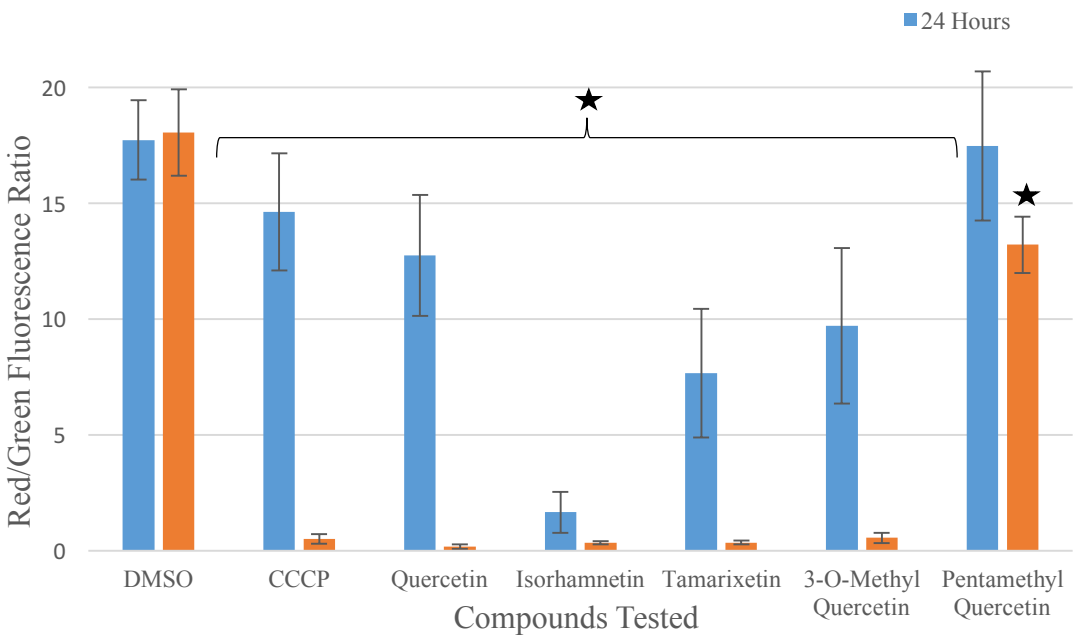
methylated derivatives of quercetin were more effective in inducing cell death than the parent quercetin molecule.

Loss of Mitochondrial Outer Membrane Potential Induced by Quercetin and Its Methylated Derivatives

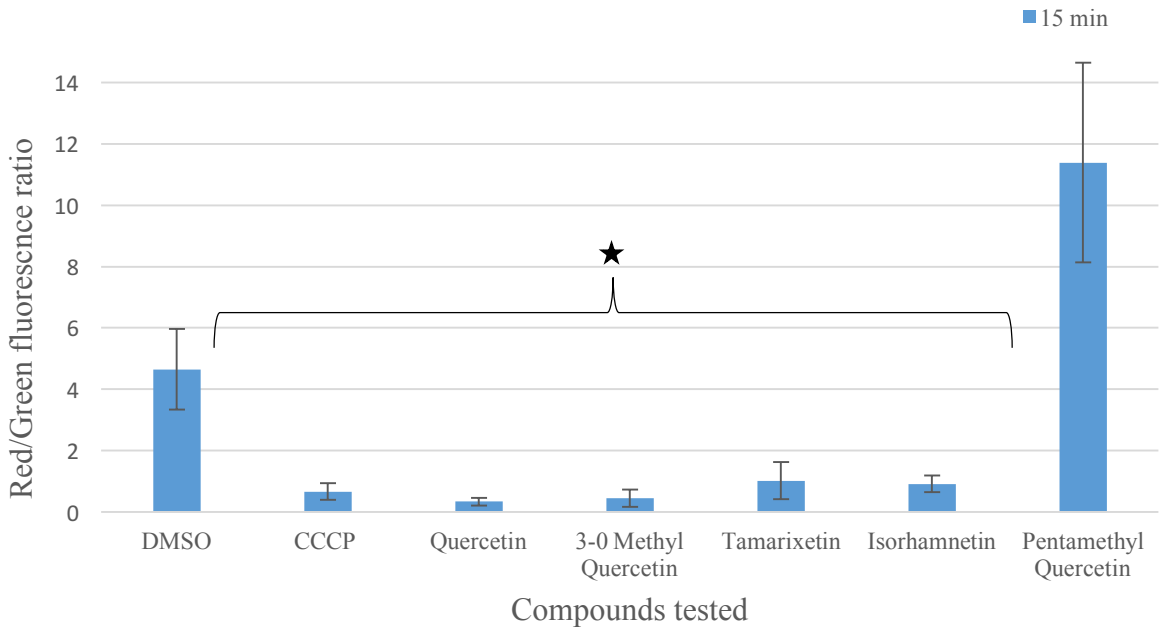
The MOMP of the three cell lines, MDA-MB 231, HeLa and MCF-10A cells, was measured after treatment with quercetin, isorhamnetin, tamarixetin, 3-*O*-methylquercetin, and pentamethyl quercetin at a concentration of 100 μM for 15 min and 24 h. A 24 h washout experiment was also conducted in MDA-MB 231 cells. The values of all compounds were compared to the positive control CCCP, a mitochondrial decoupler and the negative control (DMSO).

A loss of MOMP was observed after 15 min incubation with all compounds except pentamethyl quercetin (*Figure 3A*). The red/green fluorescence ratios were higher after 24 h compared to the 15 min incubation. No significant loss of MOMP was recorded in the 24 h washout experiment. Similar results were observed with the HeLa cells (*Figure 3B*), which showed a significant loss of MOMP in all treatments except for pentamethyl quercetin. The data from 15 min incubation with MCF-10A cells also show a significant loss of MOMP with all treatments (*Figure 3C*).

A.


24 Hours
Red/Green Fluorescence Ratio
20
15
10
5
0
DMSO
CCCP
Quercetin
Isorhamnetin
Tamarixetin
3-O-Methyl Quercetin
Pentamethyl Quercetin
Compounds Tested

B.


15 min
Red/Green fluorescnce ratio
14
12
10
8
6
4
2
0
DMSO
CCCP
Quercetin
3-0 Methyl Quercetin
Tamarixetin
Isorhamnetin
Pentamethyl Quercetin
Compounds tested

C.

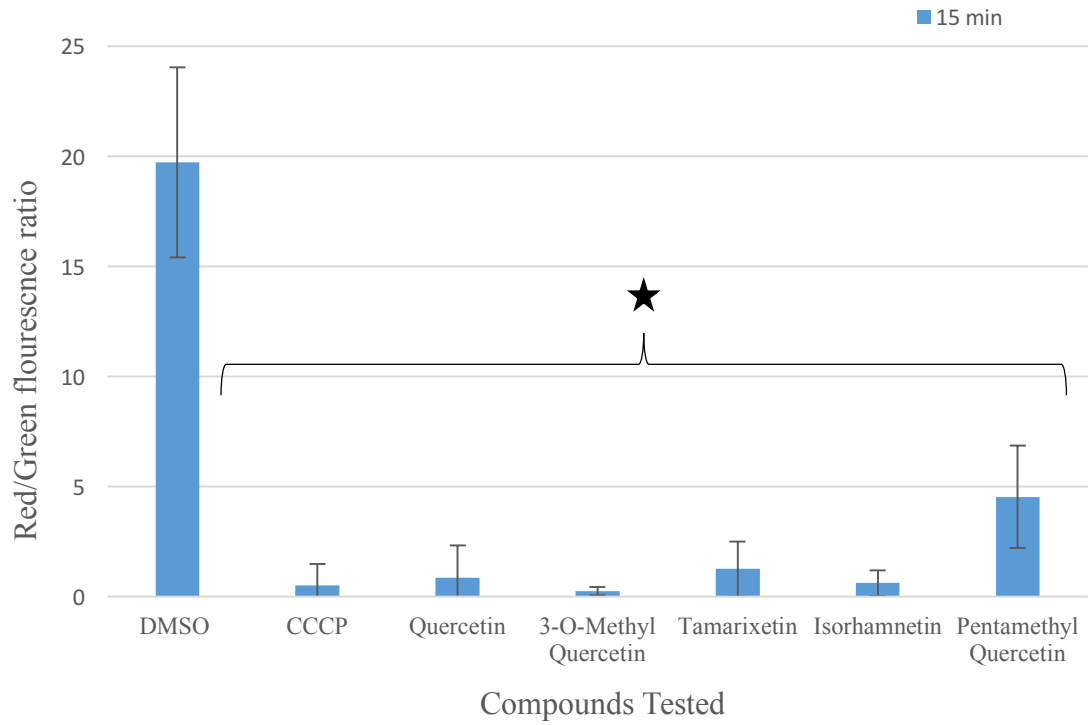


Figure 3. Effect of Quercetin and its methylated derivatives on mitochondrial outer membrane potential (MOMP). Flow cytometry data of A) MDA-MB 231 cells B) HeLa cells, and C) MCF-10A cells treated with 100 μM of quercetin and its methylated derivatives for 24 h/15 min and stained with JC-1 dye at 37°C. Carbonyl cyanide 3-cholorophenylhydrazone (CCCP) was used as positive control at a 50 μM concentration and DMSO was used as negative control. The ratio of red to green fluorescence was calculated and compared to the control and plotted on a graph. Error bars represent the standard deviation. The star indicates statistical significance, using $p \leq 0.05$ in a student t-test, n=5.

Measuring the Generation of Reactive Oxygen Species Upon Treatment with Quercetin and Its Methylated Derivatives

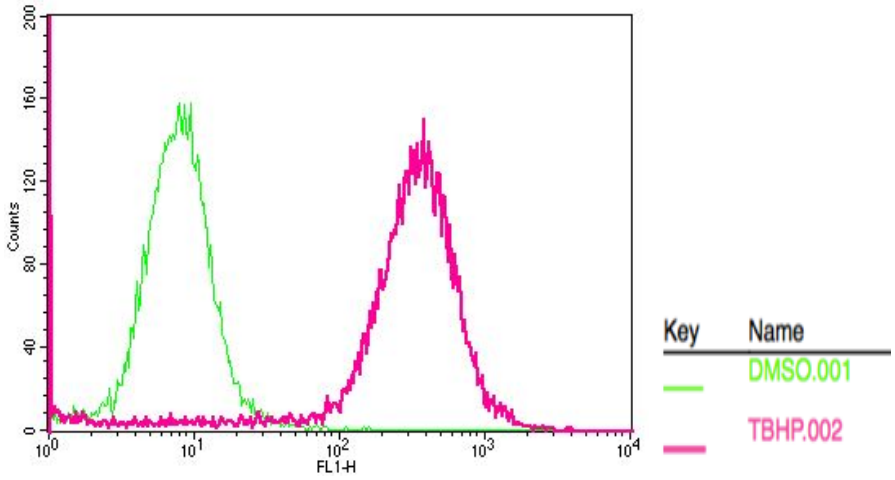
Generation of intracellular ROS is considered one of the significant moderators of apoptotic signaling in cancer treatment (Nicolini et al., 2013). Thus, the redox status of

MDA-MB 231 cells on treatment with quercetin and its methylated derivatives was determined. Cells were incubated with 100 μM quercetin, isorhamnetin, tamarixetin, 3-*O*-methylquercetin, or pentamethyl quercetin along with the CM-H_2DCFDA dye for 15 min at 37^0C. The chloromethyl derivative of H_2DCFDA is an oxidation-sensitive fluorescent dye, which acts as an indicator of ROS in cells. Tert-butyl hydroperoxide (TBHP) is a known inducer of ROS in cells and was used as a positive control. The data were evaluated in comparison to the positive control (*Figure 4A*) to determine any increase in the level of ROS upon treatment with the compounds. The compounds, along with TBHP, were also tested to determine their anti-oxidant potential.

No significant difference was evident in the levels of intracellular ROS on treatment with quercetin or isorhamnetin (*Figure 4B and 4C*). Previously a study demonstrated that the levels of ROS were not significantly different between quercetin treated cells and control cells, and our findings support these results (Chien et al., 2009). Upon individual THBP treatment of MDA-MB 231 cells with quercetin or isorhamnetin, a decrease in the levels of intracellular ROS was observed, further confirming the anti-oxidant potential of these two compounds. The levels of ROS measured upon treatment with tamarixetin and 3-O-methylquercetin (*Figure 4D and 4E*) were lower than the DMSO control indicating that no ROS was generated. The combination of TBHP with tamarixetin and 3-O-methylquercetin decreased the levels of ROS produced by TBHP, but with a weaker anti-oxidant effect than seen with quercetin. Pentamethyl quercetin (*Figure 4F*) did not show any significant change in the levels of ROS, nor did it decrease

the ROS generated by TBHP, suggesting a poor anti-oxidant activity, which is consistent with our cell viability data.

A.



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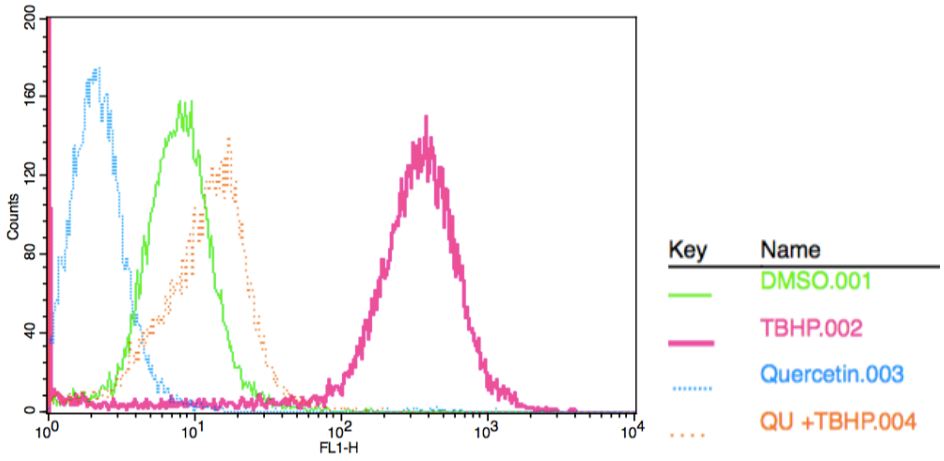

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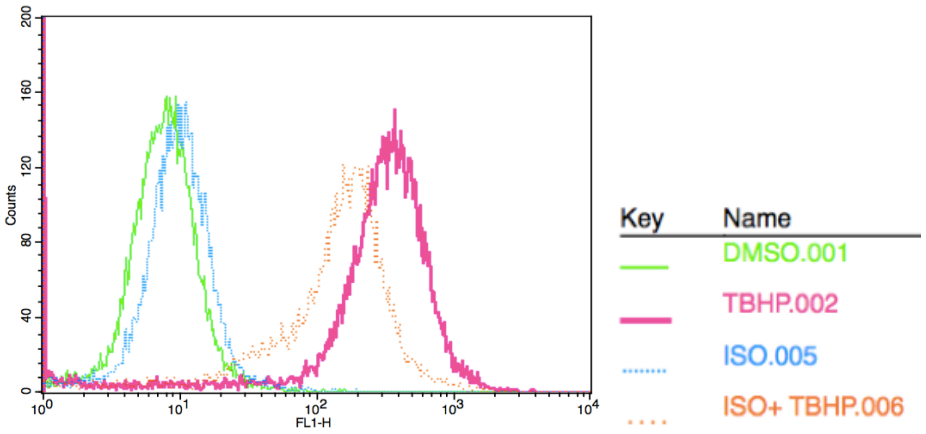
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FL1-H
Key
Name
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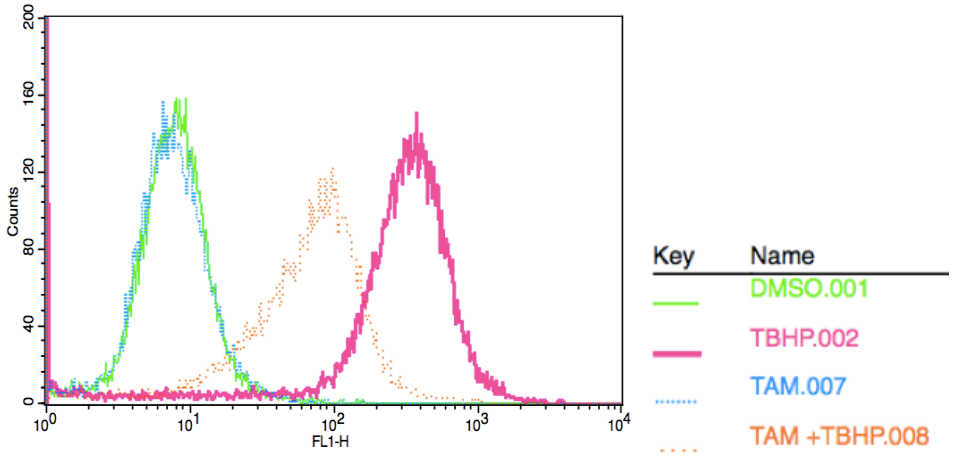
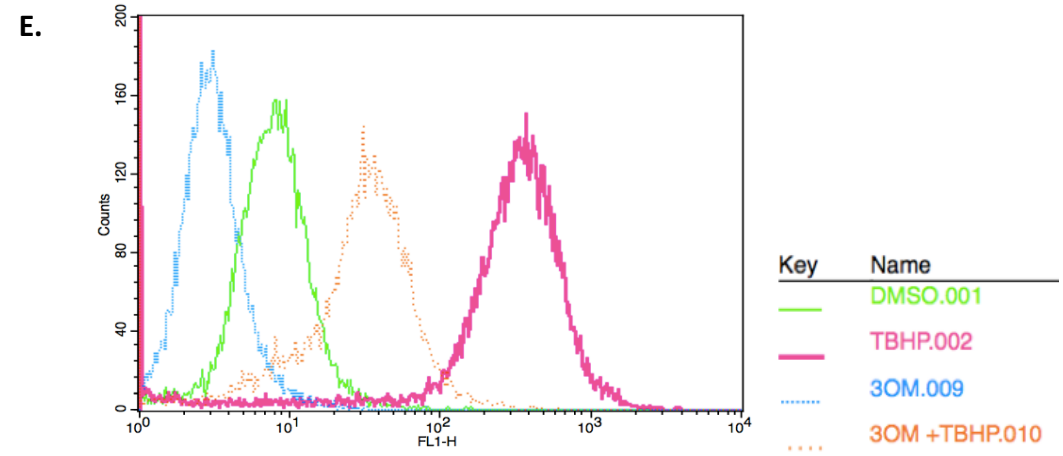
TBHP.002
ISO.005
ISO+ TBHP.006

D.


Counts
FL1-H
Key
Name
DMSO.001
TBHP.002
TAM.007
TAM +TBHP.008



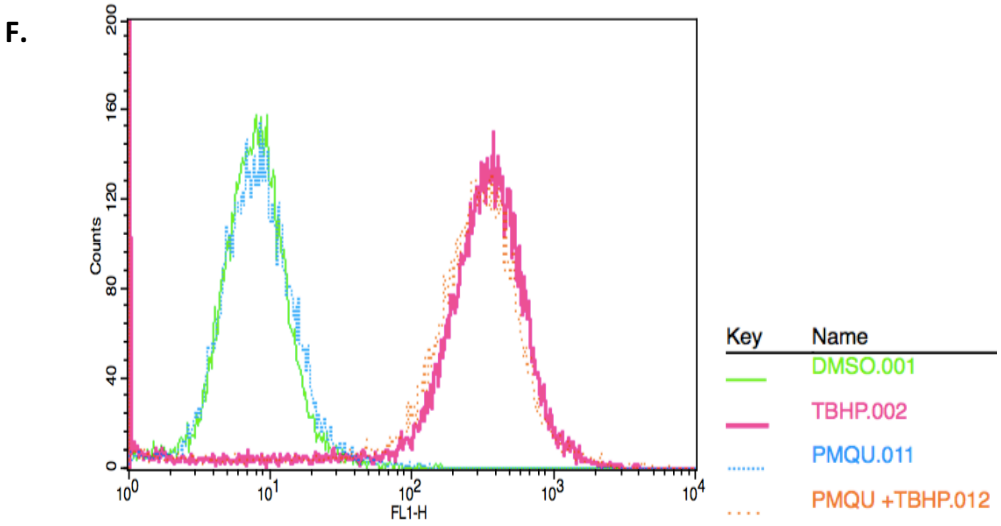
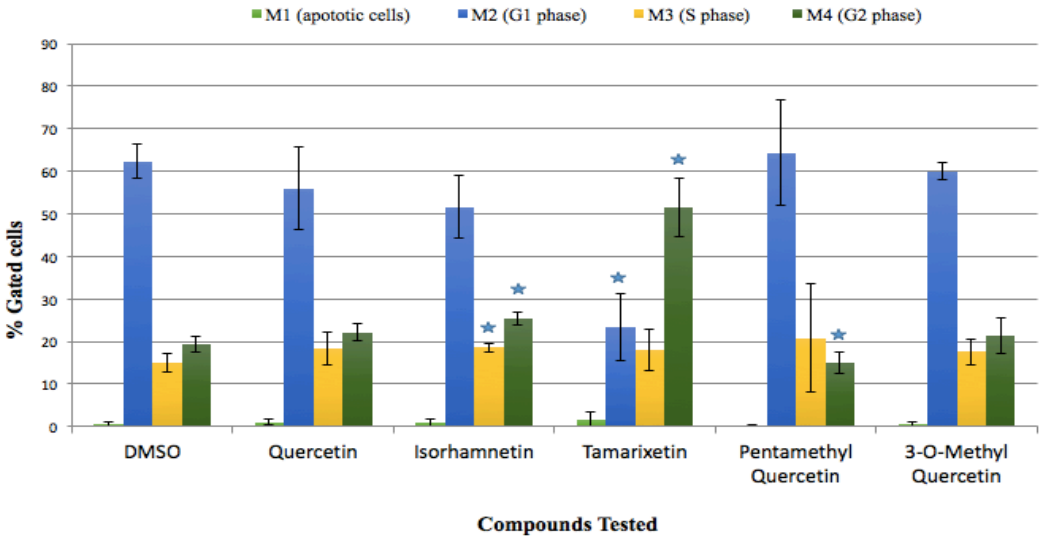


Figure 4. Flow cytometric analysis of the intracellular level of reactive oxygen species generated in MDA-MB 231 cells, displaying A) negative control DMSO and positive control TBHP and 100 μM of B) quercetin C) isorhamnetin D) tamarixetin E) 3-O-methyl quercetin, and F) pentamethyl quercetin. The treated cells were stained using CM-H_2DCFDA dye for 15 min at 37° C. The legend to the right of the graph indicates the treatments. All data were analyzed in comparison to the positive and negative control.

Induction of Cell Cycle Arrest

To assess whether the cell death induced by quercetin and its derivatives is mediated through alterations in cell cycle progression, the effect of these compounds on cell cycle phase distribution was evaluated using flow cytometry. The data obtained from this experiment were then compared to our cell death data. The MDA-MB 231 cells were treated with all compounds at a concentration of 100 µM and analyzed at 24 h and 48 h post treatment. We used DMSO as a negative control, as it displays a normal distribution of the cell cycle. Cell cycle arrest was observed at G2/M phase, 24 h post treatment using quercetin, isorhamnetin, and tamarixetin and at S phase using pentamethyl quercetin (*Figure 5A*). No significant change was evident in the cell cycle distribution with 3-*O*-methyl quercetin. The results were not significantly changed by the 48 h treatment (*Figure 5B*).

A



B

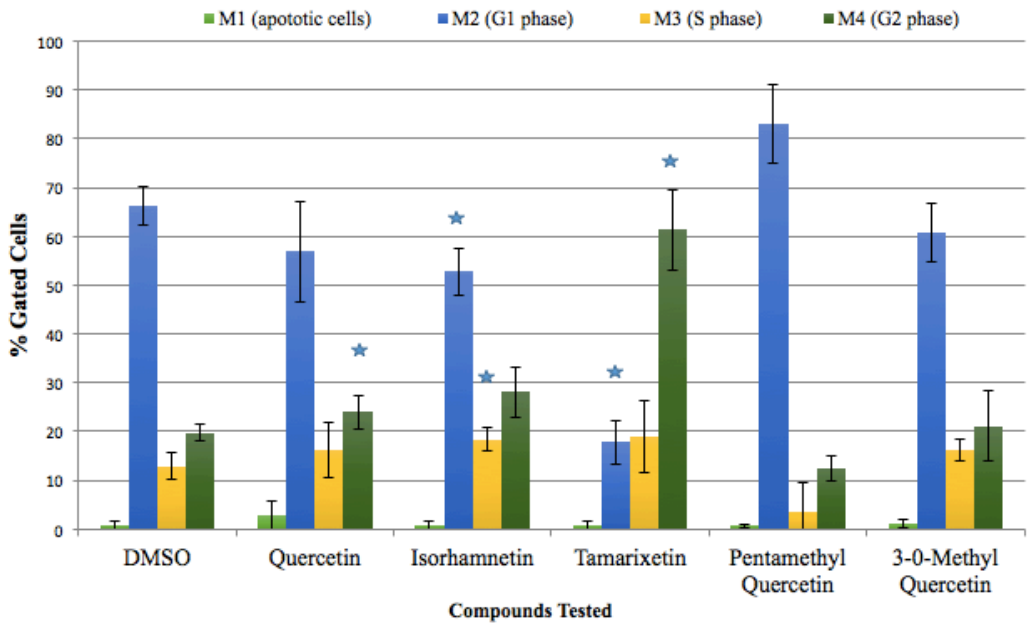


Figure 5. Cell cycle analysis of MDA-MB 231 cells after treatment with 100 μM of quercetin and its methylated derivatives. The cells were harvested and fixed in 100% EtOH, then stained with PI and incubated with RNase A at 37° C. The graphs represent data for A) 24 h treatment, and B) 48 h treatment. Error bars represent standard deviation. The star indicates statistical significance, using $p \leq 0.05$ in a student t-test, n=3.

Discussion

Quercetin and Its Methylated Derivatives Induce Dose-Dependent Dell death in MDA-MB 231 Cells

We examined the effects of quercetin and its methylated derivatives (isorhamnetin, 3-*O*-methyl quercetin, or pentamethyl quercetin) on the survival of triple negative breast cancer MDA-MB 231 cells, HeLa cells, and MCF-10A cells.

Quercetin treatment weakly induced cytotoxicity in MDA-MB 231 cells, with an IC50 of 175 μM. The data were consistent with a previous study which also showed quercetin as a weak inducer of cell death in the MDA-MB 231 cell line (Yadegarynia et al., 2012). Quercetin was more cytotoxic in HeLa cells, which suggests that the mechanism through which quercetin induces cell death may be different for various types of cancer cells. Quercetin has previously been shown to actively induce cell death in human cervical cancer, HeLa cells, through mitochondrial-mediated apoptosis and cell cycle arrest, which is carried out by p53 induction and NF-κB inhibition (Vidya Priyadarsini et al., 2010). Our data support this study. The difference in the mechanism of cell death in the MDA-MB 231 cells and HeLa cells could be because of the p53 mutation in the triple negative breast cancer MDA-MB 231 cell line, as the absence of this transcription factor results in an alternate pathway of cell death. We recorded no decrease in cell viability in MCF 10A cells on treatment with lower concentrations of quercetin, in contrast to the significant changes seen at the higher dose of 100 μM. The MCF-10A cell line is an immortalized, non-transformed, normal breast epithelial cell line. Our findings indicated that the cytotoxic effect of quercetin might be selective to tumor cells; however, more research needs to be done to determine the precise

mechanism. Isorhamnetin moderately induced cytotoxicity in MDA-MB 231 cells with an IC50 of 112.1 μM. Results from a previous study of isorhamnetin showed a similar cytotoxic potential in human colon carcinoma HCT-116 cells, with an IC50 OF 72 μM after 48 h of treatment (Jaramillo et al., 2010). We found that isorhamnetin decreased cell viability in MCF-10A cells at concentrations of 100 μM and 50 μM. Tamarixetin and 3-*O*-methyl quercetin were significant in their cytotoxic potential in all three cell lines. Both compounds were found to be potent inducers of cell death, although their cytotoxicity was much higher in HeLa cells as compared to the other cell lines. Pentamethyl quercetin showed weak induction of cytotoxicity in all three cell lines, which is unsurprising as it was previously shown to be a weak inducer of cell death in MDA-MB 231 (Yadegarynia et al., 2012).

We speculate that the five hydroxyl groups on the quercetin molecule are responsible for its ability to induce cell death, although methyl capping of one or more of these free hydroxyl groups may improve compound cytotoxicity. Pentamethyl quercetin has methylation on all five hydroxyl groups, which may explain why we found it to induce cell death weakly. If this is the case, it may also suggest that to be able to cause significant cell death, it is necessary that quercetin compounds have at least one free hydroxyl group. Using our findings, we can arrange quercetin compounds in the order of their cytotoxic potential based on the number of free hydroxyl groups, with 3-*O*-methylquercetin and tamarixetin being more strongly cytotoxic and isorhamnetin, quercetin, and pentamethyl quercetin being weakly cytotoxic.

The Induction of Cell Death is Through the Mitochondria-Mediated Pathway of Apoptosis

The loss of mitochondrial outer membrane potential (MOMP) is an indicator of the induction of apoptosis. The intrinsic pathway of apoptosis occurs due to the release of cytochrome c from the mitochondria. We measured the loss of MOMP to determine if quercetin and its methylated derivatives induce apoptosis through the intrinsic pathway mediated via the mitochondria. Our data for the 15 min treatment show that all compounds caused a significant loss of MOMP, suggesting that quercetin, isorhamnetin, tamarixetin, and 3-*O*-methyl quercetin induce cell death through the mitochondrial pathway of apoptosis. Pentamethyl quercetin did not show any significant loss of MOMP in MDA-MB 231 cells and HeLa cells. These data were consistent with the cell death data in supporting the weak cytotoxic effects of pentamethyl quercetin, further highlighting the importance of the free hydroxyl groups in cell death.

We also conducted the experiment as a 24 h treatment, washing the cells with PBS after trypsinization prior to analysis. With this treatment duration, the loss of MOMP was significantly less compared to the 15 min treatment in which the cells were not washed. Our data suggest that the effects induced by these compounds are somehow reversed on washing the cells with PBS. The exact mechanism behind this is not clear and more studies should be conducted. A previous study has shown significant loss of MOMP in human glioblastoma U373MG cells when treated with 75 μM and 100 μM of quercetin for 48 h. These cells were also trypsinized and washed prior to analysis (Kim et al., 2013). Our data were not consistent with these findings. However, from the loss of MOMP we observed in HeLa cells, we can determine that the effect of these compounds

is not cell line dependent. The data obtained from analyzing MCF-10A cells highlight that the cytotoxicity induced by these compounds may not be specific to tumor cells. Studies are currently being conducted to create targeting strategies for these compounds, which will allow us to more specifically deliver treatments and prevent the killing of healthy cells.

Quercetin and Its Methylated Derivatives Act as an Anti-Oxidant Not a Pro-oxidant

Recent studies have revealed that quercetin may behave as both as anti-oxidant and pro-oxidant based on the intracellular concentration and free radical source (Metodiewa et al., 1999). We tested quercetin and its methylated derivatives on triple negative breast cancer cell line MDA-MB 231 to determine if these flavonol compounds generated reactive oxygen species intracellularly. Based on our findings, we did not see any change in the level of reactive oxygen species (ROS) upon treatment with the flavonol compounds when compared to our positive control TBHP and negative control DMSO treatments. Our data demonstrate that quercetin, isorhamnetin, 3-*O*-methyl quercetin, and pentamethyl quercetin do not generate ROS in MDA-MB 231 cells when treated with a concentration of 100 μM for 15 min at 37^{o}C. We speculate that the compounds mentioned above do not exhibit pro-oxidant activity in the MDA-MB 231 cell line and that the pathway through which these compounds induce cell death is not by generation of ROS. Data from a previous study conducted in leukemia cells have shown tamarixetin to cause a fourfold increase in levels of ROS as compared to the control (Nicolini et al., 2013). While the data were contradictory to our results, they may suggest

that the mechanism through which tamarixetin induces cell death varies with different types of cancer.

On treating the cell with both TBHP and the flavonol compounds, a decrease in the levels of ROS was evident, indicating the anti-oxidant potential of these compounds. This potential was different for each compound tested, the highest resulting from treatment with quercetin and 3-*O*-methyl quercetin, followed by treatment with tamarixetin and isorhamnetin. There was no decrease in levels of ROS upon treatment with pentamethyl quercetin.

Alterations in Cell Cycle Distribution May Occur Prior to Cell Death After Treatment with Quercetin and Its Methylated Derivatives

Literature has shown that quercetin-induced cell death may be attributed to apoptosis mediated by cell cycle arrest. Previous studies have reported a significant arrest with quercetin at the G2/M phase of the cell cycle, with a promotion of S-phase of the cell cycle (Chien et al., 2009). G2/M arrest of cell cycle progression was also demonstrated following quercetin treatment in human leukemia U937 cells (Chou et al., 2010). Arrest at the G2/M phase of cell cycle was also evident consistently across treatment with quercetin derivatives, except for pentamethyl quercetin, which shows cell cycle arrest at the G1 phase. The G1 phase arrest by pentamethyl quercetin has been previously observed in MDA-MB 231 cell lines (Yadegarynia et al., 2012). Our study indicates that although these compounds alter the cell cycle progression, this alteration may not be associated with the observed induction of cell death. More studies should be conducted to investigate the role of cell cycle alterations in flavonol induced cell death.

Taken together, we have determined that the structure of the quercetin parent molecule likely plays a significant role in its cytotoxic potential. The increase in cell death seen upon treatment with tamarixetin and 3-O-methyl quercetin suggests that methyl capping of the free hydroxyl groups at position 3, and position 4' of the quercetin molecule improves the cytotoxic potential of the compound. However, this also compromises the anti-oxidant activity exhibited by quercetin. In this case, quercetin derivatives with fewer free hydroxyl groups will demonstrate poorer antioxidant activity than derivatives with greater hydroxyl group availability. The methyl capping on position 3' of quercetin appears to moderately affect its ability to induce cell death, suggesting that the hydroxyl group at the 3' position may not be as reactive as the one on 3 and 4'. With all five hydroxyl groups methylated, pentamethyl quercetin was found to induce the least cytotoxicity, which is consistent with the theory that at least one free hydroxyl group must be present for quercetin to be able to induce cell death. Furthermore, methyl-capping of all five hydroxyl groups may render the quercetin molecule unreactive.

We also conclude that these compounds induce cell death through the mitochondrial-mediated pathway of apoptosis. The flavonol compounds studied do not act as pro-oxidants when they induce cell death, but rather, they exhibit a radical scavenging property. Cell death may be accompanied by alterations in the cell cycle progression, but lacks validity as an indicator to determine the mechanism of apoptosis. Further studies need to be carried out to clearly understand how the structure of the quercetin molecule affects its cytotoxicity. To identify the most reactive free hydroxyl

groups, comparative analysis of additional methylated derivatives with variations in the methyl capping positions must be conducted. To underline the exact mechanism of cell death, we need to measure the levels of Bax and Bak and levels of cytochrome c in the cytosol, all of which collectively serve to indicate intrinsic apoptosis.

In conclusion, additional experiments will help us to better understand the properties of these flavonol compounds in cancer and explore their role as potential chemotherapeutic agents. This knowledge will allow us as researchers to modulate these derivatives and exploit their properties to serve as natural chemo-toxic agents in the treatment of cancer.

References

Adams, J. M., & Cory, S. (1998). The Bcl-2 protein family: Arbiters of cell survival. *Science*, *281*, 1322–1326. http://doi.org/10.1126/science.281.5381.1322

Bauer, K. R., Brown, M., Cress, R. D., Parise, C. A., & Caggiano, V. (2007). Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: A population-based study from the California Cancer Registry. *Cancer*, *109*, 1721–1728. http://doi.org/10.1002/cncr.22618

Bettega, J. M. R., Teixeira, H., Bassani, V. L., Barardi, C. R. M., & Simões, C. M. O. (2004). Evaluation of the antiherpetic activity of standardized extracts of Achyrocline satureioides. *Phytotherapy Research*, *18*, 819–823. http://doi.org/10.1002/ptr.1568

Bidère, N., Lorenzo, H. K., Carmona, S., Laforge, M., Harper, F., Dumont, C., & Senik, A. (2003). Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis. *Journal of Biological Chemistry*, *278*, 31401–31411. http://doi.org/10.1074/jbc.M301911200

Boone, E., Vanden Berghe, T., Van Loo, G., De Wilde, G., De Wael, N., Vercammen, D., … Vandenabeele, P. (2000). Structure/function analysis of p55 tumor necrosis factor receptor and Fas-associated death domain. Effect on necrosis in l929sA cells. *Journal of Biological Chemistry*, *275*, 37596–37603. http://doi.org/10.1074/jbc.M007166200

Botchkarev, V. A. (2003). Molecular mechanisms of chemotherapy-induced hair loss. *Journal of Investigative Dermatology Symposium Proceedings*, *8*, 72–75. http://doi.org/10.1046/j.1523-1747.2003.12175.x

Carini, J., Klamt, F., & Linck, V. (2014). Flavonoids from Achyrocline satureioides : promising biomolecules for anticancer therapy. *RSC Advances*, *4*, 3131–3144. http://doi.org/10.1039/c3ra43627f

Chan, F. K. M., Shisler, J., Bixby, J. G., Felices, M., Zheng, L., Appel, M., … Lenardo, M. J. (2003). A role for tumor necrosis factor receptor-2 and receptor-interacting protein in programmed necrosis and antiviral responses. *Journal of Biological Chemistry*, *278*, 51613–51621. http://doi.org/10.1074/jbc.M305633200

Chien, S.-Y., Wu, Y.-C., Chung, J.-G., Yang, J.-S., Lu, H.-F., Tsou, M.-F., … Chen, D.-

R. (2009). Quercetin-induced apoptosis acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells. *Human & Experimental Toxicology*, *28*, 493–503. http://doi.org/10.1177/0960327109107002

Chou, C.-C., Yang, J.-S., Lu, H.-F., Ip, S.-W., Lo, C., Wu, C.-C., … Chen, D.-R. (2010). Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. *Archives of Pharmacal Research*, *33*, 1181–1191. http://doi.org/10.1007/s12272-010-0808-y

Chowdhury, I., Tharakan, B., & Bhat, G. K. (2008). Caspases - An update. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, *151*, 10–27. http://doi.org/10.1016/j.cbpb.2008.05.010

Crozier, A., Lean, M. E. J., McDonald, M. S., & Black, C. (1997). Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce, and celery. *Journal of Agricultural and Food Chemistry*, *45*, 590–595. http://doi.org/10.1021/jf960339y

Daugas, E., Susin, S. A., Zamzami, N., Ferri, K. F., Irinopoulou, T., Larochette, N., … Kroemer, G. (2000). Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *14*, 729–739. http://doi.org/10.1096/fj.00-0388com

De Laurentiis, M., Cianniello, D., Caputo, R., Stanzione, B., Arpino, G., Cinieri, S., … De Placido, S. (2016). Treatment of triple negative breast cancer (TNBC): current options and future perspectives. *Cancer Treatment Reviews*, *36*, S80–S86. http://doi.org/10.1016/S0305-7372(10)70025-6

Du, X. L., Osborne, C., & Goodwin, J. S. (2002). Population-based assessment of hospitalizations for toxicity from chemotherapy in older women with breast cancer. *Journal of Clinical Oncology* , *20*, 4636–4642. http://doi.org/10.1200/JCO.2002.05.088

Duo, J., Ying, G.-G., Wang, G.-W., & Zhang, L. (2012). Quercetin inhibits human breast cancer cell proliferation and induces apoptosis via Bcl-2 and Bax regulation. *Molecular Medicine Reports*, *5*, 1453–6. http://doi.org/10.3892/mmr.2012.845

Early Breast Cancer Trialists' Collaborative Group. (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *The Lancet*, *365*, 1687–1717. http://doi.org/10.1016/S0140-6736(05)66544-0

Egert, S., Wolffram, S., Bosy-Westphal, A., Boesch-Saadatmandi, C., Wagner, A. E., Frank, J., … Mueller, M. J. (2008). Daily quercetin supplementation dose-dependently increases plasma quercetin concentrations in healthy humans. *The Journal of Nutrition*, *138*, 1615–1621. http://doi.org/138/9/1615 [pii]

Elkholi, R., Renault, T. T., Serasinghe, M. N., & Chipuk, J. E. (2014). Putting the pieces together: How is the mitochondrial pathway of apoptosis regulated in cancer and chemotherapy? *Cancer & Metabolism*, *2*, 16. http://doi.org/10.1186/2049-3002-2-16

Epand, R. F., Martinou, J. C., Montessuit, S., Epand, R. M., & Yip, C. M. (2002). Direct evidence for membrane pore formation by the apoptotic protein Bax. *Biochemical and Biophysical Research Communications*, *298*, 744–749. http://doi.org/10.1016/S0006-291X(02)02544-5

Eruslanov, E., & Kusmartsev, S. (2010). Identification of ROS using oxidized DCFDA and flow-cytometry. *Methods in Molecular Biology (Clifton, N.J.)*, *594*, 57–72. http://doi.org/10.1007/978-1-60761-411-1_4

Facts, Q. (2011). MitoProbe ^{TM} JC-1 assay kit for flow cytometry (M34152). *Cytometry*, 6–8.

Festjens, N., Vanden Berghe, T., & Vandenabeele, P. (2006). Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response. *Biochimica et Biophysica Acta - Bioenergetics*, *1757*, 1371–1387. http://doi.org/10.1016/j.bbabio.2006.06.014

Puski, G., & Francis, F. J. (1967). Flavonol glycosides in cranberries. *Journal of Food Science*, *32*, 527-530.

Fink, S. L., Cookson, B. T., Fink, S. L., & Cookson, B. T. (2005). Eukaryotic cells apoptosis , pyroptosis , and necrosis : Mechanistic description of dead and dying eukaryotic cells, *73*, 1907–1916. http://doi.org/10.1128/IAI.73.4.1907

Fulda, S., & Debatin, K.-M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*, *25*, 4798–4811. http://doi.org/10.1038/sj.onc.1209608

Galati, G., & O'Brien, P. J. (2004). Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. *Free Radical Biology and Medicine*, *37*, 287–303. http://doi.org/10.1016/j.freeradbiomed.2004.04.034

Gallus, J., Juvale, K., & Wiese, M. (2014). Characterization of 3-methoxy flavones for their interaction with ABCG2 as suggested by ATPase activity. *Biochimica et*

Biophysica Acta - Biomembranes, *1838*, 2929–2938. http://doi.org/10.1016/j.bbamem.2014.08.003

Galluzzi, L., Vanden Berghe, T., Vanlangenakker, N., Buettner, S., Eisenberg, T., Vandenabeele, P., … Kroemer, G. (2011). Programmed necrosis. From molecules to health and disease. *International Review of Cell and Molecular Biology*, 289, 1-35. Elsevier Inc. http://doi.org/10.1016/B978-0-12-386039-2.00001-8

Gluz, O., Liedtke, C., Gottschalk, N., Pusztai, L., Nitz, U., & Harbeck, N. (2009). Triple-negative breast cancer-current status and future directions. *Annals of Oncology*, *20*, 1913–1927. http://doi.org/10.1093/annonc/mdp492

Golstein, P., & Kroemer, G. (2007). Cell death by necrosis: Towards a molecular definition. *Trends in Biochemical Sciences*, *32*, 37–43. http://doi.org/10.1016/j.tibs.2006.11.001

Grunnet, L. G., Aikin, R., Tonnesen, M. F., Paraskevas, S., Blaabjerg, L., StØrling, J., … Mandrup-Poulsen, T. (2009). Proinflammatory cytokines activate the intrinsic apoptotic pathway in beta-cells. *Diabetes*, *58*, 1807–1815. http://doi.org/10.2337/db08-0178

Ha, H. C., & Snyder, S. H. (2016). Poly (ADP-Ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proceedings of the National Academy of Sciences*, *96*, 13978–13982.

Haenen, G. R., Paquay, J. B., Korthouwer, R. E., & Bast, a. (1997). Peroxynitrite scavenging by flavonoids. *Biochemical and Biophysical Research Communications*, *236*, 591–593. http://doi.org/10.1006/bbrc.1997.7016

Harwood, M., Danielewska-Nikiel, B., Borzelleca, J. F., Flamm, G. W., Williams, G. M., & Lines, T. C. (2007). A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food and Chemical Toxicology*, *45*, 2179–2205. http://doi.org/10.1016/j.fct.2007.05.015

Heim, K. E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry*, *13*, 572–584. http://doi.org/10.1016/S0955-2863(02)00208-5

Hertog, M. G., Hollman, P. C., & van de Putte, B. (1993). Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *Journal of Agriculture and Food Chemistry*, *41*, 1242–1246. http://doi.org/10.1021/jf00032a015

Hollman, P. C. H., van Trijp, J. M. P., Mengelers, M. J. B., de Vries, J. H. M., & Katan, M. B. (1997). Bioavailability of the dietary antioxidant flavonol quercetin in man. *Cancer Letters*, *114*, 139–140. http://doi.org/10.1016/S0304-3835(97)04644-2

Hurvitz, S., & Mead, M. (2015). Triple-negative breast cancer. *Current Opinion in Obstetrics and Gynecology*, *70*(17), 1. http://doi.org/10.1097/GCO.0000000000000239

Jaramillo, S., Lopez, S., Varela, L. M., Rodriguez-Arcos, R., Jimenez, A., Abia, R., … Muriana, F. J. G. (2010). The flavonol isorhamnetin exhibits cytotoxic effects on human colon cancer cells. *Journal of Agricultural and Food Chemistry*, *58*, 10869–10875. http://doi.org/10.1021/jf102669p

Kerr, J. F., Winterford, C. M., & Harmon, B. V. (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer*, *73*, 2013–2026. http://doi.org/10.1002/1097-0142(19940415)73:8<2013::AID-CNCR2820730802>3.0.CO;2-J

Kim, H., Moon, J. Y., Ahn, K. S., & Cho, S. K. (2013). Quercetin induces mitochondrial mediated apoptosis and protective autophagy in human glioblastoma U373MG Cells. *Oxidative Medicine and Cellular Longevity*, *2013*. http://doi.org/10.1155/2013/596496

Kischkel, F. C., Lawrence, D. a, Chuntharapai, A., Schow, P., Kim, K. J., & Ashkenazi, A. (2000). Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity*, *12*, 611–620. http://doi.org/10.1016/S1074-7613(00)80212-5

Koneri, K., Goi, T., Hirono, Y., Katayama, K., & Yamaguchi, A. (2007). Beclin 1 gene inhibits tumor growth in colon cancer cell lines. *Anticancer Research*, *27*, 1453–1457.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., … Melino, G. (2009). Classification of cell death: Recommendations of the nomenclature committee on cell death 2009. *Cell Death and Differentiation*, *16*, 3–11. http://doi.org/10.1038/cdd.2008.150

Kutzki, O., Park, H. S., Ernst, J. T., Orner, B. P., Yin, H., & Hamilton, A. D. (2002). Development of a potent Bcl-xL antagonist based on alpha-helix mimicry. *Journal of the American Chemical Society*, *124*, 11838–11839. http://doi.org/10.1021/ja026861k

Kuwana, T., Bouchier-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., & Newmeyer, D. D. (2005). BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and

indirectly. *Molecular Cell*, *17*, 525–535. http://doi.org/10.1016/j.molcel.2005.02.003

Lee, H. J., Lee, H. J., Lee, E. O., Ko, S. G., Bae, H. S., Kim, C. H., … Kim, S. H. (2008). Mitochondria-cytochrome C-caspase-9 cascade mediates isorhamnetin-induced apoptosis. *Cancer Letters*, *270*, 342–353. http://doi.org/10.1016/j.canlet.2008.05.040

Letan, a. (1966). The relation of structure to antioxidant activity of quercetin and some of its derivatives II. secondary (metal-complexing) activity. *Journal of Food Science*, *31*, 395–399.

Levine, B., & Yuan, J. (2005). Autophagy in cell death: An innocent convict? *Journal of Clinical Investigation*, *115*, 2679–2688. http://doi.org/10.1172/JCI26390

Li, C., Yang, D., Zhao, Y., Qiu, Y., Cao, X., Yu, Y., … Yin, X. (2015). Inhibitory effects of isorhamnetin on the invasion of human breast carcinoma cells by downregulating the expression and activity of matrix metalloproteinase-2/9. *Nutrition and Cancer*, *67*, 1191–1200. http://doi.org/10.1080/01635581.2015.1073763

Li, L. Y., Luo, X., & Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*, *412*(6842), 95–99. http://doi.org/10.1038/35083620

Li, Z., & Gao, Q. (2013). Induction of apoptosis in HT-29 cells by quercetin through mitochondria-mediated apoptotic pathway. *Animal Cells and Systems*, *17*, 147–153. http://doi.org/10.1080/19768354.2013.793210

Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., & Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*, *402*(6762), 672–676. http://doi.org/10.1038/45257

Lockshin, R. A., & Zakeri, Z. (2004). Caspase-independent cell death? *Oncogene*, *23*, 2766–2773. http://doi.org/10.1038/sj.onc.1207514

Lum, J. J., DeBerardinis, R. J., & Thompson, C. B. (2005). Autophagy in metazoans: cell survival in the land of plenty. *Nature Reviews. Molecular Cell Biology*, *6*, 439–48. http://doi.org/10.1038/nrm1660

Martin, S. J. (2014). Caspases: Executioners of apoptosis. *Pathobiology of Human Disease*, *16*, 145–152. http://doi.org/10.1016/B978-0-12-386456-7.01411-8

Metodiewa, D., Jaiswal, A. K., Cenas, N., Dickancaité, E., & Segura-Aguilar, J. (1999). Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. *Free Radical Biology and Medicine*, *26*, 107–116. http://doi.org/10.1016/S0891-5849(98)00167-1

Michalak, E. M., Villunger, A., Adams, J. M., & Strasser, A. (2008). In several cell types tumour suppressor p53 induces apoptosis largely via Puma but Noxa can contribute. *Cell Death and Differentiation*, *15*, 1019–29. http://doi.org/10.1038/cdd.2008.16

Murakami, A., Ashida, H., & Terao, J. (2008). Multitargeted cancer prevention by quercetin. *Cancer Letters*, *269*, 315–325. http://doi.org/10.1016/j.canlet.2008.03.046

Murota, K., & Terao, J. (2003). Antioxidative flavonoid quercetin: Implication of its intestinal absorption and metabolism. *Archives of Biochemistry and Biophysics*, *417*, 12–17. http://doi.org/10.1016/S0003-9861(03)00284-4

Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H., … Tsujimoto, Y. (2005). Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature*, *434*(7033), 652–658. Retrieved from http://dx.doi.org/10.1038/nature03317

Nicolini, F., Burmistrova, O., Marrero, M. T., Torres, F., Hernández, C., Quintana, J., & Estévez, F. (2013). Induction of G2 /M phase arrest and apoptosis by the flavonoid tamarixetin on human leukemia cells. *Molecular Carcinogenesis*, *950*(April 2013), 1–12. http://doi.org/10.1002/mc.22055

Norberg, E., Orrenius, S., & Zhivotovsky, B. (2010). Mitochondrial regulation of cell death: Processing of apoptosis-inducing factor (AIF). *Biochemical and Biophysical Research Communications*, *396*, 95–100. http://doi.org/10.1016/j.bbrc.2010.02.163

Noteborn, H. P. J. M., Jansen, E., Benito, S., & Mengelers, M. J. B. (1997). Oral absorption and metabolism of quercetin and sugar-conjugated derivatives in specific transport systems, *Cancer Letters*, *114*, 175-177.

O'Brien, M. A., & Kirby, R. (2008). Apoptosis: A review of pro-apoptotic and anti-apoptotic pathways and dysregulation in disease. *Journal of Veterinary Emergency and Critical Care*, *18*, 572–585. http://doi.org/10.1111/j.1476-4431.2008.00363.x

Ossovskaya, V., Wang, Y., Budoff, a., Xu, Q., Lituev, a., Potapova, O., … Daraselia, N. (2011). Exploring molecular pathways of triple-negative breast cancer. *Genes & Cancer*, *2*, 870–879. http://doi.org/10.1177/1947601911432496

Pandurangan, N., Bose, C., & Banerji, A. (2011). Synthesis and antioxygenic activities of seabuckthorn flavone-3-ols and analogs. *Bioorganic and Medicinal Chemistry Letters*, *21*, 5328–5330. http://doi.org/10.1016/j.bmcl.2011.07.008

Park, Y., & Lee, C. Y. (1996). Identification of isorhamnetin 4′-Glucoside in onions. *Journal of Agricultural and Food Chemistry*, *44*, 34–36.

Pastorino, J. G., Simbula, G., Yamamoto, K., Glascott, P. A., Rothman, R. J., & Farber, J. L. (1996). The cytotoxicity of tumor necrosis factor depends on induction of the mitochondrial permeability transition. *Journal of Biological Chemistry*, *271*, 29792–29798. http://doi.org/10.1074/jbc.271.47.29792

Pelicano, H., Feng, L., Zhou, Y., Carew, J. S., Hileman, E. O., Plunkett, W., … Huang, P. (2003). Inhibition of mitochondrial respiration: A novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. *Journal of Biological Chemistry*, *278*, 37832–37839. http://doi.org/10.1074/jbc.M301546200

Peng, W., Li, Y., Zhu, C., Han, X., & Yu, B. (2005). Synthesis of tamarixetin and isorhamnetin 3-O-neohesperidoside. *Carbohydrate Research*, *340*, 1682–1688. http://doi.org/10.1016/j.carres.2005.04.021

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products*, *63*, 1035–1042. http://doi.org/10.1021/np9904509

Polster, B. M., Basañez, G., Etxebarria, A., Hardwick, J. M., & Nicholls, D. G. (2005). Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. *Journal of Biological Chemistry*, *280*, 6447–6454. http://doi.org/10.1074/jbc.M413269200

Pozarowski, P., & Darzynkiewicz, Z. (2004). Analysis of cell cycle by flow cytometry. *Methods in Molecular Biology (Clifton, N.J.)*, *281*, 301–311. http://doi.org/10.1385/1-59259-811-0:301

Puthalakath, H., O'Reilly, L. A., Gunn, P., Lee, L., Kelly, P. N., Huntington, N. D., … Strasser, A. (2007). ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell*, *129*, 1337–1349. http://doi.org/10.1016/j.cell.2007.04.027

Ren, D., Tu, H.-C., Kim, H., Wang, G. X., Bean, G. R., Takeuchi, O., … Cheng, E. H.-Y. (2010). BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program. *Science (New York, N.Y.)*, *330*, 1390–3. http://doi.org/10.1126/science.1190217

Ren, W., Qiao, Z., Wang, H., Zhu, L., & Zhang, L. (2003). Flavonoids: Promising anticancer agents. *Medicinal Research Reviews*, *23*, 519–534. http://doi.org/10.1002/med.10033

Russo, M., Spagnuolo, C., Tedesco, I., Bilotto, S., & Russo, G. L. (2012). The flavonoid quercetin in disease prevention and therapy: Facts and fancies. *Biochemical Pharmacology*, *83*, 6–15. http://doi.org/10.1016/j.bcp.2011.08.010

Saelens, X., Festjens, N., Vande Walle, L., van Gurp, M., van Loo, G., & Vandenabeele, P. (2004). Toxic proteins released from mitochondria in cell death. *Oncogene*, *23*, 2861–2874. http://doi.org/10.1038/sj.onc.1207523

Schmidt, S., Rainer, J., Ploner, C., Presul, E., Riml, S., & Kofler, R. (2004). Glucocorticoid-induced apoptosis and glucocorticoid resistance: Molecular mechanisms and clinical relevance. *Cell Death and Differentiation*, *11*, S45–S55. http://doi.org/10.1038/sj.cdd.4401456

Schwingel, L. C., Schwingel, G. O., Storch, N., Barreto, F., & Bassani, V. L. (2014). 3-O-Methylquercetin from organic Nicotiana tabacum L. trichomes: Influence of the variety, cultivation and extraction parameters. *Industrial Crops and Products*, *55*, 56–62. http://doi.org/10.1016/j.indcrop.2014.01.054

Scorrano, L., & Korsmeyer, S. J. (2003). Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. *Biochemical and Biophysical Research Communications*, *304*, 437–444. http://doi.org/10.1016/S0006-291X(03)00615-6

Sharma, A., & Gupta, H. (2010). Quercetin- A flavanoid. *Chron Young Sci, 1, 10-5*.

Sharma, N., Dobhal, M., Joshi, Y., & Chahar, M. (2011). Flavonoids: A versatile source of anticancer drugs. *Pharmacognosy Reviews*, *5*(9), 1. http://doi.org/10.4103/0973-7847.79093

Siegel, R. L., Miller, K. D., & Jemal, A. (2016). Cancer statistics, *66*, 7–30. http://doi.org/10.3322/caac.21332.

Siegel, R., Miller, K., & Jemal, A. (2015). Cancer statistics , 2015 . *CA Cancer J Clin*, *65*, 29. http://doi.org/10.3322/caac.21254.

Siegelin, M. D., Reuss, D. E., Habel, A., Rami, A., & von Deimling, A. (2009). Quercetin promotes degradation of survivin and thereby enhances death-receptor-mediated apoptosis in glioma cells. *Neuro-Oncology*, *11*, 122–31. http://doi.org/10.1215/15228517-2008-085

Smaili, S. S., Hsu, Y. T., Sanders, K. M., Russell, J. T., & Youle, R. J. (2001). Bax translocation to mitochondria subsequent to a rapid loss of mitochondrial membrane potential. *Cell Death and Differentiation*, *8*, 909–920. http://doi.org/10.1038/sj.cdd.4400889

Spencer, J. P. E., Kuhnle, G. G. C., Williams, R. J., & Rice-Evans, C. (2003). Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites. *The Biochemical Journal*, *372*, 173–81. http://doi.org/10.1042/BJ20021972

Sun, B., Sun, G. B., Xiao, J., Chen, R. C., Wang, X., Wu, Y., … Sun, X. B. (2012). Isorhamnetin inhibits H 2O 2-induced activation of the intrinsic apoptotic pathway in H9c2 cardiomyocytes through scavenging reactive oxygen species and ERK inactivation. *Journal of Cellular Biochemistry*, *113*, 473–485. http://doi.org/10.1002/jcb.23371

Tsujimura, M., & September, R. (1930). On tea catechin isolated from green tea. *Journal of the Agricultural Chemical Society of Japan*, 6, 62-69.

Ustün, O., Ozçelik, B., Akyön, Y., Abbasoglu, U., & Yesilada, E. (2006). Flavonoids with anti-Helicobacter pylori activity from Cistus laurifolius leaves. *Journal of Ethnopharmacology*, *108*, 457–461. http://doi.org/10.1016/j.jep.2006.06.001

van Acker, S. A. B. E., de Groot, M. J., van den Berg, D.-J., Tromp, M. N. J. L., Donné-Op den Kelder, G., Van Der Vijgh, W. J. F., & Bast, A. (1996). A quantum chemical explanation of the antioxidant activity of flavonoids. *Chemical Research in Toxicology*, *9*, 1305–12. http://doi.org/10.1021/tx9600964

van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., … Vandenabeele, P. (2001). Endonuclease G: A mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. *Cell Death and Differentiation*, *8*, 1136–1142. http://doi.org/10.1038/sj.cdd.4400944

Vercammen, D., Brouckaert, G., Denecker, G., Van de Craen, M., Declercq, W., Fiers, W., & Vandenabeele, P. (1998). Dual signaling of the Fas receptor: Initiation of both apoptotic and necrotic cell death pathways. *The Journal of Experimental Medicine*, *188*, 919–30. http://doi.org/10.1084/jem.188.5.919

Vidya Priyadarsini, R., Senthil Murugan, R., Maitreyi, S., Ramalingam, K., Karunagaran, D., & Nagini, S. (2010). The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells through p53 induction and NF-kB inhibition. *European Journal of Pharmacology*, *649*, 84–91. http://doi.org/10.1016/j.ejphar.2010.09.020

Wach, A., Pyrzyńska, K., & Biesaga, M. (2007). Quercetin content in some food and herbal samples. *Food Chemistry*, *100*, 699–704. http://doi.org/10.1016/j.foodchem.2005.10.028

Walensky, L. D., & Gavathiotis, E. (2011). BAX unleashed: The biochemical transformation of an inactive cytosolic monomer into a toxic mitochondrial pore. *Trends in Biochemical Sciences*, *36*, 642–652. http://doi.org/10.1016/j.tibs.2011.08.009

Walle, T. (2009). Methylation of dietary flavones increases their metabolic stability and chemopreventive effects. *International Journal of Molecular Sciences*, *10*, 5002–5019. http://doi.org/10.3390/ijms10115002

Walle, T., Ta, N., Kawamori, T., Wen, X., Tsuji, P. A., & Walle, U. K. (2007). Cancer chemopreventive properties of orally bioavailable flavonoids-Methylated versus unmethylated flavones. *Biochemical Pharmacology*, *73*, 1288–1296. http://doi.org/10.1016/j.bcp.2006.12.028

Wang, S., & El-Deiry, W. S. (2003). TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene*, *22*, 8628–33. http://doi.org/10.1038/sj.onc.1207232

Wang, Y., Cao, J., & Zeng, S. (2005). Involvement of P-glycoprotein in regulating cellular levels of Ginkgo flavonols: Quercetin, kaempferol, and isorhamnetin. *The Journal of Pharmacy and Pharmacology*, *57*, 751–8. http://doi.org/10.1211/0022357056299

Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., … Korsmeyer, S. J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. *Genes and Development*, *14*, 2060–2071. http://doi.org/10.1101/gad.14.16.2060

Wen, X., & Walle, T. (2006). Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. *Drug Metabolism and Disposition*, *34*, 1786–1792. http://doi.org/10.1124/dmd.106.011122

Wu, X.-N., Yang, Z.-H., Wang, X.-K., Zhang, Y., Wan, H., Song, Y., … Han, J. (2014). Distinct roles of RIP1-RIP3 hetero- and RIP3-RIP3 homo-interaction in mediating necroptosis. *Cell Death and Differentiation*, *21*, 1709–20. http://doi.org/10.1038/cdd.2014.77

Xu, Y., Huang, S., Liu, Z. G., & Han, J. (2006). Poly(ADP-ribose) polymerase-1 signaling to mitochondria in necrotic cell death requires RIP1/TRAF2-mediated JNK1 activation. *Journal of Biological Chemistry*, *281*, 8788–8795. http://doi.org/10.1074/jbc.M508135200

Yadegarynia, S., Pham, A., Ng, A., Nguyen, D., Lialiutska, T., Bortolazzo, A., … White, J. B. (2012). Profiling flavonoid cytotoxicity in human breast cancer cell Lines: determination of structure-function relationships. *NPC Natural Product Communications*, 7, 1295-1304.

Yan-Hwa, C., Chang, C. L., & Hsu, H. F. (2000). Flavonoid content of several vegetables and their antioxidant activity. *Journal of the Science of Food and Agriculture*, *80*, 561–566. http://doi.org/10.1002/(SICI)1097-0010(200004)80:5<561::AID-

JSFA574>3.0.CO;2-#

Yang, Q. H., Church-Hajduk, R., Ren, J., Newton, M. L., & Du, C. (2003). Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. *Genes and Development*, *17*, 1487–1496. http://doi.org/10.1101/gad.1097903

Zhang, Q., Zhao, X.-H., & Wang, Z.-J. (2009). Cytotoxicity of flavones and flavonols to a human esophageal squamous cell carcinoma cell line (KYSE-510) by induction of G2/M arrest and apoptosis. *Toxicology in Vitro : An International Journal Published in Association with BIBRA*, *23*, 797–807. http://doi.org/10.1016/j.tiv.2009.04.007

Zu, Y., Li, C., Fu, Y., & Zhao, C. (2006). Simultaneous determination of catechin, rutin, quercetin kaempferol and isorhamnetin in the extract of sea buckthorn (Hippophae rhamnoides L.) leaves by RP-HPLC with DAD. *Journal of Pharmaceutical and Biomedical Analysis*, *41*, 714–719. http://doi.org/10.1016/j.jpba.2005.04.052