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Profiling flavonoid cytotoxicity in human breast cancer cell lines

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PROFILING FLAVONOID CYTOTOXICITY IN HUMAN BREAST CANCER CELL LINES

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ABSTRACT

PROFILING FLAVONOID CYTOTOXICITY IN HUMAN
BREAST CANCER CELL LINES

by
Sina Yadegarynia

Flavonoids are part of a large family of polyphenols that are found extensively in fruits and vegetables. This class of compounds has been of considerable medical interest due to their anti-inflammatory and anti-cancer activities. Although extensive effort has been made to identify the biological effects responsible for the chemopreventive activity of these compounds, the exact molecular mechanisms involved are not fully understood. In this study, we focused on the cytotoxic effects of fourteen different flavonoids against a series of breast cancer cell lines and evaluated the induction of cell cycle arrest at G1 or G2/M phase as result of such treatment. We also assessed a possible structure-function relationship for cellular cytotoxicity based on the various chemical structures of flavonoids. The results showed that several flavonoids were cytotoxic in all cell lines even in the absence of certain signaling pathways. In addition, only some flavonoids were able to induce cell cycle arrest, suggesting their cytotoxic potential may be independent of their ability to block cells at G1 or G2/M phases. Our results enabled identification of certain structural properties that are important for the anticancer activity of flavonoids. Finally, these results suggested that cytotoxicity does not depend on a particular signaling pathway.

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LIST OF ABBREVIATIONS

Akt: serine/threonine protein kinase

ANOVA: analysis of variance

AP-1: activator protein-1

Bak: Bcl-2-homologous antagonist/killer

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma-2

Bcl-XL: B-cell lymphoma-extra large

BRCA1 or 2: breast cancer type 1 or 2 susceptibility gene

CDK: cyclin-dependant kinases

cFLIP: cellular FLICE inhibitory protein

COX-2: cyxlooxygenase-2

CTG: CellTiter-Glo® luminescent cell viability assay

CYP: cytochrome P450 superfamily

DMEM: Dulbecco's modified eagle medium

DMSO: dimethyl sulfoxide

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ESR1: estrogen receptor-1

GST: glutathione S-transferase

HER2/ERBB2: human epidermal growth factor receptor-2

HMEC: human mammary epithelial cell

IFN-γ: interferon-gamma

IL-2: interleukin-2

IκBα/IKK: inhibitor of kappa-B-alpha

MAPK: mitogen-activated protein kinases

MMP: matrix metalloproteases

MTT Assay: methylthiazol tetrazolium assay

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NOS: inducible nitric oxide synthase

P53: protein 53-kilo Daltons

PARP: poly ADP-ribose polymerase family

PGE2: prostaglandin E2

PI3K: phosphatidylinositol 3-kinase

PKC: protein kinase C

PLK-1: polo-like kinase-1

PR: progesterone receptor

ROS: reactive oxygen species

RPMI: Roswell Park Memorial Institute medium

RyR-1: type-1 ryanodine receptor

STAT3: signal transducer and activator of transcription-3

TBE: trypan blue exclusion

TNF: tumor necrosis factors

TRAF1: TNF receptor-associated factor-1

VEGF: vascular endothelial growth factor

INTRODUCTION

Cancer has been thought to be a preventable disease due to its slow development and progression, taking many years to become invasive in a step-by-step manner [1]. Such property provides a great opportunity not only for early detection, but also for prevention of the disease progression. Despite this, breast cancer is the most frequently diagnosed cancer in women worldwide and ranks second as a cause of cancer death [2]. It has been estimated that more than two-thirds of human cancers could be prevented through lifestyle modifications, such as dietary habits [3]. As early as 1676, when Dr. Wiseman proposed that cancer might arise from “an error in diet,” diet has been considered an important factor in cancer development [4].

Over the past several decades, there has been a particular interest in the role of flavonoids in cancer prevention. Flavonoids are naturally occurring polyphenols widely distributed in fruits, vegetables, and beverages including teas and wine [4]. They represent a large portion of the compounds found in plants with more than 5,000 varieties [5]. Flavonoids are reported to have a range of biological activities including anti-oxidant, anti-inflammatory, and anti-tumorigenic properties. In particular, flavonoids are active at different stages of cancer development by protecting DNA from oxidative damage, activating carcinogen metabolism and detoxification, preventing cellular proliferation, and/or inducing cellular cytotoxicity [6-10].

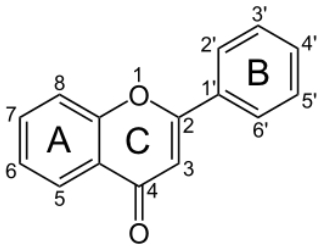
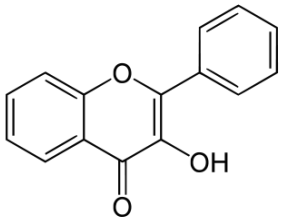
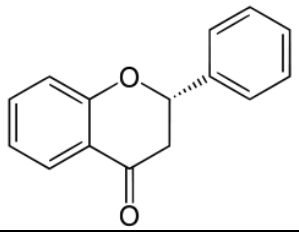
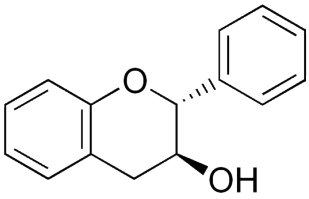
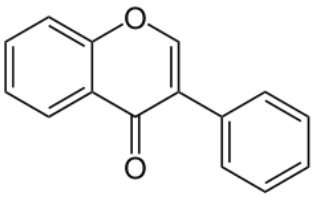
A survey of the literature shows a plethora of effects of flavonoids on tumor and normal cell types, but the exact molecular mechanisms of flavonoid action are not fully understood. Consequently, a careful characterization is needed to establish a reference point for further analysis of flavonoids and potential derivatives, which may provide a novel mechanism targeting clinical treatment and prevention of cancer.

Table 1. Some of the signaling components of the cell lines in this study. A (+) indicates present, (-) indicates absent, and (+/-) indicates weak expression. IDC: Invasive Ductal Carcinoma; AC: Adenocarcinoma; ESR1: estrogen receptor-1; PR: progesterone receptor; HER2: human epidermal growth factor receptor-2.

Cell Line	Tumor Type	Invasiveness	Gene Cluster Subtype	p53	ESR1	PR	HER2
BT-474	IDC	Strong	Luminal	Temperature sensitive (E285K)	+	+	++
MCF-7	IDC	Weak	Luminal	Wild type	+	+	+
MDA-MB-231	AC	Strong	Basal	Nonfunctional (R280K)	-	-	-
SK-BR-3	AC	Strong	Luminal	Missense (R175H)	-	-	++
ZR-75-1	IDC	Moderate	Luminal	Wild type	+/-	+	+/-

We, therefore, conducted a study to characterize the effects of fourteen flavonoids on a variety of human breast cancer cell lines (Table 1 and 2). It has been determined that these commercially available breast cancer cell lines mirror the genomic, transcriptional, and biological heterogeneity of the primary tumors [11]. These cell lines were selected based upon the presence of mutations

Table 2. Sub-classes and chemical structures of flavonoids in this study.
OMe indicates methylated analogues, methylapigenin: 5,7,4'-trimethoxyflavone.

Subclasses and Structures	Name	Substitution							
		5	6	7	8	2'	3'	4'	5'
Flavones 	Apigenin	OH	H	OH	H	H	H	OH	H
	Methylapigenin	OMe	H	OMe	H	H	H	OMe	H
	Chrysin	OH	H	OH	H	H	H	H	H
	Luteolin	OH	H	OH	H	H	OH	OH	H
	Methylluteolin	OMe	H	OMe	H	H	OMe	OMe	H
Flavonols 	Kaempherol	OH	H	OH	H	H	H	OH	H
	Myricetin	OH	H	OH	H	H	OH	OH	OH
	Quercetin	OH	H	OH	H	H	OH	OH	H
	Methylquercetin	OMe	H	OMe	H	H	OMe	OMe	H
Flavanones 	Naringenin	OH	H	OH	H	H	H	OH	H
	Methylnaringenin	OMe	H	OMe	H	H	H	OMe	H
Flavanol 	(+)-Catechin	OH	H	OH	H	H	OH	OH	H
Isoflavones 	Daidzein	H	H	OH	H	H	H	OH	H
	Genistein	OH	H	OH	H	H	H	OH	H

Modified from Yadegarynia et al. [12]

in various signaling pathways. Mutations are reported in estrogen receptor-1 (ESR1), human epidermal growth factor-2 (HER2/ERBB2), progesterone receptor, and tumor suppressor protein (p53). These cell lines have been used in many studies to individually investigate the role of each flavonoid (Table 3). For comparison with a non-transformed cell type, we used normal human mammary epithelial cells (HMEC) with a lifespan of only 15 to 20 passages in culture. HMECs are not immortalized, unlike MCF10A which have been previously used in studies [13]. Such information may be used to help elucidate the specific signaling pathways utilized by flavonoids for the induction of cellular cytotoxicity.

Table 3. A survey of literature for effects of the flavonoids on breast cancer cell line proliferation and cytotoxicity.

Cell Line	Flavonoid Used	Reference
BT-474	Apigenin, Genistein	[14, 15]
MCF-7	Apigenin, Catechin, Genistein, Kaempherol, Myricetin, Naringenin, Quercetin	[16-24]
MDA-MB-231	Apigenin, Genistein, Kaempherol, Naringenin, Quercetin	[6, 17, 25-37]
SK-BR-3	Apigenin, Genistein, Quercetin	[38-40]
ZR-75-1	Apigenin, Genistein, Kaempherol, Quercetin	[41-45]
MCF10A/HMEC	Apigenin, Daidzein, Genistein, Quercetin, Naringenin	[13, 24, 46-49]

In this study, we showed that some flavonoids induced cell death in all cell lines tested, including HMECs. Furthermore, we suggest that flavonoids might

induce cellular cytotoxicity through a generalized, signaling pathway-independent, mechanism.

LITERATURE REVIEW

Chemistry of Flavonoids

Plants are rich sources of chemically diverse compounds, many with beneficial properties to human health. Consequently, about 50% of the anticancer therapeutic agents known are derived from plants [50]. For example, compounds such as Taxol and vinca alkaloids act to destabilize the microtubules of cancer cells, preventing the rapid proliferation of tumors [51]. Polyphenolic compounds make up one of the most abundant groups of compounds in the plant kingdom [9]. They are secondary metabolites involved in many important functions in plants. Some of these functions include UV protection, defense against biotic and abiotic stresses, pigmentation, and normal growth and development [52]. Polyphonels are divided into 10 general classes with more than 8,000 compounds identified to date. The most abundant occurring polyphenols are flavonoids, accounting for about 60% of the polyphenols. Flavonoids are divided into six sub-classes based on their chemical structure, including flavanols, flavones, flavanones, flavonols, isoflavones, and anthocyanidins.

The chemical structure of flavonoids is characterized by a diphenylpropane carbon skeleton of C6-C3-C6, where two benzene rings are linked by a 3-carbon chain forming a heterocyclic pyran or pyrone ring with an oxygen. Differences in the number and arrangement of the hydroxyl groups lead

to variations within each group. Flavonoids may be hydroxylated at positions 3, 5, 7, 3', 4', and/or 5' (Table 2). Except for isoflavones, where the B-ring is attached at the 3-position on the pyran, the rest of the sub-classes have the B-ring attached at the 2-position. The flavones contain a 2,3-unsaturation in the C-ring, and the flavonols possess both the 2,3-unsaturation and a hydroxyl group at the 3-position. The flavanones have a saturated 2,3-bond, and the flavanols also possess a hydroxyl group at the saturated 3-positions. The names and structures of the flavonoids in the study are listed in Table 2.

The anti-oxidant activity of these compounds is due to the presence of these phenolic hydroxyl groups, which create their electron-donating property against free radicals [53]. At the same time, flavonoids are frequently found attached to sugars almost exclusively as β-glycosides, where a hydroxyl group at positions 3 or 7 is substituted by various glycosides [54, 55]. Glucose is the most common residue attached, which ultimately increases the water solubility of flavonoids in plants [52, 56]. The deglycosylation of flavonoids may be an important first step for their absorption in the body, and this rate is dependant on both the structure of the polyphenol and the position or the nature of the attached sugars [55].

Consequently, due to such diversity, flavonoids are able to interact with many targets and influence various signaling pathways. This further emphasizes the importance of studying their molecular chemistry.

Flavonoids in Food

In the 1930s, scientists Rusznyák and Szent-Györgyi showed that a substance from lemon peel reduced capillary permeability and was effective for purpura treatment [57]. They named it “Vitamin P,” P for permeability, and later reported that it consisted of a mixture of polyphenols. Flavonoids lost their status as vitamins in the 1950s, when it was shown that their removal from the diet did not cause any abnormalities. However, studies since the 1980s have been providing strong implications regarding their protective effects against many chronic diseases, such as coronary heart disease and cancer. As the result, the World Health Organization (WHO) recommended an intake of about 400 g/day of fruits and vegetables, which may save up to 2.7 million lives annually [58].

Flavonoids are still considered to be a non-essential dietary components found ubiquitously in foods and beverages of plant origin, such as vegetables, fruits, teas, wine, and chocolate (Table 4). Sub-classes of flavonoids, however, do not seem to be uniformly distributed in many foods. For example, grapefruit juice has been shown to contain around 200-850 mg/L of total flavonoids, among which naringenin is about 145-638 mg/L and the most abundant flavonoid [59]. Orange juice, however, mainly contains the flavanone hesperidin at about 200-450 mg/L [60]. Green tea and red wine are rich sources of flavanol catechins, as high as 1,000 mg/L, while soy foods are rich source of isoflavones, such as genistein and daidzein [61].

Table 4. Sub-classes and common dietary sources of flavonoids.

Flavonoid sub-class	Major Food Sources
Flavonols	Onions, spinach, cherries, apples, broccoli, kale, tomato, berries, almond, tea, red wine
Flavones	Parsley, thyme, celery, peppers, rosemary
Isoflavones	Soybeans, legumes, peanuts, fava beans, red clover
Flavanols	Apples, tea, red wine, chocolate
Flavanones	Oranges, grapes, lemons, psoralea
Anthocyanidins	Berries, grapes, cherries, plums, cashews, hazelnuts, eggplant

It has been estimated that the total amount of flavonoid consumed in the western diet is about 1 g/day [62], but some studies indicate that this amount can vary widely [63]. This may be due to the great diversity of flavonoids and the limited data on their content in foods. Another complication is the fact that the flavonoid content is influenced by many other factors, including season, climate, sunlight, and food preparation [64]. However, in 2011 the U.S. Department of Agriculture (USDA) compiled and published an updated database on the 26 most commonly occurring flavonoids in over 500 foods [65].

Flavonoids as Chemopreventative Agents

Even though cancer is attributed to genetic mutations, such as the BRCA1 and BRCA2 genes in breast cancer, known genetic defects only account for up to 10% of all cases. On the other hand, lifestyle factors, including diet, smoking, and physical activity, account for the majority of cancer cases [66, 67]. A lot of the epidemiological data from case-control and cohort studies have found an

inverse relationship between the risk of developing certain cancers and the consumption of vegetables and fruit [62, 68-70]. The American Institute for Cancer Research published a comprehensive report in 2008, which concluded that there was convincing evidence for a statistically significant decrease in development of certain cancers with consumption of fruits and vegetables [71].

Additionally, lung, colon, prostate, and breast cancer have been shown to be more common in Western rather than in Eastern countries [7]. More specifically, it has been reported that China and Japan, where the daily consumption of soy products reaches up to 100 mg as opposed to a few milligrams in Western countries, have one third the incidence of prostate and breast cancer [72].

Much compelling data highlights the important role of flavonoids as chemopreventative agents. Through extensive studies, flavonoids have been shown to possess many biochemical and pharmacological actions that may significantly affect cellular systems. Numerous studies report on the anti-flammatory, anti-oxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities of these compounds [73] (Table 5).

Chemoprevention has been defined as the prevention, inhibition, or reversal of carcinogenesis during the progression of cancer, before cellular invasion across the basement membrane by pharmacological agents like flavonoids [74]. As a result of this slow development and progression, cancer has been thought to be a preventable disease.

Table 5. Proposed mechanisms for chemoprevention by phytochemicals.

Antioxidant activity to reduce the role of ROS and other free radicals
Inhibition of cell proliferation, oncogene expression, signal transduction pathways, inflammation, cell adhesion and invasion, nitrosation and nitration
Enzyme inhibition: -Phase I enzyme (blocking activation of carcinogens) -Cyclooxygenase-2 -Inducible nitric oxide synthase -Xanthine oxidase
Induction of cell differentiation, cell-cycle arrest, apoptosis, tumor suppressor gene expression
Induction of enzymes and enhancement of detoxification -Phase II enzymes -Glutathione peroxidase -Catalase -Superoxide dismutase
Enhancement of immune response
Antiangiogenesis
Prevention of DNA adduct formation or DNA intercalation
Regulation of estrogen and steroid hormone metabolism

Modified from Liu et al. [75]

Furthermore, carcinogenesis is a very complex multistep process that includes tumor initiation, promotion, and progression from the initial exposure to carcinogen and the ultimate development of cancer [76] (Figure 1). As chemopreventive agents, flavonoids are active at different stages of cancer development interfering with the overall process through various mechanisms such as modulating mitogenic signaling, survival/apoptotic signaling, cell-cycle regulation, angiogenesis, and metastatic effects in the cells [77]. Studies show that targets in various signal transduction pathways vary depending on the origin of cancer. For example, flavonoids may inhibit the activity of DNA topoisomerase I/II [78], release of cytochrome c from mitochondria and subsequent activation of caspases-3, 8 and 9 [79, 80], down-regulation of Bcl-2 and Bcl-xl expression

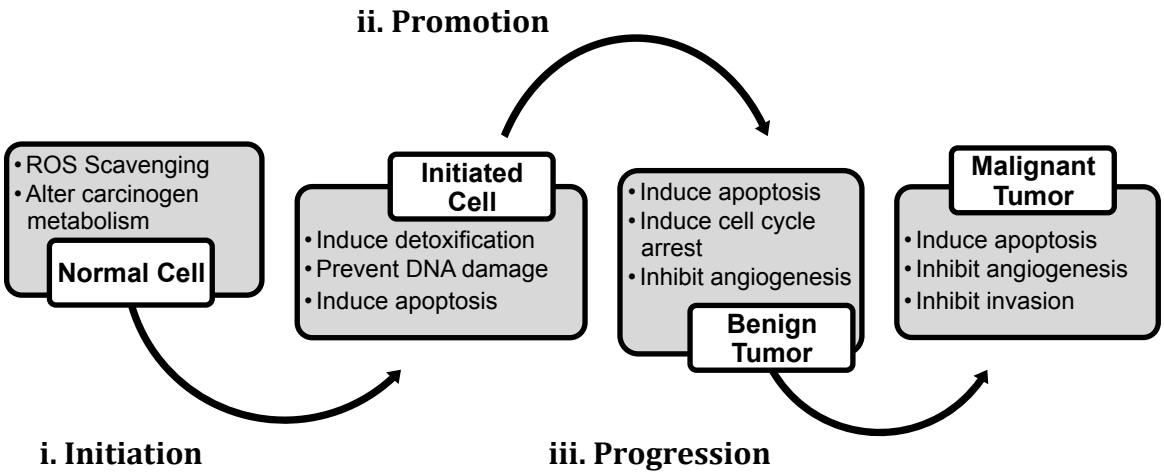


Figure 1. Model of carcinogenesis and potential consequences of flavonoids on cancer progression. Carcinogenesis is a complex multistep process that can be activated by various agents including ROS. Steps: i. Initiation: the exposure or uptake of the carcinogen by a cell and its interaction with the DNA. ii. Promotion: the persistence and replication of the abnormal cells, originating preneoplastic cells. iii. Progression: uncontrollable growth of tumors with gradual conversion of premalignant cells to neoplasia with potential for metastasis and angiogenesis [9]. Carcinogens are known to affect many signaling pathways, such as: modulation of transcription factors (e.g. NF-κB, AP-1, STAT3), apoptotic proteins (e.g. Akt, Bcl-2, Bcl-XL, caspases, PARP), protein kinases (e.g. EGFR, HER2, MAPK), cell cycle protein (e.g. cyclins, cyclin-dependant kinases), cell adhesion molecules, COX-2, and growth factor signaling pathways [7]. Flavonoids may interact with many cellular molecules and interfere with the growth and progression of the tumor. For example, kaempherol can stop initiation by inhibiting the metabolic activation of the carcinogens by phase I enzymes (CYPs) or their interaction with DNA. Naringenin and quercetin, on the other hand, can stimulate detoxification of carcinogens by activating phase II enzymes. Genistein can interfere with promotion and progression by inducing cell cycle arrest, apoptosis and/or inhibiting metastasis and angiogenesis [81].

and/or up-regulation of Bax and Bak expression leading to apoptosis [80]. While specific molecules have been identified to be involved in the process, the induction and regulation still remains unexplained.

One of the well studied roles of flavonoids is their anti-oxidant potential against Reactive Oxygen Species (ROS), which cause DNA damage and promote carcinogenesis in biological systems [82]. Since an imbalance between production of ROS and the body's anti-oxidant defenses can cause many chronic diseases, the anti-oxidant activity of flavonoids has been regarded as the main biological activity for many years. However, recent bioavailability data has challenged this notion. It has been shown that the concentrations of flavonoids are limited due to their low absorption by the body and short half-life in plasma. In addition, extensive metabolism by the body significantly alters their redox potential. As a result, their concentrations in the tissue are lower than endogenous anti-oxidants, limiting such efficacy *in vivo* [52, 70].

Many other studies have, therefore, linked the anti-cancer function of flavonoids to modulation of various molecular targets involved in many intracellular signaling pathways such as cell signaling, mitogenic signaling, cell proliferation, cell cycle regulation, angiogenesis, and metastatic events (Table 6). For example, flavonols and flavonones interfere with the activation of carcinogens by inhibiting cytochrome P450 enzyme of CYP1A family [69]. Some flavonoids also interfere with the activities of many other enzymes such as β-glucuronidase, lipoxygenase, cyclooxygenase (COX), nitric oxide synthase

(NOSs), monooxygenase, thyroid peroxidase (TPO), xanthine oxidase (XOs), mitochondrial succinoxidase and NADH-oxidase, phosphodiesterase (PDE), phospholipase A2 (PLA2), and protein kinase [83, 84].

Table 6. Some of the molecular targets of flavonoids.

Anti-Apoptotic Proteins	Apoptotic Proteins	Cell-Cycle Proteins	Growth Factor Pathways	Transcription Factors	Protein Kinases
↓Bcl-2	↑Caspases	↓Cyclin D1, E	↓TNF	↓NF-κB	↓IKK
↓Bcl-XL	3, 7, 8, 9	↑p21/WAF	↓EGF	↓AP-1	↓EGFR
↓Survivin	↑PARP	↑p27Kip/Cip	↓IFN-γ	↓STAT1,3,5	↓HER2
↓TRAF1	↑Bax	↓CDK1,2,4,6,7	↓IL-1,2,6,8	↓β-catenin	↓Akt
↓cFLIP			↓Erythropoietin	↑p53	↓MAPK

Modified from Aggarwal et al. [7]

Molecular Targets of Flavonoids

Flavonoids have been shown to possess many biochemical and pharmacological actions that may significantly affect cellular systems by modulating various proteins in signaling cascades (Table 6). While specific molecules have been identified to be involved in these processes, the induction and regulation of many remain unexplained. Some of the molecular targets are highlighted here.

Nuclear Factor-κB (NF-κB) is a family of proteins that are pro-inflammatory transcription factors that can promote carcinogenesis [85]. Upon activation by ROS, cytokines, and other carcinogens, NF-κB is translocated into the nucleus and is able to regulate expression of more than 200 genes, which may suppress apoptosis and promote proliferation, invasion, metastasis, and inflammation [86]. Studies have suggested that apigenin, catechin, genistein, quercetin,

kaempherol, and luteolin are potent inhibitors of the NF-κB signaling cascade through various mechanisms such as inhibition of Bcl-2, Bcl-XL, cyclin D1, matrix metalloproteases (MMP), and vascular endothelial growth factor (VEGF) [7, 87, 88]. Activation of the serine/threonine protein kinase (Akt) has also been shown to support cancer cell survival through activation of NF-κB and inhibition of apoptosis [89]. Genistein has been shown to inhibit Akt activation through epidermal growth factor (EGF) inhibition [7].

Activator Protein-1 (AP-1) is also a transcriptional activator that regulates expression of several genes involved with apoptosis, cell proliferation, cell transformation, inflammation, and innate immune response [90]. For example, AP-1 may activate cyclin D1 and repress tumor-suppressor genes such as p53. Catechin and quercetin have been reported to suppress the activation of AP-1 [91, 92].

In addition, defect in the regulation of cell cycle also leads to cancer. For example, overexpression of major cell cycle regulators, such as cyclins and cyclin-dependant kinases (Cdk), leads to promotion of carcinogenesis [93]. Prostate, lung, and breast cancer have been shown to overexpress cyclin D1, which is regulated by NF-κB [94-96]. As a result, NF-κB inhibitors such as genistein and apigenin are able to induce cell cycle arrest at gap-2 (G2) phase and halt the proliferation of cancer cells [7, 97].

Apoptosis is also an essential part of homeostasis that involves a series of events inducing programmed cell death. Defects in the regulation of apoptosis

have also been linked to several factors including NF-κB and AP-1. Flavonoids have been shown to induce apoptosis in some cancer cell lines, while their molecular mechanism is not fully understood. For example, genistein causes apoptosis in MDA-MB-468 cells, but not in MCF-7, ZR-75, or MDA-231 cells [98]. Suggested mechanisms include inhibition of NF-κB and AP-1, DNA topoisomerase I/II activity, regulation of heat shock proteins, and release of cyctochrome c with activation of caspases-9 and 4 [8, 99]. Catechin, on the other hand, causes apoptosis by down regulating the expression of apoptosis suppressor proteins, such as Bcl-2 and Bcl-XL [100].

Tumor-suppressor p53 is a transcription factor also involved in many cell processes including DNA repair, cell cycle control, and apoptosis. Upon activation, p53 expresses genes that induce apoptosis, cause cell cycle arrest, or inhibit angiogenesis [86]. Many flavonoids, therefore, have been shown to activate p53 or its target genes. For example, luteolin induces cell cycle arrest and apoptosis by activating p53, p21, and Bax genes [86].

Finally, many cancers, including liver, breast, lung, and skin, contain overexpression of the enzyme cyclooxygenase-2 (COX-2), which leads to inflammation [101]. Several transcription factors, such as NF-κB and AP-1, cause this overexpression. Therefore, flavonoids such as luteolin, genistein, apigenin, and catechin inhibit these transcriptional factors and suppress COX-2 transcription.

Bioavailability of Flavonoids

Bioavailability refers to the amount of any substance that reaches the plasma unchanged. Thus bioavailability of flavonoids determines their activity *in vivo*. Most of polyphenols are present in the form of esters, glycosides, or polymers (called tannins [63]) produced by the plants or as a result of food processing, which also increases their water solubility [52, 60]. Therefore, there is little chance of absorption by passive diffusion, ATP pumps, or transporters when orally administered [52]. In order to be absorbed by the body, they must be hydrolyzed or deglycosylated by the intestinal enzymes or the colonic microflora [102]. Even though the exact modifications of flavonoids during metabolism are not yet fully understood, polyphenols are further broken down and modified by methylation, sulphation, and glucuronidation in the liver and/or kidney [103]. This is also a common metabolic detoxification process that facilitates biliary and urinary elimination of xenobiotics by increasing their hydrophilicity. It has also been reported that for many of the polyphenols the plasma baseline levels are reached within 24 hours of consumption [104].

Flavonoids undergo extensive phase I deglycosylation and phase II metabolism by biotransformation enzymes in the small intestine epithelial cells and liver [83, 105]. Three general processes are involved: conjugation with thiols, oxidative metabolism, and P450-related metabolism [83]. Extensive conjugation of the free hydroxyl groups is thought to be the main reason for low oral bioavailability of flavonoids *in vivo* [106]. As the result, polyphenols are

present in low concentrations after consumption and are eliminated shortly afterwards. These metabolic modifications of flavonoids alter the structure and ultimately the redox potential of these compounds. Further studies are needed to better understand their bioavailability *in vivo*.

Moreover, intestinal absorption of polyphenols has been shown to vary greatly. For example, *in vivo* studies have reported a 0-60% absorption of the original dose with a half-life of 2-28 hours in the body [107]. Many factors such as the sugar moiety or gut microflora affect account for these variations. In addition, the amount of flavonoids absorbed in the intestine varies depending on the sub-classes. For example, only 0.2-0.9% of tea catechins are absorbed as opposed to 20% for quercetin and isoflavones [69]. Isoflavones appear to have the highest bioavailability, while flavonols appear to have the lowest bioavailability but the highest half-life in plasma. Flavanols appear to have the shortest half-life [107, 108]. In addition, bioavailability of flavonoids also varies based on the source of food consumed. For example, quercetin absorption from onion is four times that of apple or tea [69].

It is important to note that the most common flavonoids in the diet may not be the most active *in vivo*, and that the metabolites in the plasma may also not be the original compound. As a result, many inconsistencies on the bioavailability of flavonoids have been reported. Compiling comprehensive and reliable data *in vivo* is thus essential, yet has proven to be challenging.

Higher Bioavailability and Metabolic Stability of Methylated Flavonoids

Methylated flavonoids have been studied less extensively due to the absence of hydroxyl groups, and because ultimately they lack anti-oxidant properties. However, recent studies have shown that methylated flavonoids were metabolically stable and experienced slower hepatic metabolism compare to the unmethylated compounds [109]. Studies in rats have shown that methylchrysin had a higher oral bioavailability and tissue accumulation, particularly in the liver, compared to the unmethylated compound [110]. Methylated compounds have also been shown to have a higher rate of intestinal permeability [106].

Although most of the compounds studied are synthetic, some methylated flavonoids have been identified in plants. For example, methylapigenin is present in citrus fruits and methylchrysin is present in fruits and leaves of neotropical nutmeg species [110]. Since more *in vivo* studies relate the chemoprevention of flavonoids to the modulation of cellular signaling pathways, as opposed to their anti-oxidant effect, greater attention should be given toward methylated polyphenols.

It is important to mention that a different mechanism of action has been suggested for the methylated analogues of flavonoids, when comparing their cell cycle arrest data. For example, apigenin have been shown to induce arrest in the G2/M phase, while methylapigenin and methylchrysin arrest most cells in the G1 phase [110]. Methylated flavones have also been shown to have a higher potency, which could be attributed to their higher accumulation in the cell [111].

Consequently, methylation of flavonoids not only increases their oral bioavailability, but also their metabolic stability and tissue accumulation. In addition, methylation of the hydroxyl groups has shown to not interfere with the anti-proliferative capacity of flavonoids [112]. Taken together, methylated flavonoids may prove to be more effective *in vivo*. However, more extensive studies on their mechanisms of action are still required.

Structure-Function Relationship of Flavonoids

It has been suggested that the structure of flavonoids dictates their bioactivity. In addition, that specific structural features are involved in whether or not flavonoids can act as a potent inhibitors of molecular targets such as protein kinase C (PKC), tyrosine kinase, and PI 3-kinase [113]. There is still no clear consensus on structure-function relationships based on the various structures of sub-classes. While some studies have found no obvious relationship [72, 114], others have highlighted important structural features. For example, the presence of 2,3-unsaturation on the C-ring and the number and substitution of hydroxyl groups on the A and B-rings have shown to greatly affect the anti-oxidant and anti-cancer activity of these compounds [83, 115].

One of the important structural features of flavonoids is the presence of 2,3-unsaturation in the C-ring (Figure 2). The presence of this double bond has shown to enhance the anti-proliferative effect of the flavonoids [83, 112, 115]. In addition to the presence of this double bond, number and substitution of hydroxyl groups appear to affect the anti-oxidant potential of flavonoids. For example,

three adjacent hydroxyl groups on the A or B-ring (e.g., myricetin with 3', 4', 5'-OH groups) results in a strong anti-proliferative effect [112]. Therefore, it has been suggested that more hydroxyl groups lead to better ROS scavenging and anti-inflammatory activity [116]. In terms of positions, the hydroxyl group at 4' in the B-ring improves the anti-proliferative effect of the flavonoids, evident when comparing apigenin (with 4'-OH group) and chrysin (without 4'-OH) [117]. Flavonoids with 5- and 7-OH groups in the A-ring (e.g., apigenin, luteolin, quercetin, and kaempherol) significantly inhibit lipogenesis [118]. Kaempherol, myricetin, and quercetin (with 3, 5, and 7-OH groups) have been shown to have higher anti-oxidant activity [119].

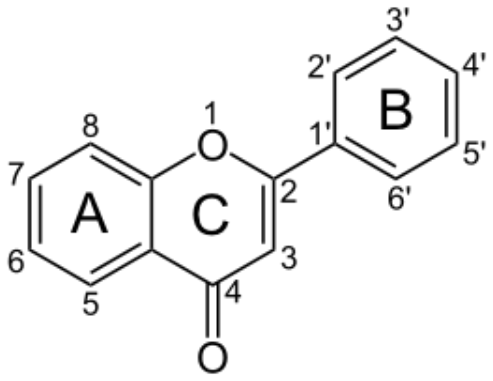


Figure 2. General structure of flavones.

Therefore, many researchers have concluded that the presence of a particular molecular structure leads to inhibition of several kinases, which ultimately induces cell cycle arrest and apoptosis. It has been suggested that the presence of the 4-oxo group, the 2,3-double bond in the C-ring, and the 3' and 4'-

OH in the B-ring (so-called catechol group) enhances the anti-oxidant and anti-proliferation activity of flavonoids [53, 73, 112, 116-118, 120].

Biphasic Effects of Flavonoids

Several flavonoids have been shown to have a biphasic effect on apoptosis. For example, apigenin and chrysin were shown to stimulate proliferation at low concentrations, while their methylated analogs did not [110]. The authors concluded that at lower concentrations, apigenin activates ERα-mediated gene transcription and caused growth stimulation. At higher concentrations, on the other hand, apigenin down regulates protein levels of ERα and inhibits protein kinases p38, MAPK, PKA, and Akt, leading to growth inhibition [16]. Similarly, genistein has been shown to stimulate growth of ER-positive cancer cells, such as MCF7, at lower concentrations by acting as an estrogen agonist [39]. At higher concentrations, however, genistein inhibits cell growth by being an estrogen antagonist and inhibiting tyrosine kinase activity [121, 122]. These results highlight the importance of more extensive studies into the biphasic effect of some flavonoids, especially in ER-positive cancer cells.

Flavonoids Used in the Study

Flavonoids have been shown to modulate many molecular targets affecting various cellular pathways within cells. Some of the specific molecular targets of flavonoids used in our study and their effects on various pathways are listed in Table 7.

Table 7. Molecular targets of flavonoids used in this study.

Flavonoid	Molecular targets
Apigenin	↓ NF-κB, ↑ IκBα
Catechin	↑ RyR1, ↓ NF-κB
Chrysin	↓CDK6/cycD, ↓TBK1
Daidzein	↓ NF-κB, ↓ STAT-1, ↓ iNOS
Genistein	↓ NF-κB, ↓ NF-κB-DNA binding, ↑ IκBα, ↓ IL-8
Kaempherol	↓ PGE2, ↓ COX-2, ↓ NF-κB
Luteolin	↓ NF-κB, ↓TBK1
Myricetin	↓ COX-2, ↓ NF-κB
Naringenin	↓ iNOS, ↓ NO, ↓ NF-κB, ↓ STAT-1
Quercetin	↓ NF-κB, ↓ AP-1

Data gathered from [123-127]

Apigenin, chrysin, and luteolin are flavones found in limited quantities in leafy vegetables and other herbs. Apigenin and luteolin have been reported to induce cell cycle arrest at G2/M phase. They also induce apoptosis through various pathways including activation of caspases-3, 6, and 9, suppression of Akt and NF-κB, and induction of tumor suppressor p53 [41, 128-130]. Chrysin has been reported to increase the expression of PPARs, causing cytoplasmic lipid accumulation [131].

Flavonols are the most abundant flavonoid found in leafy vegetables, apples, onions, broccoli, and berries. Kaempherol, myricetin, and quercetin fall into this sub-class of flavonoids. Kaempherol has been shown to induce apoptosis through mitochondria-dependent pathways and induce cell cycle arrest through down-regulation of serine/threonine protein kinase (PLK-1) [132]. Kaempherol has also been reported to have a minimal apoptotic effect in MDA-MB-231 cells, as opposed to MCF-7 cancer cell line [33]. Myricetin has been

shown to suppress topoisomerase II [133]. Quercetin is the most ubiquitous dietary flavonoid with a daily intake of 25-30 mg in Western countries [66]. Quercetin has been shown to induce cell cycle arrest at the G1 or G2/M phase, depending on the cell lines [40], and cause anti-proliferation by increasing tumor suppressor p53, activation of caspases-6, 8, and 9, and suppression of NF-κB, COX-2, and Akt [66, 99, 134].

Naringenin is a flavanone found predominately in citrus fruits and their juices. Naringenin has been shown to impair glucose uptake and inhibit cellular proliferation *in vitro* [129, 135]. It has also been noted that naringenin causes apoptosis through a p53-independent induction of caspase-3, activation of p38/MAPK, and inactivation of phosphatidylinositol 3-kinase (PI3K) and Akt [123, 135-137].

Catechin is a flavanol found extensively in tea, apple, grapes, chocolate, and red wine. Many studies have highlighted the chemopreventative potential of catechins derived from various green and black teas against several cancers including cervical, prostate, and hepatic malignancies [66].

Genistein and daidzein are naturally abundant isoflavones found in soy products, tea, fruits, and vegetables. They are commonly known as phytoestrogens, with estrogenic activity in the cell [21]. Since approximately 70% of all breast cancers express ER [39], ER status is an important factor in chemopreventative potential of phytoestrogens and requires careful consideration. It has been reported that some breast cancers are dependent on

estrogen for sustained growth, and such phytoestrogens bind to the ER and activate it [138]. It has been shown that both isoflavones modulate multiple signaling pathways and are able to induce apoptosis via a caspase-3 mediated pathway [139]. Daidzein has been shown to disrupt mitochondrial membrane potential leading to cytochrome c release and to induce cell cycle arrest at the G1 and G2/M phases by up regulating expression of p27 [18, 140]. Therefore, daidzein disrupts tumorogenesis through a cell cycle mediated pathway. Genistein has been shown to down regulate Bcl-2, up regulate Bax, and inhibit proliferation by inducing apoptosis through the MAPK pathway [121, 141].

MATERIALS AND METHODS

Materials

Flavonoids and iso-flavonoids were purchased from Indofine or Alexis Biochemicals and stocks were prepared at 50 mM in dimethyl sulphoxide (DMSO) with the exception of methylluteolin, which was prepared at 25 mM in DMSO. The compounds were used at 25, 50, 100, and 200 μM for all experiments. The maximum DMSO concentration used in experiments was 0.4% (200 μM flavonoid treatment). DMSO at this level did not have any effect on assays conducted compared to non-DMSO treatment (data not included). Propidium iodide was purchased from Sigma. RNase was purchased from Fisher. Trypan Blue was purchased from Hyclone.

Cell Culture

The following human breast cancer cell lines were purchased from the American Type Culture Collection (ATCC): BT-474, MCF-7, MDA-MB-231, SK-BR-3, and ZR-75-1. BT-474, SK-BR-3, and ZR-75-1 were routinely maintained in RPMI 1640 with L-glutamine (HyClone) and supplemented with 10% bovine growth serum (BGS) and 1X antibiotic/antimycotic (HyClone) containing 10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of Amphotericin B per milliliter. MDA-MB-231 and MCF-7 cells were maintained in DMEM with high-glucose, L-glutamine, and sodium pyruvate, supplemented with 10% BGS and 1X antibiotic/antimycotic. All cell lines were maintained at 37°C in a humidified

atmosphere containing 5% CO_2 and passaged based on recommended dilutions and confluencies from ATCC. Human Mammary Epithelial Cells (HMEC) were purchased from Lonza and were maintained in the recommended media with supplements from Lonza.

Cell Viability Assays

Cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (CTG, Promega) or Trypan Blue Exclusion Assay (TBE, HyClone). For the CTG assay, 0.5×10^4 cells per well were seeded in 96-well white plates (BD Falcon). After 24h of incubation, media were changed and cells were exposed to various concentrations of flavonoids and iso-flavonoids. The CTG assays were initiated at 24, 48, and 72h after exposure to compounds, and cells were processed following the manufacturer's recommend protocol. Briefly, cells were incubated at room temperature on a variable speed shaker for 10 min with the CTG reagent, mixed briefly by pipetting, and centrifuged at 300 xg to remove bubbles. Plates were read in a Veritas 96 Well Luminometer (Turner Biosystems) or a Glo-Max II Multimode Plate Reader (Promega).

For the TBE assay, 0.5×10^5 cells per well were seeded in 6-well culture dishes. After 24h of incubation, media were changed, and cells were treated with 100 μM flavonoid or DMSO as a control. Cells were harvested by trypsinization at 24, 48, and 72h post treatment and resuspended in 1 mL of media. Cells were briefly vortexed, and an equal volume of cell suspension was combined with an

equal volume of 0.4% Trypan Blue in PBS. Cells lacking Trypan Blue were counted using a hemocytometer, and the percentage of live cells was calculated.

Cell Cycle Analysis

Progression through the cell cycle was analyzed following a previously published protocol [142]. Briefly, MDA-MB-231 cells were seeded at 0.5 x 10^5 cells per well in 6-well culture dishes. After 24h of incubation, cells were treated with 100 μM of various flavonoids. Cells were harvested via trypsinization 24 and 48h after treatment, washed with cold PBS, and processed for cell cycle analysis. The cells were fixed in absolute ethanol and stored at −20°C for later analysis. The fixed cells were centrifuged at 1000 rpm and washed with cold PBS twice. RNase A (20 μg/mL final concentration) and Propidium iodide staining solution (50 μg/mL final concentration) was added to the cells. The cells were incubated for 30 min at 37°C in the dark. The cells were analyzed using a FACSCalibur instrument (BD Biosciences) equipped with CellQuest, and 10,000 events were collected for analysis.

Statistical Analysis

Statistical data were analyzed for the CTG assay using a four-factor ANOVA model for drug, cell-line, drug concentration, and time. F-tests followed by Tukey's multiple comparison adjustment were utilized to identify statistically significant differences in cell death. To compare cell death as measured by CTG and TBE, p-values were calculated from two-sided Student t-tests followed by

Hochberg multiple comparison adjustments. Only adjusted p-values of <0.05 were considered statistically significant.

For comparison of cell viability to cell cycle arrest, 95% confidence intervals were computed for G2 fold change of one (no change) and plotted against cell viability as determined by TBE assays. Dose-response data were analyzed using sigmoidal curve fits in Prism (GraphPadSoftware, Inc) with variable slope to determine IC50 values. The top part of the curve was set to 100% response (0% viability) and the bottom part of the curve to 0% (100% viability). The IC50 values are reported with 95% confidence intervals.

For comparison between two groups in all other assays, the data were analyzed using the two-sided, two independent sample Student t-test with 95% confidence intervals reported. A p-value of <0.05 was regarded as statistically significant.

RESULTS

Flavonoid-Induce Cellular Cytotoxicity

We measured cell viability of five human breast cancer cell lines (Table 1) and human mammary epithelial cells (HMEC) after treatment with fourteen different flavonoids (Table 2) using two assays. First, we used Promega's CellTiter-Glo® (CTG) Luminescence assay, which indirectly determines the number of viable cells by quantifying the amount of ATP present in a metabolically active cell [143]. This assay has been previously used to measure amount of cytotoxicity. The CTG assay utilizes a luciferase reaction producing light output to measure the amount of intracellular ATP. This assay was chosen over the conventional MTT or MTS assay because flavonoids have been shown to absorb light at the same wavelength as MTT and MTS assays [144, 145], which may interfere with the proper interpretation of the data. Secondly, we used a classical trypan blue exclusion (TBE) assay to measure the number of viable cells. After treatment with flavonoids, live cells do not take up trypan blue dye because the membrane is intact, and the cells appear clear under the microscope.

Cellular viability was measured at 24, 48, and 72h after treatment with flavonoids at four concentrations of 25, 50, 100, and 200 μM (see Appendix B). Figures 3 through 6 present the cellular viability measured by the CTG assay after 72h of treatment with the four concentrations. Cellular viability decreased

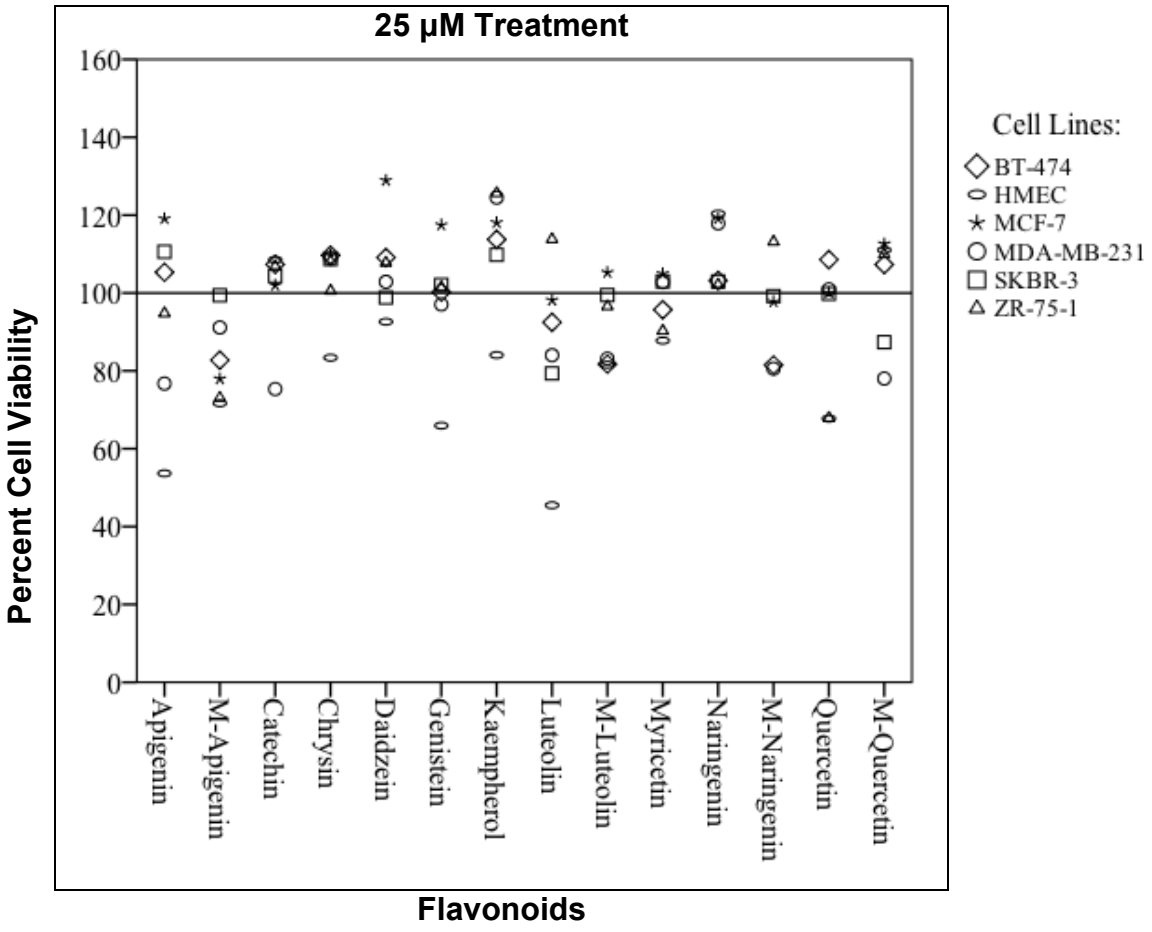


Figure 3. Analysis of cell viability for five breast cancer cell lines and one primary cell line after treatment with 25 µM of flavonoids. The CTG assay was performed 72h after treatment. The DMSO-treated cell group was set to 100% cell viability, indicated by a horizontal line. Flavonoids used in the study are indicated along the X-axis. The Y-axis represents the percent cell viability.

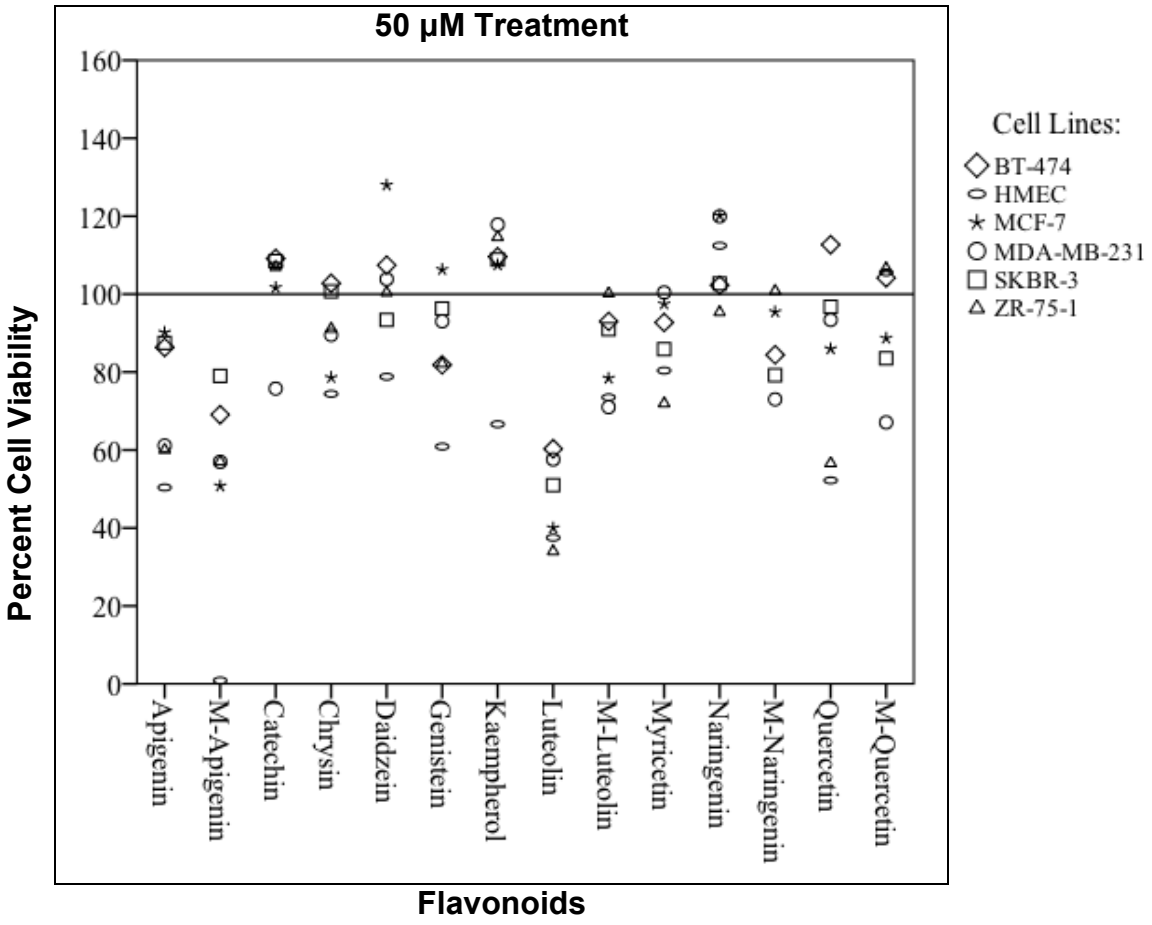


Figure 4. Analysis of cell viability for five breast cancer cell lines and one primary cell line after treatment with 50 µM of flavonoids. The CTG assay was performed 72h after treatment. The DMSO-treated cell group was set to 100% cell viability, indicated by a horizontal line. Flavonoids used in the study are indicated along the X-axis. The Y-axis represents the percent cell viability.

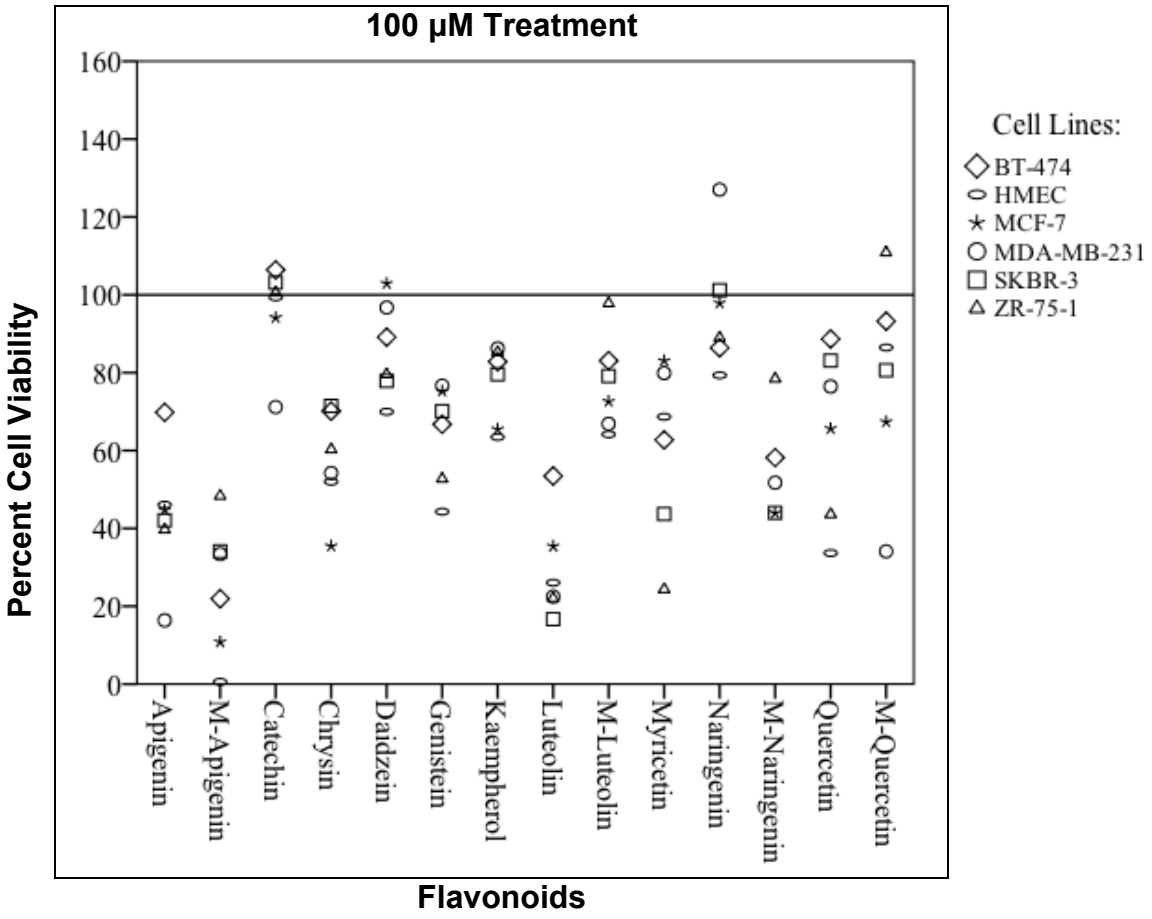


Figure 5. Analysis of cell viability for five breast cancer cell lines and one primary cell line after treatment with 100 µM of flavonoid. The CTG assay was performed 72h after treatment. The DMSO-treated cell group was set to 100% cell viability, indicated by a horizontal line. Flavonoids used in the study are indicated along the X-axis. The Y-axis represents the percent cell viability.

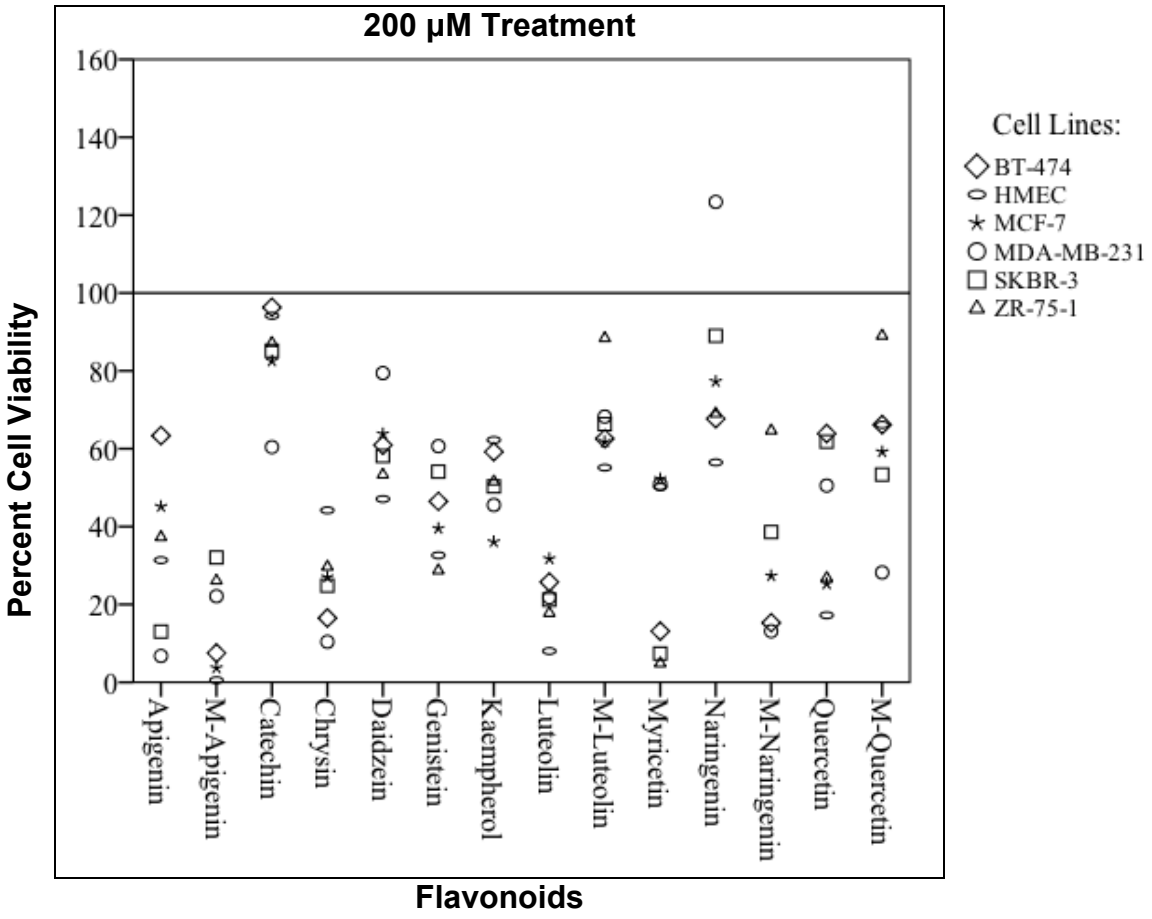


Figure 6. Analysis of cell viability for five breast cancer cell lines and one primary cell line after treatment with 200 µM of flavonoids. The CTG assay was performed 72h after treatment. The DMSO-treated cell group was set to 100% cell viability, indicated by a horizontal line. Flavonoids used in the study are indicated along the X-axis. The Y-axis represents the percent cell viability.

over time and increasing flavonoid concentration. Data were analyzed for the CTG assay using a four-factor ANOVA model for drug, cell line, drug concentration, and time. F-tests followed by Tukey's multiple comparison procedure were used to find statistically significant differences in cell death. An ANOVA table with the prospective p-values for this comparison is presented in Appendix A. There was a significant time-dose effect on cytotoxicity by the flavonoids in all cancer cell lines, as well as HMECs, tested. Maximum cell death was measured at 72h after initial treatment ($p<0.001$). Surprisingly, the data suggested that some flavonoids stimulated cellular proliferation, especially at the lower concentrations with data points at a higher than 100% cell viability as shown in Figure 3 (also see Appendix B).

Becaues the CTG assay determines the number of viable cells indirectly, by quantifying the amount of ATP, we also conducted the TBE assay in order to measure the actual number of viable cells. By comparing these data with the CTG, we were able to identify wether a viability of higher than 100% was due to stimulation of the cells or was a result of the increase in the amount of ATP produced by the cells. We chose to measure only cellular viability after 72h of treatment with flavonoids since this time point showed the maximum effect, as determined in the CTG assays. These results suggested that the two assays, CTG and TBE, did not show the same amount of cellular viability (Figure 7). The majority of the data points lie on the left side of the line indicating a lower TBE percentage and higher CTG percentage. The results appear as a bar graph in

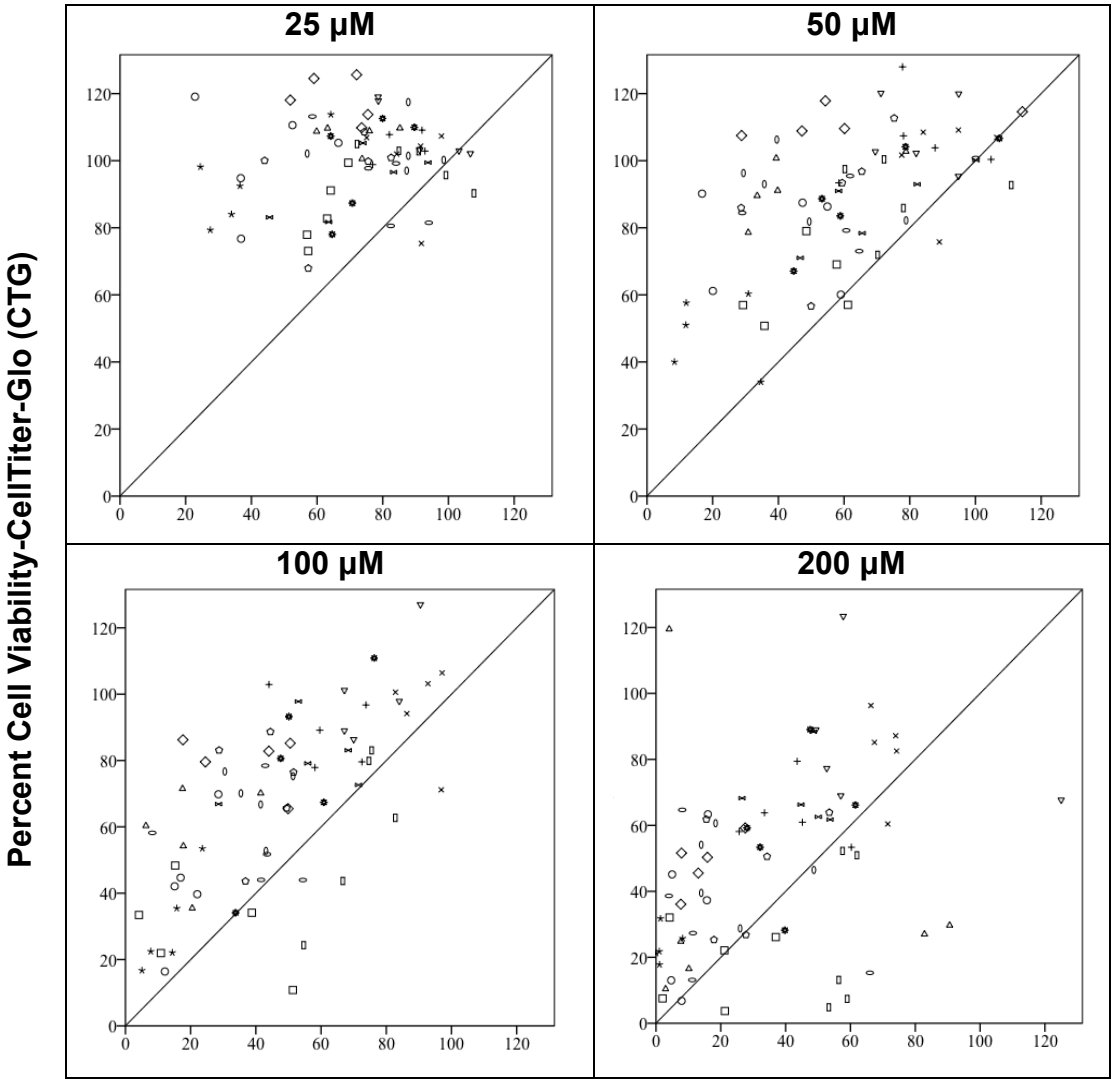


Figure 7. Comparative analysis of cell viability in CTG and TBE after 72h of flavonoid treatment at four different concentrations. The key to flavonoids is provided on the right. The X-axis represents percent cell viability from TBE. The Y-axis represents percent cell viability from CTG.

Appendix C. Apigenin, chrysin, genistein, kaempherol, luteolin, and quercetin consistently showed a higher cell viability using the CTG assay than the TBE assay for all cell lines tested. This suggested that these compounds were capable of increasing the amount of ATP present in the cells.

In addition, IC50 curves were calculated for all flavonoids after 72h of treatment using the results from the TBE assay. Table 8 shows the calculated IC50s with 95% confidence interval ranges indicated in parenthesis. Table 9 is a summary of the findings regarding cell death in breast cancer cell lines. Categories are based on the percent viability by TBE in combination with the IC50 data and grouped flavonoids based on how effectively they induced cellular cytotoxicity. Apigenin, m-apigenin, and luteolin were the most effective at inducing cytotoxicity in all cell lines tested. In certain cell lines, quercetin, m-quercetin, m-luteolin, kaempherol and chrysin were also very effective at inducing cytotoxicity but were ineffective in other cell lines. Genistein, m-naringenin, and daidzein were very weak inducers of cytotoxicity in all breast cancer cell lines. Finally, myricetin, naringenin, and catechin were unable to induce cytotoxicity in any of the breast cancer cell lines tested. These results indicated that not all flavonoids were capable of inducing cytotoxicity in breast cancer cell lines.

Table 8. IC50 data for flavonoid-induced cell death after 72h. Values are reported in μM. Values in the parenthesis are the range for the 95% confidence interval. A value of >200 μM indicates that flavonoid did not induce cellular death. M-apigenin represents methylapigenin.

	BT-474	MCF-7	MDA-MB-231	SK-BR-3	ZR-75-1
Apigenin	49 (43-56)	2 (0.33-16)	14 (11-19)	32 (23-46)	14 (43-48)
M-apigenin	42 (35-52)	40 (17-94)	33 (27-40)	53 (36-77)	38 (18-62)
Catechin	>200	>200	>200	>200	>200
Chrysin	80 (66-95)	34 (28-40)	40 (35-45)	34 (27-42)	59 (48-72)
Daidzein	154 (129-182)	102 (80-130)	179 (144-222)	97 (69-139)	>200
Genistein	97 (61-154)	74 (52-105)	51 (43-61)	30 (18-53)	81 (68-96)
Kaempherol	77 (62-96)	25 (16-40)	38 (31-47)	48 (40-58)	80 (61-106)
Luteolin	13 (6-27)	5 (14-20)	16 (14-20)	13 (9-20)	32 (28-37)
M-luteolin	>200	>200	21 (12-38)	133 (87-200)	150 (100-225)
Myricetin	>200	>200	>200	>200	160 (77-330)
Naringenin	>200	>200	>200	>200	>200
M-naringenin	69 (58-82)	102 (64-161)	75 (59-96)	72 (54-96)	40 (22-74)
Quercetin	>200	102 (62-161)	101 (82-124)	64 (55-75)	47 (29-77)
M-quercetin	>200	105 (64-171)	54 (39-75)	78 (50-123)	>200

Table 9. Summary of findings for cell death using the TBE assay and IC50 values. Categories were created based on strong killing (>80% and IC50<50 μM), weak killing (20-70% and IC50<100 μM), or no killing (<20%).

Kills Strongly (>80%)		Kills Weakly	Does Not Kill
Non-Selective	**Selective**	**(20-70%)**	**(>20%)**
Apigenin	Chrysin	Daidzein	Naringenin
Methylapigenin	Genistein	Methylnaringenin	Catechin
Luteolin	Kaempherol		Myricetin
	Methylluteolin		
	Quercetin		
	Methylquercetin		

Cell Cycle Arrest does not Correlate with Cell Death

The ability of flavonoids to induce cell cycle arrest was assessed by flow cytometry, and the data were compared to cellular viability by the TBE assay. All breast cancer cell lines were treated with 100 μM flavonoid and processed at 24 and 48h post-treatment. Figure 8 represents fold change in G2/M DNA content of flavonoid-treated cells from DMSO-treated control compared to the cell viability data calculated by the TBE assay. Points to the right of the confidence intervals indicate a significant increase in DNA content due to an arrest in the G2/M phase. Points to the left of the confidence intervals indicate a decrease in DNA content due to an arrest in the G1 phase. Table 10 summarizes the cell cycle arrest findings. Besides catechin, all the flavonoids arrested the cell cycle of the different breast cancer cell lines either at the G1 phase or the G2/M phase, and the arrests were statistically significant ($p<0.05$).

The majority of flavonoid-induced cell cycle arrest was in the G2/M phase with the exception of m-quercetin and m-naringenin, which consistently arrested

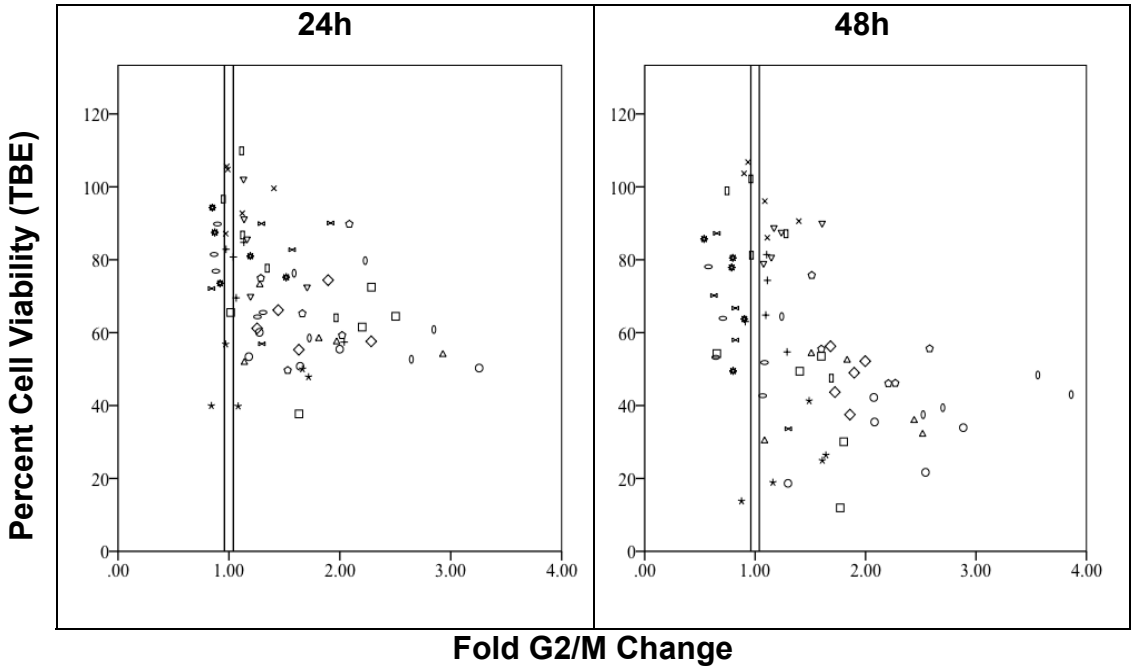


Figure 8. Cell cycle arrest does not correlate with cell viability. We measured cell cycle arrest at 24 and 48h treatment with 100 μM flavonoids and plotted the results against cellular viability as measured by TBE assay. The X-axis has the fold G2/M DNA content change compared to DMSO-treated control. The Y-axis represents the cell viability from the TBE assay. The key to the flavonoids is provided on the right. Vertical bars indicate the 95% confidence interval for no significant change in G2/M DNA content in treated cells.

cell lines in G1 phase. It has been previously shown that methylated versions of various flavonoids arrest cells in the G1 phase [110], and our data supported these findings.

It is noteworthy that cell cycle arrest did not occur in all cell lines for a particular flavonoid (Table 10). For example, apigenin and chrysin arrested all breast cancer cell lines in G2/M phase with the exception of SK-BR-3 cells. Another group reported that apigenin is able to induce cell cycle arrest in SK-BR-3 cells [41] . We were unable to reproduce this result. However, apigenin does induce cytotoxicity in SK-BR-3 cells and is similar to what was reported by this group. Therefore, it remains inconclusive whether or not cell cycle arrest occurs upon apigenin treatment in SK-BR-3 cells.

Kaempherol and quercetin were the only two flavonoids that consistently arrested all cell lines tested at both 24 and 48h. Kaempherol is also a very strong inducer of cellular cytotoxicity in all cell lines, whereas quercetin is more selective in its cytotoxicity. Naringenin, daidzein, m-luteolin, and myricetin are all compounds that were not able to induce cellular cytotoxicity at very high levels. All of these compounds were able to induce cell cycle arrest in some cell lines, but not others. Taken together, these results suggest that cell cycle arrest may not be a strong indicator of whether or not a flavonoid is able to induce cellular cytotoxicity. From these data, we can conclude that flavonoids are able to exert their effects regardless of cell line used.

Table 10. Summary of cell cycle arrest identified in all breast cancer cell lines after 24 and 48h with 100 µM flavonoid treatment. NE indicates that there was no cell cycle arrest detected that was statistically significant. G2 corresponds to arrest during the G2/M phase, G1 corresponds to arrest during the G1 phase, and when indicated was statically significant ($p<0.05$). Results shown represent the averages of at least three independent experiments.

	BT-474		MCF-7		MDA-MB-231		SK-BR-3		ZR-75-1	
	24	48	24	48	24	48	24	48	24	48
Apigenin	G2	G2	G2	G2	G2	G2	NE	NE	G2	G2
M-apigenin	G2	NE	G2	NE	G2	G2	NE	G2	G2	G2
Catechin	NE	NE	G2	NE	NE	NE	NE	NE	NE	NE
Chrysin	G2	G2	G2	G2	G2	G2	NE	NE	G2	G2
Daidzein	NE	NE	G2	NE	NE	NE	NE	G1	NE	NE
Genistein	G2	G2	G2	NE	G2	G2	G2	G2	G2	G2
Kaempherol	G2	G2	G2	G2	G2	G2	G2	G2	G2	G2
Luteolin	G2	G2	G2	G2	G2	G2	NE	G1	G2	G2
M-luteolin	G2	G1	NE	G1	NE	NE	G1	G1	NE	G1
Myricetin	NE	NE	NE	G1	G2	NE	G2	G2	G2	G2
Naringenin	G2	G2	G2	NE	G2	G2	NE	NE	NE	NE
M-naringenin	NE	NE	NE	G1	G1	NE	G1	G1	NE	G1
Quercetin	G2	G2	G2	G2	G2	G2	G2	GE	G2	G2
M-quercetin	NE	G1	G1	G1	G1	G1	G1	G1	NE	G1

DISCUSSION

Flavonoid-Induce Cytotoxicity and Apoptosis Appear to be Independent of a Particular Signaling Pathway

We undertook a comprehensive study evaluating flavonoid-induced cellular cytotoxicity. We used a variety of breast cancer cell lines and flavonoids to characterize this cellular cytotoxicity. We chose the cell lines based on their lack of expression in various signaling pathways (Table 1). Some of these same cell lines have been used in previous studies, listed in Table 3, to characterize the effect of flavonoids on cellular proliferation and cytotoxicity. We chose flavonoids from various sub-classes (Table 2) in order to determine if there was a structural-functional relationship within the chemical structures for induction of cytotoxicity.

Apigenin, m-apigenin, and luteolin induced the strongest cytotoxicity in all breast cancer cell lines tested. Becasue these compounds induced cytotoxicity equally well on all cell lines, we concluded that the mechanism of action was independent of a particular signaling pathway component, such as HER2, p53, and EGFR.

The isoflavonoids are considered to be phytoestrogens because of their structural similarity to the mammalian steroid hormone 17β-estradiol. Both have been reported to bind to the estrogen receptor and activate it [138]. Some breast cancers have been shown to be dependent on the estrogen receptor for

sustained growth. Therefore, circulating phytoestrogens could be harmful by stimulating growth of breast cancer. Interestingly, consumption of foods rich in phytoestrogens correlates with reduced risk in breast cancer [148]. Our studies indicate that daidzein is a weak inducer of cellular cytotoxicity regardless of the cell line tested. However, genistein appears to be selective for ER-negative cell lines in the ability to induce cytotoxicity.

We have shown that the same flavonoids, which induce cytotoxicity in breast cancer cells, are able to induce cytotoxicity in HMECs. This is in contrast to previous reports in the literature, which have indicated that genistein is unable to decrease cell viability in MCF10A cells (Table 3). However, it should be noted that all studies we have seen in the literature generally have not done a comparison of HMECs and MCF10A cells. Our results suggest that flavonoid action is through a generalized mechanism and that flavonoids may not directly target cancer cells as previously suggested, although this still remains controversial.

We also see cellular cytotoxicity occurring with the methylated derivatives of some of the flavonoids. Of the methylated flavonoids, methylapigenin was able to induce cytotoxicity at the equivalent level of apigenin, whereas methylluteolin and methylquercetin were not. Previous studies have shown that the methylated versions of various flavonoids tend to be more potent than their unmethylated counterparts [106].

By comparing CTG and TBE data, we were also able to show that some flavonoids increase the amount of ATP within the cell. It is important to consider that CTG determines number of viable cells according to the amount of ATP in the cell suspension, and may result in an overestimation of cell viability for these flavonoids. It has also been reported that MTT and MTS-based methods result in an underestimation of cytotoxicity due to reduction of MTT and MTS by some flavonoids and formation of formazan [144]. These results further demonstrate the need for a careful consideration of the methods utilized for evaluating cellular proliferation of flavonoids.

Our studies indicate that flavonoid induction of cellular cytotoxicity may be occurring through a non-classical apoptotic mechanism. We have previously reported that flavonoids are able to inhibit caspase-3 and caspase-7 at similar concentrations that induce cytotoxicity in our breast cancer cell lines [149]. We also showed that flavonoids are cytotoxic to cells, which lack caspase-3 and caspase-7. Taken together, these results do not suggest a particular signaling pathway required for the chemopreventative property of flavonoids. Instead, the data may suggest that the classical apoptosis may not be the primary form of cellular cytotoxicity and that the importance of individual signaling component modulating cell growth may be cancer cell type specific. However, more extensive research into the role of flavonoids in each signaling pathway is still required.

Structure-Function Relationship of Flavonoid Effects

We suggest that flavonoids that are able to induce cytotoxicity may do so in a generalized cellular mechanism. We have been able to identify structural features, which we believe is important in flavonoid-induced cytotoxicity. Our data suggested that the flavones and some flavonols were most cytotoxic, while the flavanones tested were far less effective at killing cancer cells. This further suggests that the planarity of the benzopyran nucleus may be a key structural element in cytotoxicity. For example, we noted that both naringenin and (+)-catechin were identical to apigenin and quercetin, respectively, with the exception of the 2,3-double bond on the C-ring. However, both naringenin and (+)-catechin did not induce cytotoxicity in cell lines tested. Isoflavonoids were weaker in their ability to induce cytotoxicity. These observations suggest that flavonoid cytotoxicity requires the compounds to be planar in nature, possess the 2,3-double bond on the C-ring and the B-ring to be attached at the 2-position of the benzopyran core. We also believe that the planarity of the flavonoid(s) may be important in their ability to cross the cell membrane and enter the cytosol and that the B-ring's position is important for binding to a protein target.

Future Perspective

Significant progress has been made during the past thirty years on not only efficacy, but also identifying many molecular mechanisms altered by flavonoids in various cancers. Medicinal plants have played pivotal roles in the development of new drugs to treat human diseases. Some of the earliest forms

of chemotherapy originate from natural products derived from both plants and marine organisms. It is no surprise to see resurgence in the investigation of natural products for their anti-cancer use. Flavonoids have been shown to be potent bioactive molecules that possess anticarcinogenic effects. Flavonoids have also emerged as potential chemopreventative candidates for cancer. Despite this promise, contradictory results regarding molecular mechanisms of action have been reported from many laboratories. Although results from *in vitro* experiments are not always predictive of medicinal utility, they constitute a valuable tool for studying the effects of the drug candidate on molecular targets involved in tumor growth and survival. In addition, much attention has been given to the gene targets altered by these chemopreventative agents.

Many other techniques, especially cDNA microarray, may provide great insight into such gene-drug relationship. cDNA microarray enables researchers to identify many of altered gene expression as result of treating cells with flavonoids. This will provide bases for analysis of the functional groups and molecules involved in many process such as tumor growth and progression, cell cycle, apoptosis, DNA damage, inflammation and even metabolic alterations/ activations. It is also noteworthy that many researchers study the protective mechanism of such compounds. Since these dietary compounds are not classified as drugs, flavonoids do not require FDA approval to be available for consumption [150]. Therefore, a thorough evaluation of their toxicity and drug interactions is necessary to ensure their safety for the public.

More extensive studies are also needed on absorption and bioavailability of flavonoids in the body. In addition, the majority of dietary compounds contain a mixture flavonoids and other polyphenols. It has been shown that some flavonoids may have synergistic effects, whereas individually they are not effective chemopreventative agents [151]. This is more relevant as bioavailability data limit the concentration of flavonoids *in vivo*. Therefore, further extensive studies are necessary.

Consequently, more studies are clearly needed to resolve the conflicting data, to more fully understand the mechanism(s) of anti-cancer activity of flavonoids, and to evaluate their potential as therapeutic agents. As proteins that interact with flavonoids are identified, these discoveries will provide the basis for rational drug design.

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APPENDICES

Appendix A. Modified 4-Way ANOVA Table for the CTG Cell Viability Data.

Source	p-value
Drug	< 1E-300
Dose	< 1E-300
Time	< 1E-300
Cell Line	2.417E-261
Drug*Dose	< 1E-300
Drug*Time	3.187E-209
Drug*Cell Line	< 1E-300
Dose*Time	1.1998E-80
Dose*Cell Line	9.1602E-76
Time*Cell Line	2.0637E-49
Drug*Dose*Time	4.7378E-32
Drug*Dose*Cell Line	< 1E-300
Drug*Time*Cell Line	8.5134E-77
Dose*Time*Cell Line	1.9173E-07
Drug*Dose*Time*Cell Line	2.694E-06

R^2= 0.758

Table 11. Data analysis was conducted for the CTG assay using a four-factor ANOVA model for drug, dose, time, and cell line. F-tests followed by Tukey's multiple comparison adjustment were utilized to identify statistically significant differences in cell death.

Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Measured by CTG Assay.

9A

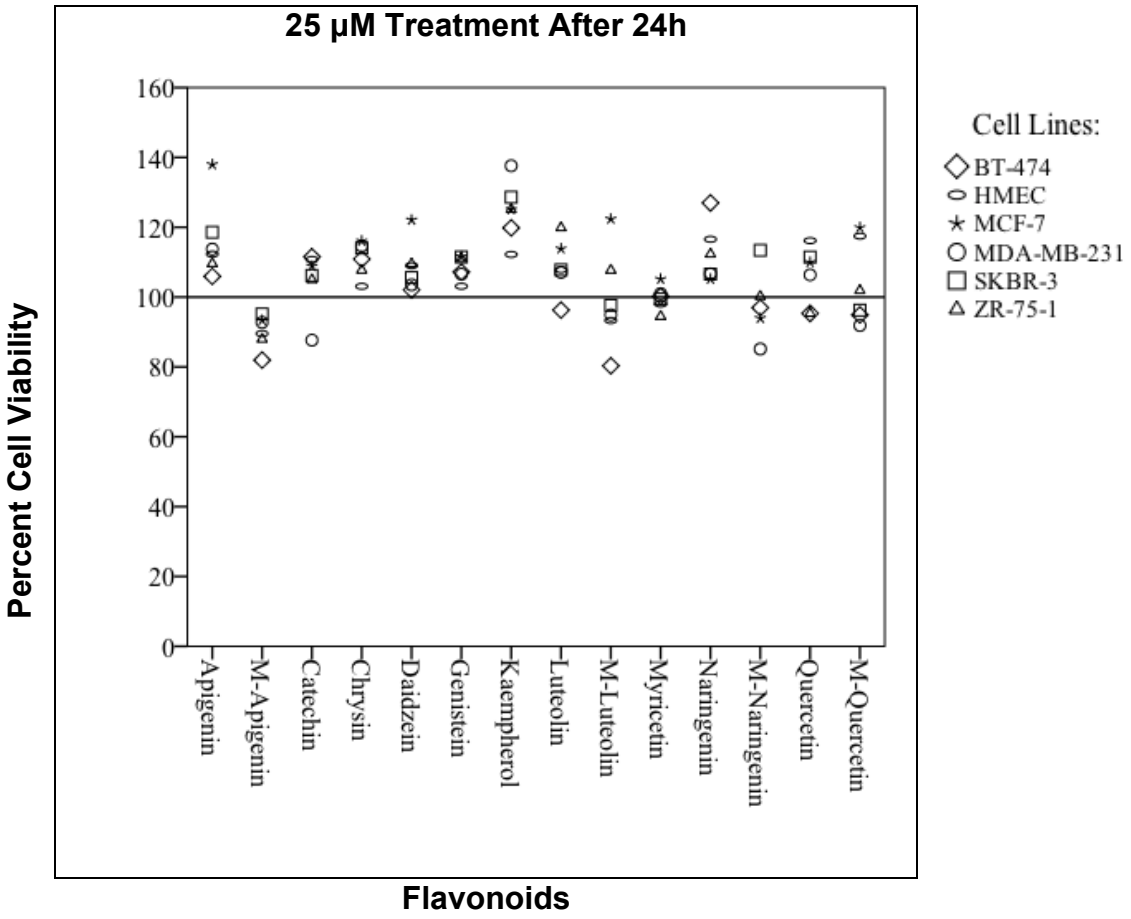
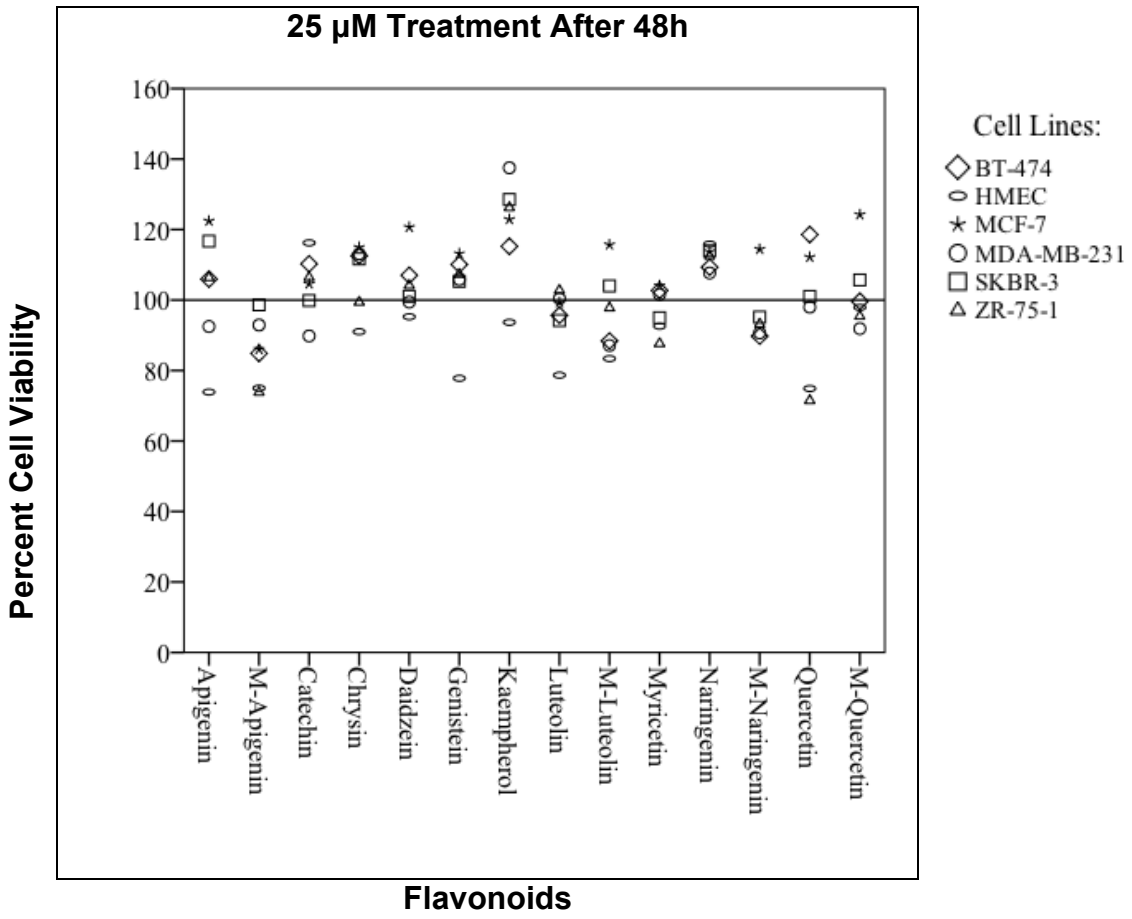


Figure 9. The CTG assay was performed after cells were treated with each flavonoids. The DMSO-treated cell group was set to 100% cell viability, which is indicated by a horizontal line. Figures 9A-9L represent the CTG data for concentrations of 25, 50, 100, 200 µM after 24, 48, 72h treatments. The key to cell lines tested is provided on the right. Flavonoids used in the study are indicated along the X-axis. The Y-axis represents the percent cell viability.

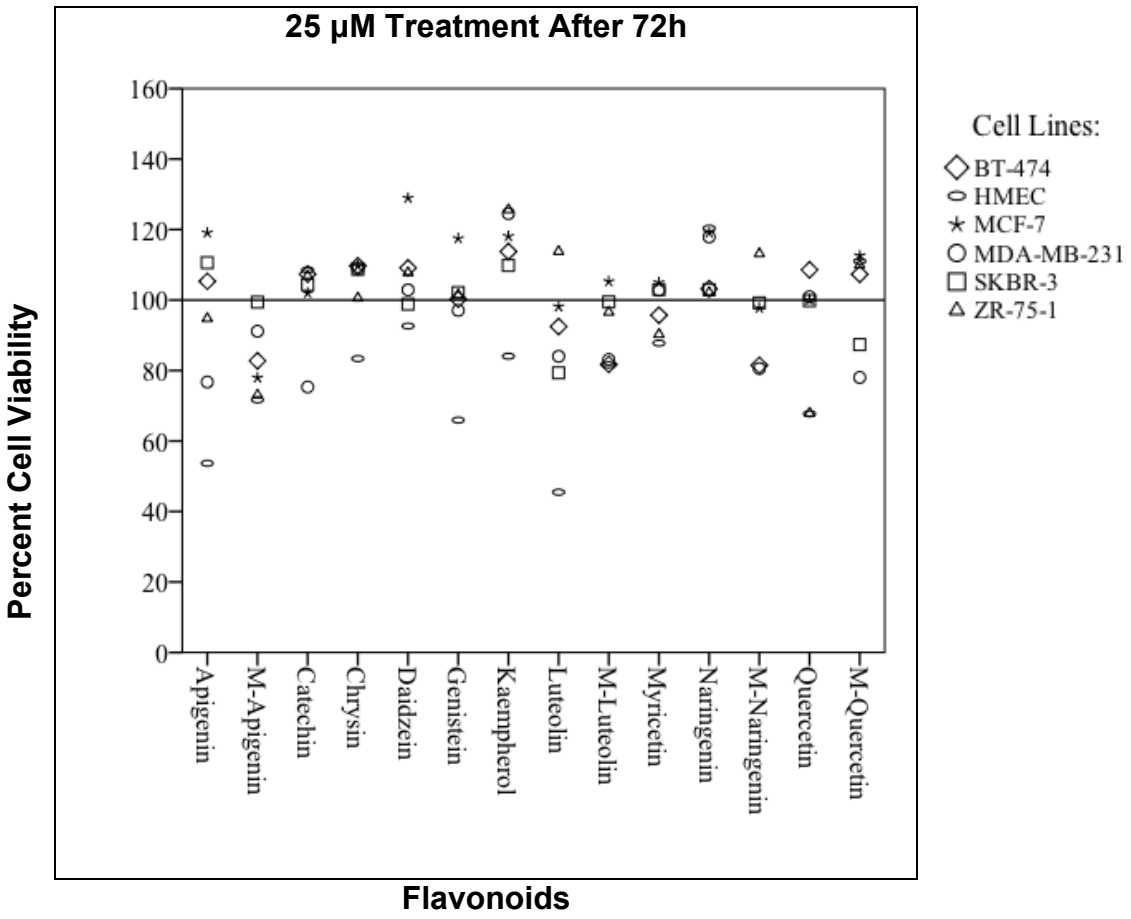
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9B



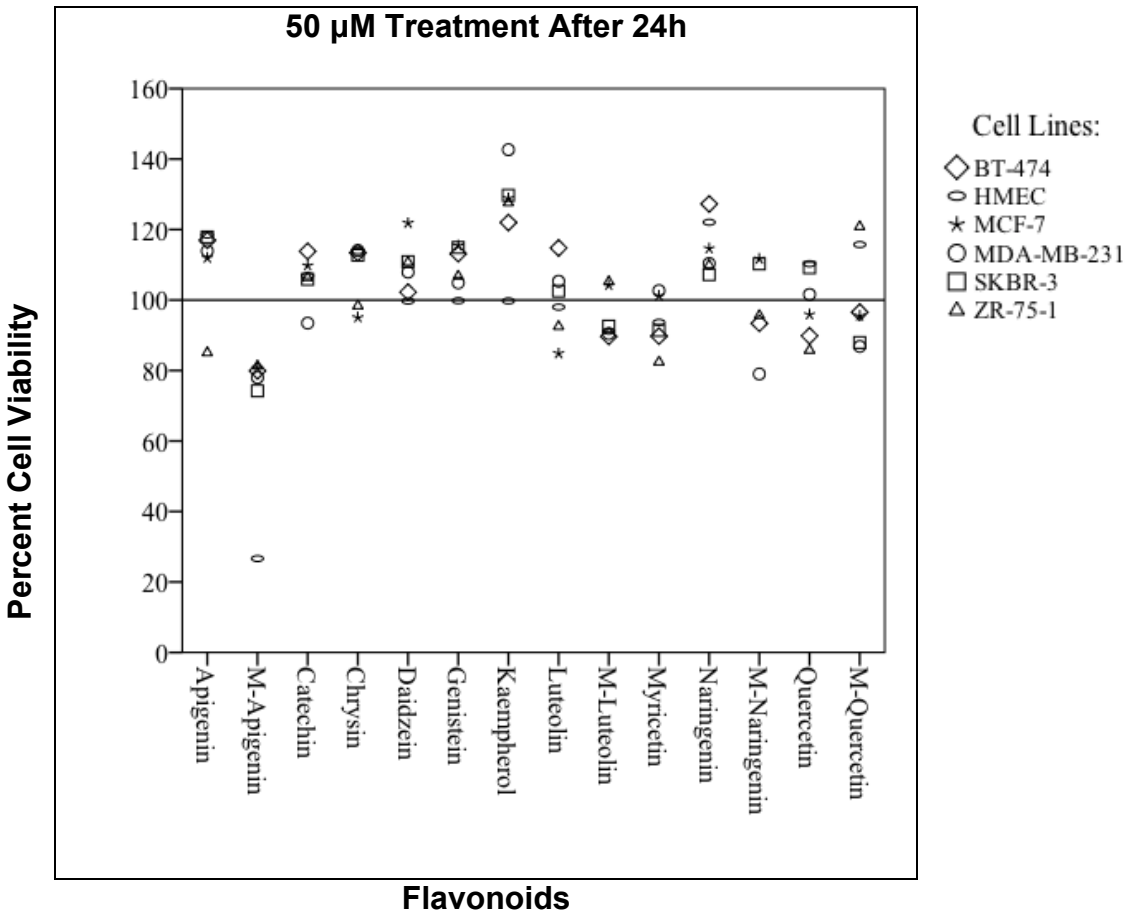
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9C



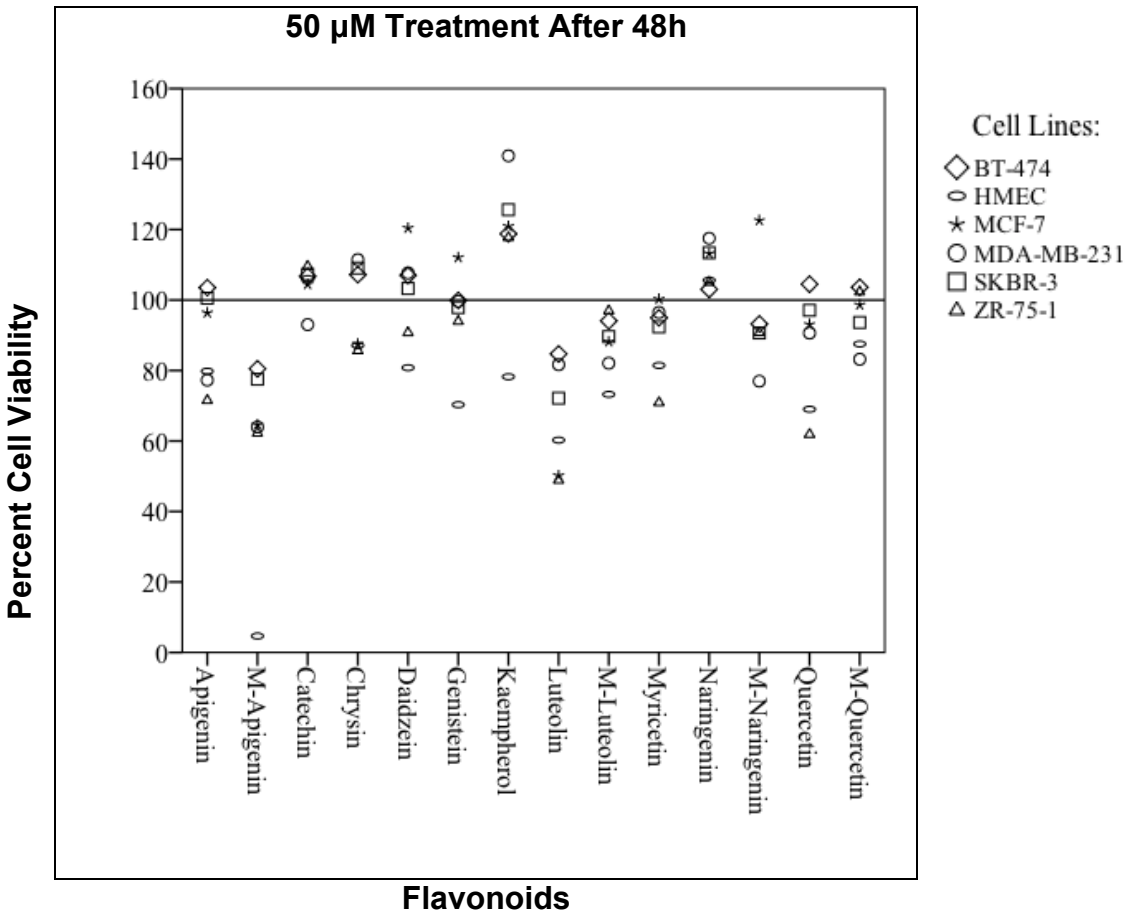
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9D



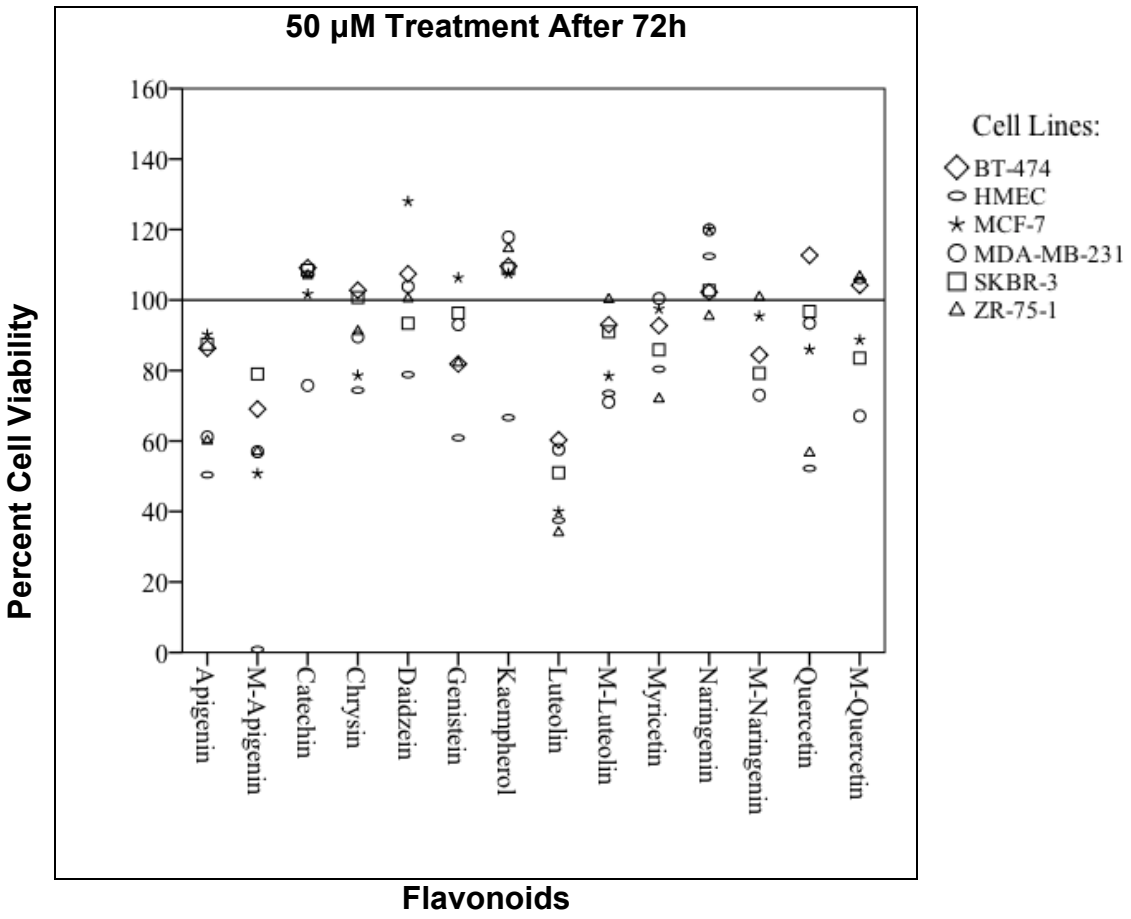
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9E



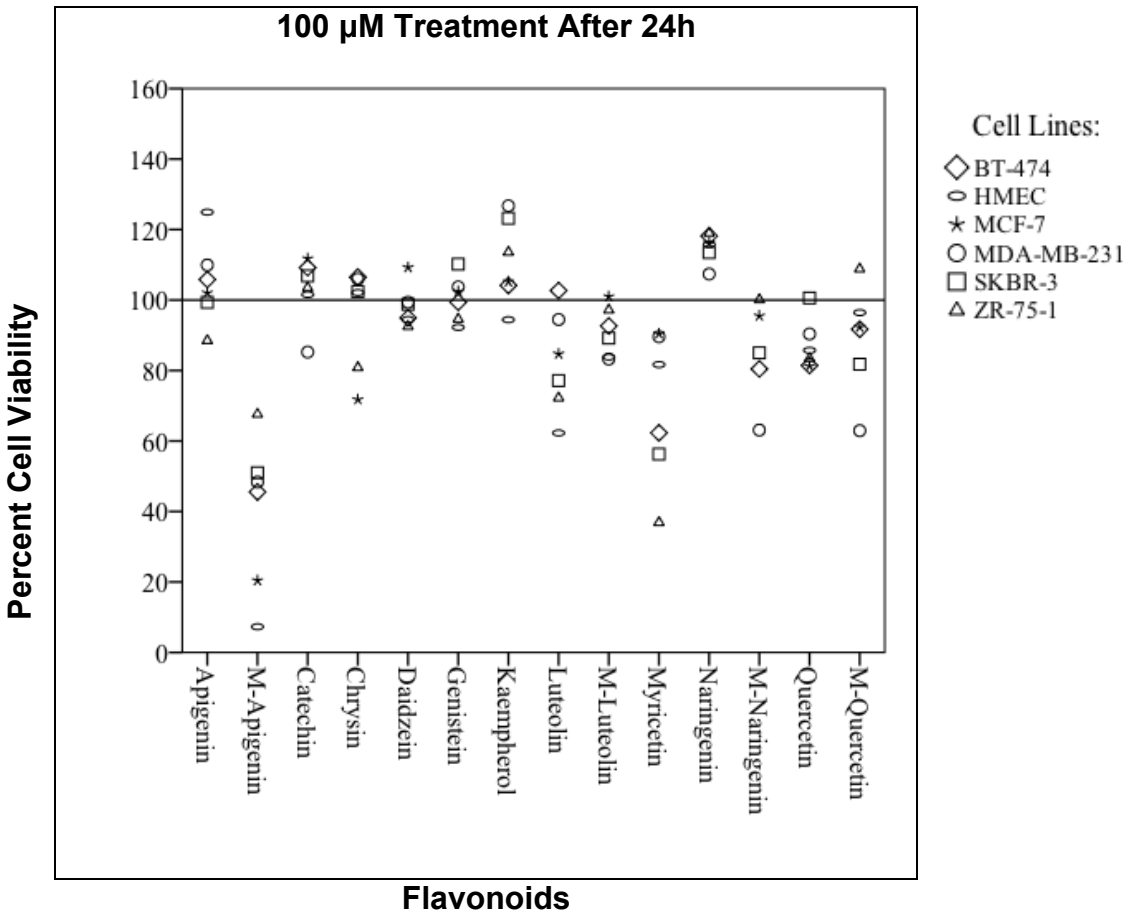
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9F



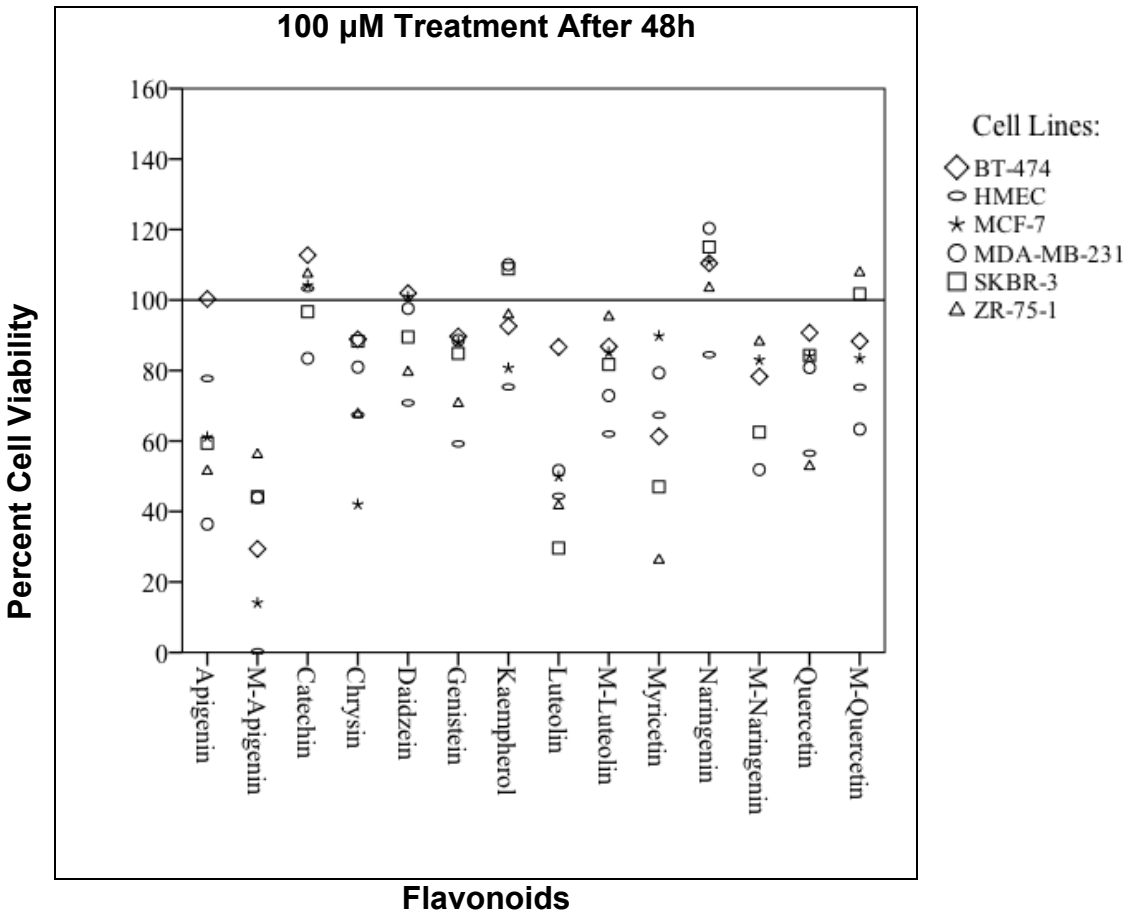
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9G



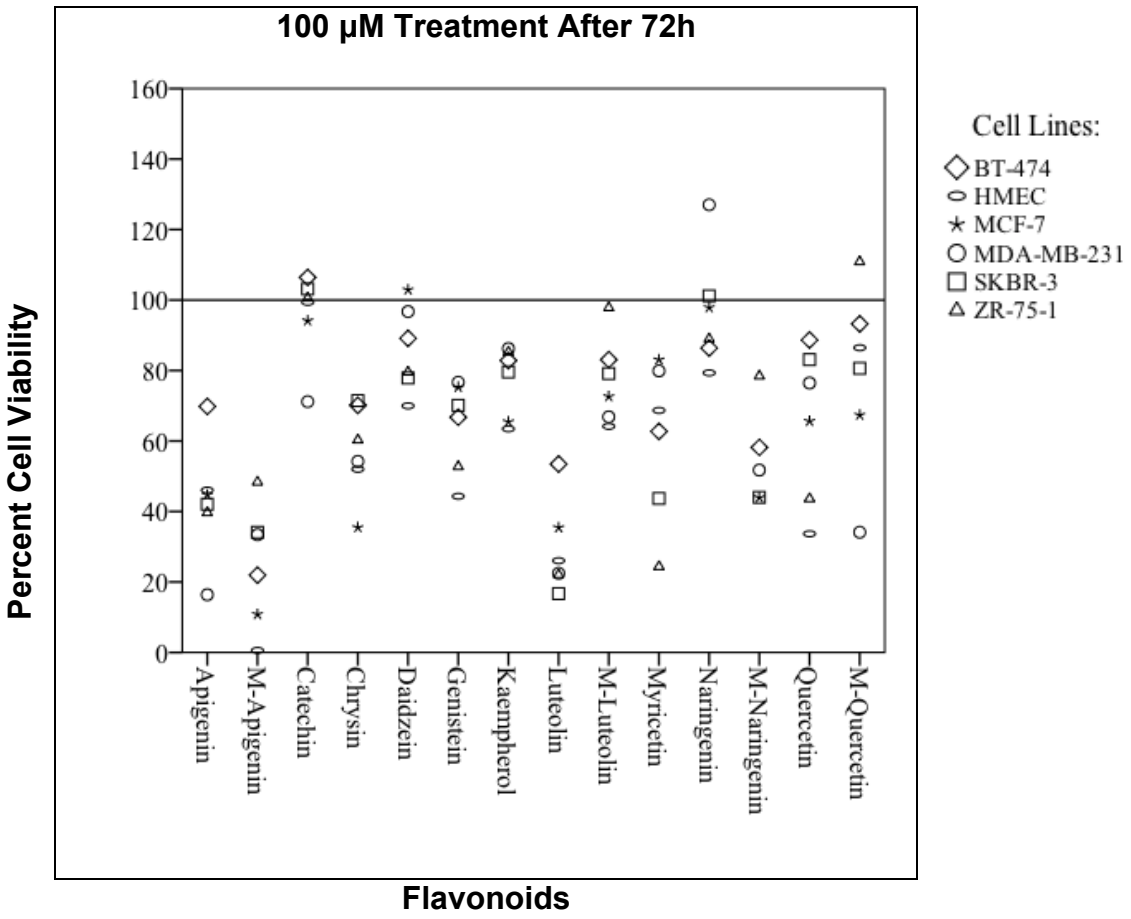
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9H



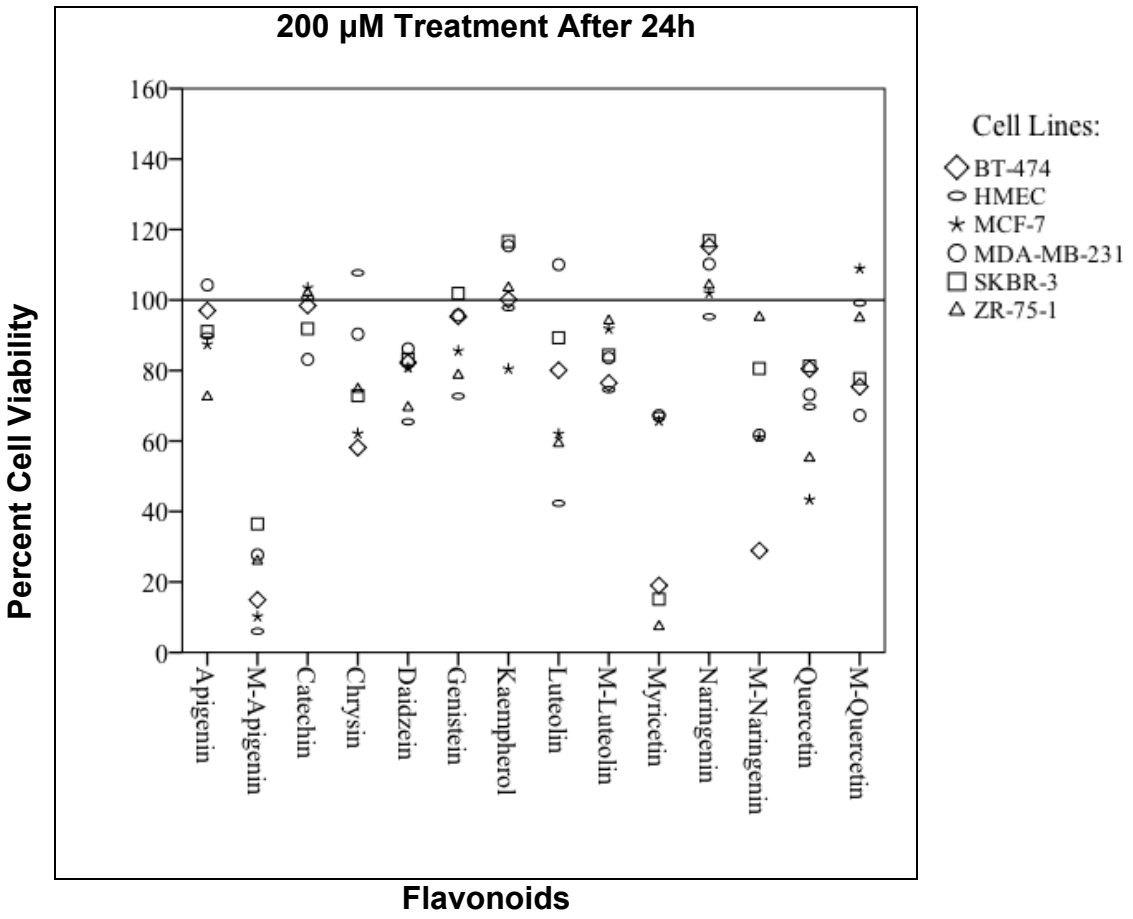
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9l



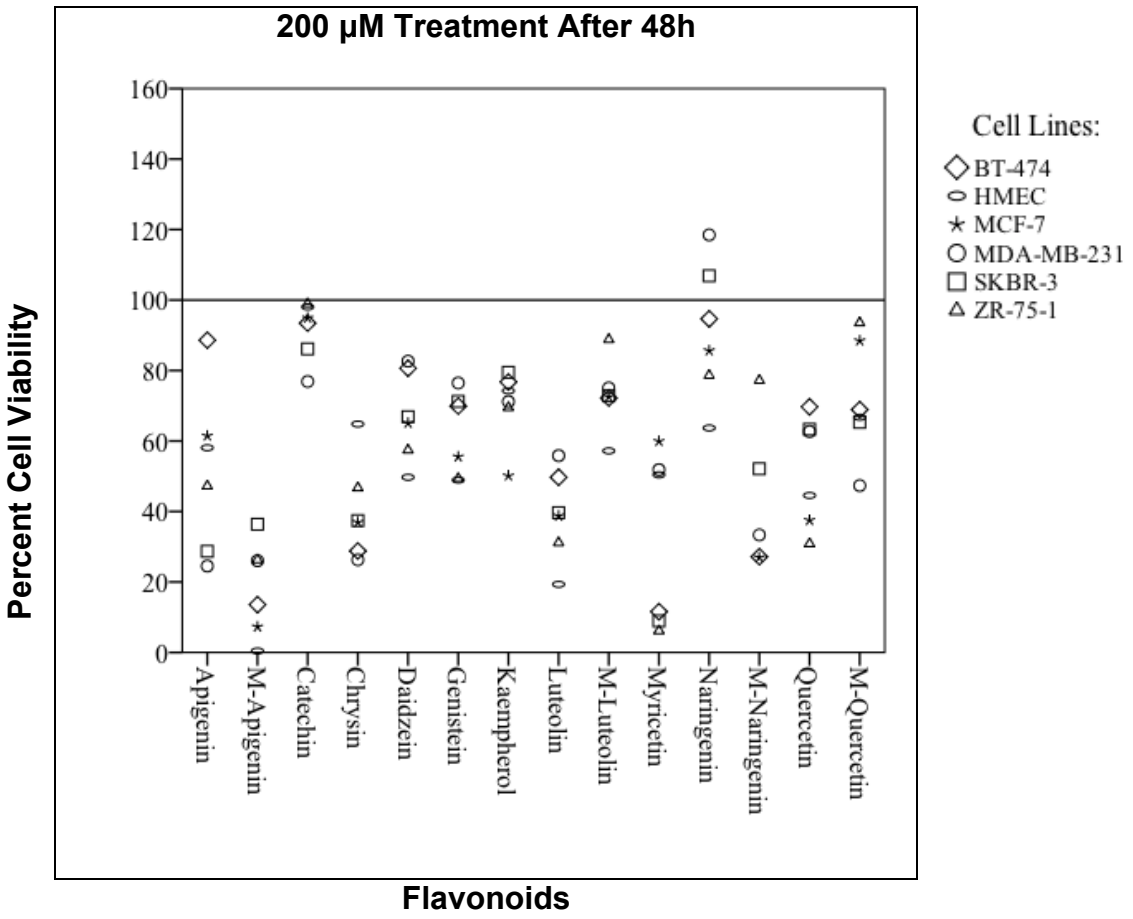
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9J



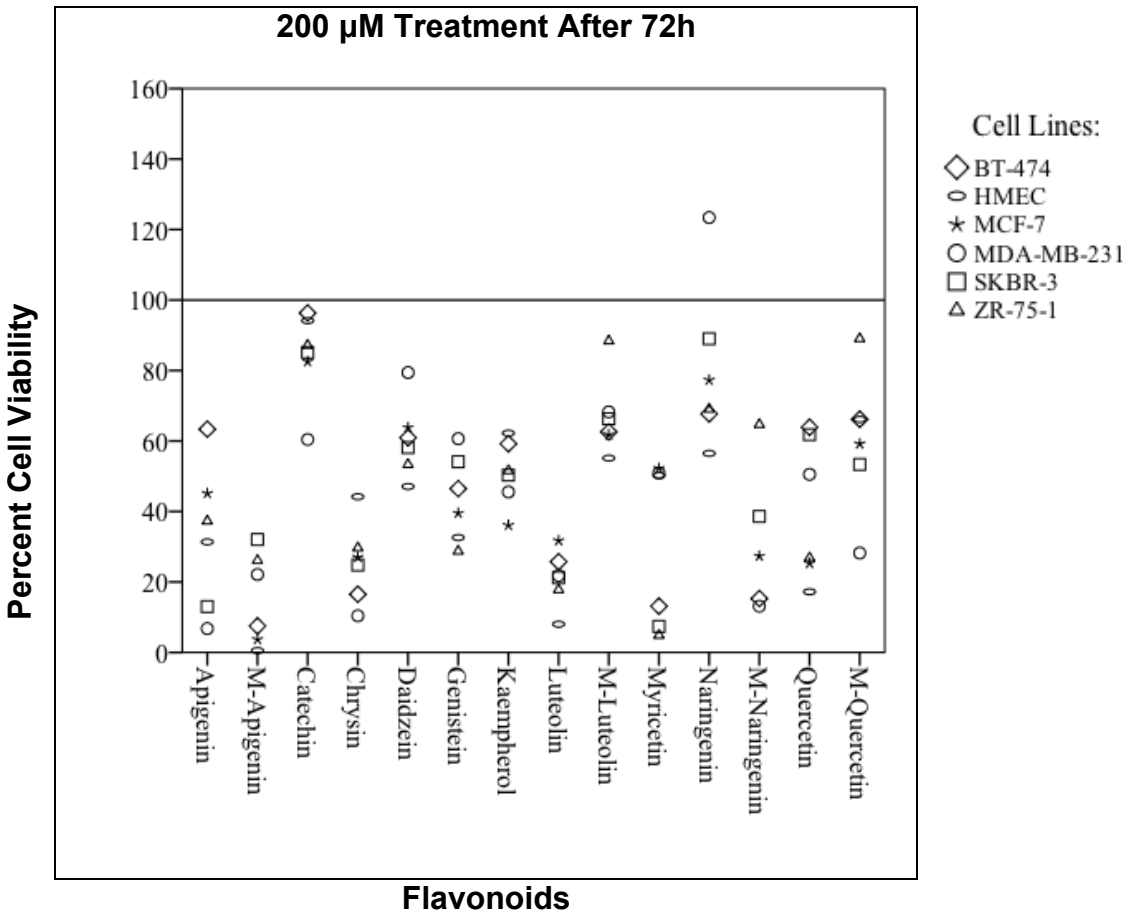
Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9K



Appendix B. Cell Viability of Breast Cancer Cell Lines after Flavonoid Treatment Via CTG Assay.

9L



Appendix C. Comparinson of CTG and TBE Assays for Measuring Cell Viability with 100 µM Flavonoid for 72h.

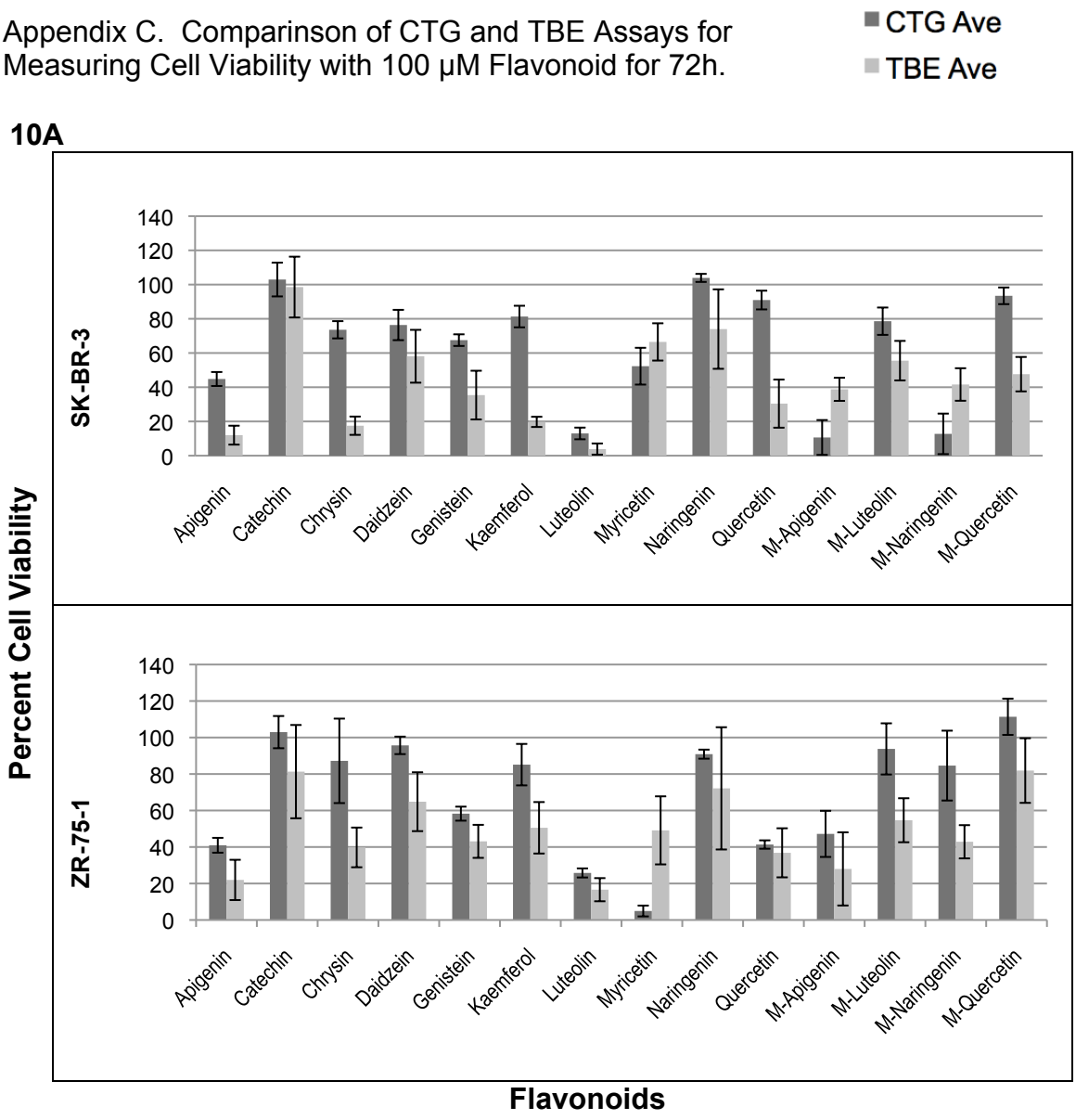
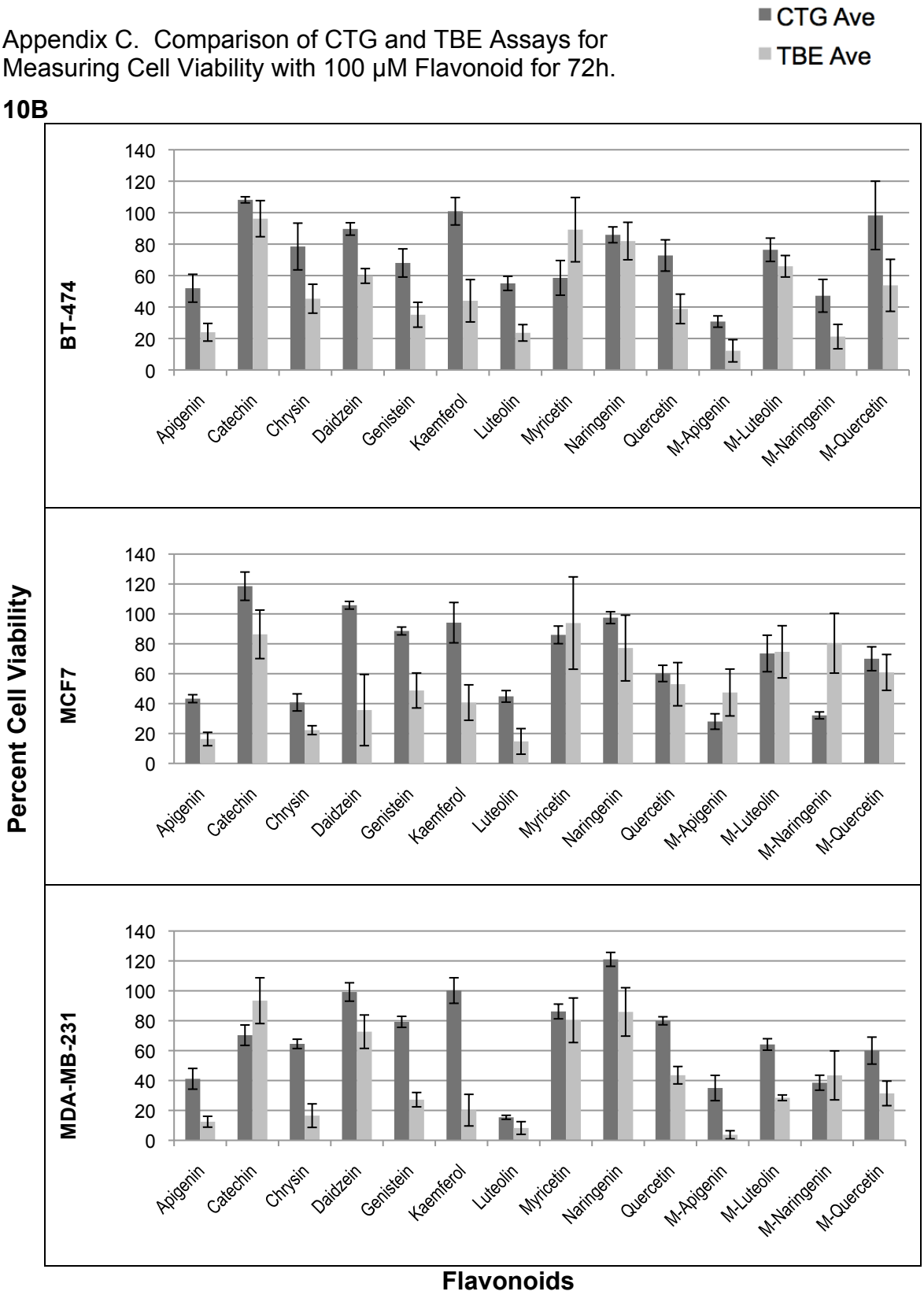


Figure 10. Bar graphs comparing cell viability between CTG and TBE assays were created for the cells treated with 100 µM after 72h, and standard deviations are indicated. Results shown are for all flavonoid treatments in SK-BR-3 and ZR-75-1 (A), and BT-474, MCF7, and MDA-MB-231 (B).

Appendix C. Comparison of CTG and TBE Assays for Measuring Cell Viability with 100 µM Flavonoid for 72h.

10B



Appendix D. Summary of Significant Differences Between Data for CTG and TBE Assays.

	BT-474	MCF-7	MDA-MB-231	SK-BR-3	Zr-75-1
Apigenin	200,100,50,25	200,100,50,25	50,25	200,100,50,25	25
M-apigenin	25	100,25	25	200	200,100,25
Catechin	200	ND	ND	ND	25
Chrysin	25	200,100,50,25	100,50,25	100,50,25	200,50,25
Daidzein	200,100,50	200,100,50	200,100,50	50	ND
Genistein	50	50	200,100,50	200,100,50,25	ND
Kaempherol	200,50,25	200,50,25	200,100,50,25	200,100,50,25	200,100,50,25
Luteolin	100,50,25	200,100,15	200,50,25	200,50,25	200,100
M-luteolin	ND	25	200,100,50,25	100,50	200,100
Myricetin	200	50,25	25	200	ND
Naringenin	ND	50	200,50,25	200,100,50	200,100,50,25
M-naringenin	ND	50	ND	200	200,100,50,25
Quercetin	100,50,25	50	200,100,50,25	200,100,50	ND
M-quercetin	ND	200,50	25	100	200,100

Table 12. Summary of statistical significant differences (p-values <0.05) noted between CTG and TBE assays. Results shown are after Hochberg multiple comparison adjustment. ND corresponds to no significant difference.