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Determining the Intracellular Concentrations of Flavonoids in MDA-MB-231 Cells Using HPLC-Coupled Mass Spectrometry

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DETERMINING THE INTRACELLULAR CONCENTRATIONS OF FLAVONOIDS IN MDA-MB-231 CELLS USING HPLC-COUPLED MASS SPECTROMETRY

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ABSTRACT

DETERMINING THE INTRACELLULAR CONCENTRATIONS OF FLAVONOIDS IN MDA-MB-231 CELLS USING HPLC-COUPLED MASS SPECTROMETRY

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Flavonoids are polyphenolic compounds found in a wide variety of plants. In recent years, flavonoids have been found to be beneficial to human health. Our lab has investigated the ability of flavonoids to induce cytotoxicity in human breast cancer cell lines. We have discovered that some compounds can induce cytotoxicity very readily, whereas others cannot. We hypothesized that this difference is attributable to a compound's ability to transverse the cell membrane. Therefore, we used LC-MS to measure the amount of each flavonoid that is able to enter into MDA-MB-231 cells, human breast cancer cells. We tested 14 flavonoids. MDA-MB-231 cells were plated and treated with 100 μM flavonoids for 24 and 48 hr. After treatment, cells were extracted using methanol. Flavonoids from extracts were characterized and quantitated by LC-MS. We found that intracellular flavonoid concentration increased over time for some of the flavonoids. We could not detect quercetin, myricetin, and catechin in cell extracts. However, quercetin was found to be a moderate inducer of cytotoxicity, whereas myricetin and catechin were not. We found that quercetin dimerized over time in the cell medium only in the presence of serum. The quercetin dimer could not be detected in cellular extracts, which suggested that it was unable to cross the membrane. Additionally, synthetic quercetin dimer did not induce cell death. Our results suggest that only fresh quercetin killed cells; once quercetin dimerized, it could not induce cell death.

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Lastly, I would like to thank my family for supporting me throughout my life. Their love provided my inspiration and was my driving force. I own them everything and wish I could show them how much I love and appreciate them.

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List of Abbreviations

ACN: acetonitrile

Api: apigenin

ATP: adenosine triposphate

BSA: bovine serum albumin

Cat: catechin

Chrys: chrysin

Daid: daidzein

DMF: dimethoxyflavone

DMEM: Dulbecco's modification of Eagle's medium

DMSO: dimethyl sulfoxide

EGFR: epidermal growth factor receptor

ESI: electrospray ionization

FAK: focal adhesion kinase

FBS: fetal bovine serum

HBSS: Hanks's buffered salt solution

HPLC: high performance liquid chromatography

IC: inhibitory concentration

Kaemp: kaempferol

LC: liquid chromatography

Lut: luteolin

M-Api: methyl-apigenin

M-Lut: methyl-luteolin

M-Nar: methyl-naringenin

M-Quer: methyl-quercetin

MeOH: methanol

MMP: matrix metalloproteinase

MS: mass spectrometry

Myr: myricetin

m/z: mass per charge

Nar: naringenin

NMR: nuclear magnetic resonance

PARP: poly ADP ribose polymerase

PBS: phosphate buffered saline

PKC: protein kinase C

POD: peroxidase

PPO: polyphenol oxidase

PSIG: pound per square inch

Q-3-O-GP: quercetin-3-O-glucopyranoside

Quer: Quercetin

RPM: revolution per minute

TGF: transforming growth factor

TLC: thin layer chromatography

TMF: trimethoxyflavone

TOF: time-of-flight

Introduction

Cancer is a disease in which cells grow out of control. These cells become cancerous due to damage of their DNA, which ultimately leads to mutations. They continue to reproduce, forming new abnormal cells. Cancer cells also have the ability to invade other tissues in the body. In the United States, one in eight women will develop invasive breast cancer in the course of her lifetime. Breast cancer death rates are second highest, just below lung cancer. Currently, there are multiple treatments for cancers including surgery, radiation therapy, chemotherapy, and complementary medicine. However, these therapies do not guarantee a cure and can have many negative side effects (http://www.breastcancer.org).

Flavonoids are compounds originally identified in plants. The consumption of fruits and vegetables introduces flavonoids into the human body. To date, over 4,000 different flavonoids have been identified (Ramos, 2007). Studies show that intake of foods containing flavonoids reduces the risk of cancer. They are responsible for numerous biological activities including inhibition of cell growth, inhibition of protein kinase activity, inhibition of apoptosis, inhibition of MMP secretion, inhibition of tumor cell invasion, and inhibition of adhesion and spreading of cells; flavonoids also have anti-angiogenic properties (Kanadaswami et al., 2005).

Our group has been studying the ability of flavonoids to induce cell death in a variety of breast cancer cell lines. The results show that not all are successful. The cytotoxicity results vary for each flavonoid. We hypothesized that this difference can be explained by the amount of flavonoid that permeates the cell.

In this study, we used liquid chromatography coupled with mass spectrometry to determine the amount of flavonoid within cells after an incubation period. With the resultant data, we correlated intracellular concentration of flavonoids and cytotoxicity after incubation.

Literature Review

Flavonoids: Structure

Found in a wide distribution of plants, flavonoids are polyphenolic compounds that perform multiple functions. They are considered members of secondary metabolites that are not directly responsible for growth, photosynthesis, reproduction, or any other primary function of plants (Ramos, 2007). They play a role in plant pigmentation and provide resistance to bacteria, fungi, viruses, and insects; they also provide tolerance to freezing temperatures, salt, and metals (Cohen, Sakihama, & Yamasaki, 2001). In recent years, flavonoids have been found to be beneficial to human health because of their anti-viral, anti-allergic, anti-platelet, antioxidant, and anti-tumor properties (Ramos, 2007). To date, over 4,000 flavonoids have been identified. They all have the similar general structural of diphenylpropanes, C_6-C_3-C_6, two benzene rings joined together by a three-carbon chain with an oxygen bridge (Fig. 1). Flavonoids are distinguished from one another by the presence of hydroxyl and methyl groups at various carbons (Fig. 2). Although these compounds are closely related to each other structurally, they could have different biological effects.

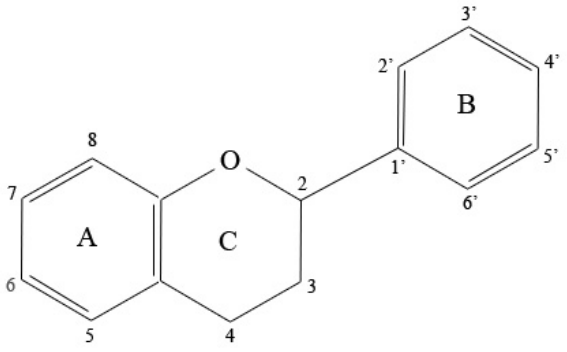


Fig. 1. General structure and numbering system of flavonoid.

Flavonoids are divided into different classes based on their molecular structures. The major groups are flavones, flavonols, flavanones, flavanols, and isoflavones (Manach, Williamson, Morand, Scalbert, & Rememsy, 2005). Representatives of the flavones group include apigenin, methyl-apigenin, chrysin, luteolin, and methyl-luteolin. Flavones are mainly found in herbs such as celery and parsley. Compounds in this group have a backbone structure of 2-phenyl-1-benzopyran-4-one.

Flavonols are the largest flavonoid group and include quercetin, methyl-quercetin, myricetin, and kaempferol. The backbone structure of flavonols is 3-hydroxy-2-phenylchromen-4-one. They can be found in onions, apples, tomatoes, tea, and red wine. This group has been extensively studied because of its prevalence in the human diet (Manach et al., 2005).

The representative flavonoids in the flavanones group are naringenin and methyl-naringenin. This group has a backbone structure of 2,3-dihydro-2-phenyl-4H-1-benzopyran-4-one. Naringenin and methyl-naringenin can be found in citrus fruits such as oranges and grapefruits. Catechin, a flavonoid in the flavanol group, is abundant in chocolate, beans, apples, tea, and red wine. Flavanols have a 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton. This structure has two chiral carbons, allowing each flavanol to have four different diastereoisomers (Manach et al., 2005).

Isoflavones can be found only in soybean-derived products. Examples include daidzein and genistein. The backbone structure for these isoflavones is 3-phenyl-4H-1-benzopyr-4-one. This class is distinct from the other classes because the attachment of the B ring is at carbon number 3 instead of 2 on the carbon bridge (Manach et al., 2005).

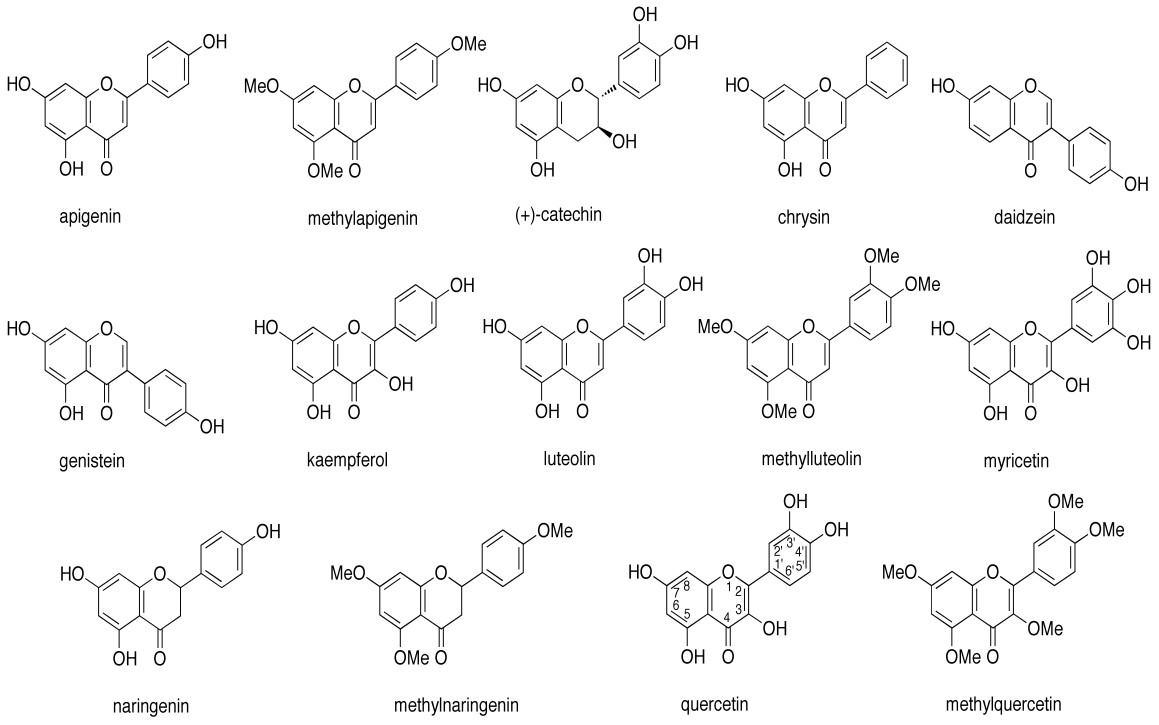


Fig. 2. Structures of flavonoids used in this study. The flavonoids may be hydroxylated at position 3, 5, 7, 3', 4', and/or 5'. Carbon numbering is indicated on quercetin.

Flavonoids without sugar molecules attached are called aglycones. Flavonoids with one or more sugar molecules, where attached hydroxyl groups are replaced with glycosides, are called flavonoid glycosides. Most of the flavonoids found in natural products have a sugar molecule attached with the exception of catechin (Manach et al., 2005). The attachment of glycosides plays an important role in the bioavailability of flavonoids to the human body (Hollman, de Vries, van Lee-uwen, Mengelers, & Katan 1995). Aside from glycosides, flavonoid hydroxyl groups can be capped with methyl groups; these flavonoids are called O-methylated flavonoids or methoxyflavonoids. The attachment of methyl groups can also play an important role in flavonoid cell permeability, solubility, and anticancer properties (Walle, 2004).

Bioavailability of Flavonoids

Initially, research presupposed that the absorption of flavonoids into the human body through food intake was negligible because most are attached to sugar. Flavonoid aglycones were thought to be the only kind that could pass through the wall of the gut; there were no known enzymes in the gut that could cleave the glycoside bonds (Kuhnau, 1976). However, more recent studies show that the absorption of quercetin glycosides from onions is higher than the absorption of quercetin aglycone *in vivo*, 52% and 24% for quercetin glycosides and quercetin aglycone, respectively (Hollman et al., 1995). This suggests that the glycoside moiety enhances absorption. A study from Day et al. (1998) showed that the absorption of quercetin aglycone from tea is half that of the absorption of quercetin glycosides found in onions.

Flavonoids in the diet are absorbed from the gastrointestinal tract (Crespy et al., 1999). Medical flavonoids for cancer treatment can be directly injected into tissue such as the skin, lungs, throat, and the vascular system (Ross & Kasum, 2002). Within the intestine, bacterial enzymes remove the glycosides from aglycone, and only 15% of flavonoid aglycones are absorbed into epithelial cells and carried to the lymph (Day et al., 1998; Spencer et al., 1999). Flavonoids are absorbed into cells by passive diffusion, or by the $Na^{\pm}$ dependent transport ATPase (Sharma, Kaushal, Sareen, Singh, & Bhatia, 1981). Then, the lymph carrying flavonoids enters the blood stream near the liver. Eighty percent of flavonoids are absorbed by the liver because parts of them are attached to the serum albumin (Casley-Smith & Casley-Smith, 1986; Podhajcer, Friedlander, &Graziani, 1980). Within the liver, flavonoids travel to the Golgi apparatus to be

degraded oxidatively (Griffiths & Smith, 1972). However, some have already undergone oxidative degradation in the intestine by bacterial enzymes (Winter, Moore, Dowell, & Bokkenheuser, 1989). The products are then released by organic acid transporters into the blood (Graefe, Derendorf, & Veit, 1999). Non-absorbed flavonoids leave the body through feces and urine (Choudhury, Srai, Debnam, & Rice-Evans, 1999). The half-life of flavonoids in the body has been estimated to be between 1 and 2 hr. However, there are not enough data to support this estimate (Honcha, Platte, Oesch, & Friedberg, 1995). The rate of flavonoid absorption and bioprocessing is still not very well characterized (Havsteen, 2005). Further studies are required to fully understand the bioavailability of flavonoids.

Flavonoids can be absorbed into systemic circulation; however, the concentration *in vivo* is not high enough to fully perform their cellular biological functions. The main reason for low oral bioavailability of flavonoids is the conjugation of the free hydroxyl group (Walle, 2004). Therefore, the methyl groups that cap the free hydroxyl groups might play an important role in flavonoid absorption. Absorption of three unmethylated versus methylated flavones in Caco-2 cells was observed by Wen & Walle (2006). Methylated compounds permeated cells more extensively than unmethylated compounds. Wen et al. attribute this property to higher metabolic stability of methylated compounds. The bioavailability between chrysin and 5,7-dimethoxyflavone (5,7-DMF), a similar structure to chrysin except for the methyl groups on carbons 5 and 7, was observed *in vivo* in rats. After 1 hr, only 5,7-DMF was detected in the liver, lungs, kidney tissue, and

plasma with peak concentration at 2.3 μM. Chrysin was not detected in any tissues after the first hour (Walle, Kawamori, Wen, Tsuji, & Walle, 2007).

The Anticancer Activities of Flavonoids

Many experimental studies have explored the effects of flavonoids on general health. Consumption of foods that contain flavonoids has been shown to reduce the risk of some diseases, such as cancer (Ramos, 2007). High soy diets rich in isoflavones inhibit tumor development in animal models (Watson, Jiyang, & Jones, 2000). Isoflavones, such as genistein, can also inhibit the promotion or progression of cancer by removing damaged or initiated cells. Flavones have been shown to reduce human colon carcinoma cell line proliferation (Wenzel, Kuntz, & Brendel, 2000). Flavonols such as quercetin and kaempferol can decrease lipogenesis in prostate and breast cancer cells (Brusselmans, Vrolix, Verhoeven, & Swinnen, 2004). Phenolic compounds have been demonstrated to induce apoptosis (Ramos, Alia, Bravo, & Goya, 2005). However, these biological activities are still not very well characterized.

Flavonoid anticancer activities include inhibition of cell growth, inhibition of protein kinase activities, inhibition of apoptosis, inhibition of MMP secretion, inhibition of tumor cell invasion, and inhibition of adhesion and spreading of cells; flavonoids also have anti-angiogenic properties (Kanadaswami et al., 2005). Protein kinases are required for the proliferation of cancer cells. Flavonoids have been reported to inhibit three major kinases: protein kinase C (PKC), epidermal growth factor receptor (EGFR), and focal adhesion kinase (FAK). Quercetin is the most efficient inhibitor of PKC, and PKC plays a role in cell multiplication, apoptosis, and transformation (Gamet-Payrastre et al., 1999).

Quercetin and luteolin have been demonstrated to be the most active inhibitors of partially purified rat brain PKC. Luteolin competitively blocks the ATP binding site on the catalytic unit of PKC (Ferriola, Cody, & Middleton, 1989). EGFR tyrosine kinase is a cell surface glycoprotein that binds EGF, and EGF stimulates the growth of tumor cells (Carpenter & Cohen, 1990). If the amount of EGFR tyrosine kinase activity was reduced, tumor cell growth would be inhibited (Lee et al., 2002). Genistein, luteolin, and quercetin have also been shown to suppress EGFR tyrosine kinase activity (Constantinou, Kiguchi, & Huberman, 1990; Lee et al., 2002). FAK is an important regulator of the cell-signaling pathway for cell survival, cell cycle, and cell mobility (Schlaepfer, Hauck, & Sieg, 1999). In tumor cells, FAK is overexpressed; inactivation of FAK suppresses cell proliferation and mobility. Tumor cells treated with luteolin and quercetin exhibit a reduction of FAK expression (Lee et al., 2004).

Apoptosis, programed cell death, plays an important role in embryonic development, metamorphosis, hormone-dependent atrophy, and maintenance of tissue homeostasis (Kanadaswami et al., 2005). Apoptosis is one of the pathways used by antitumor agents to stop the growth of tumor cells. Flavonoids have been shown to induce apoptosis. Moreover, genistein, quercetin, and luteolin have been shown to induce apoptosis in the HL-60 cell line (Huang et al., 1999; Traganos, Ardelt, Halko, Bruno, & Darzynkiewicz, 1992; Wei et al., 1994). In leukemia HL-60 cells, genistein caused apoptosis within 8 hr. EGF, TGF-α, and basic fibroblast growth factor, which have the ability to suppress apoptosis, were completely blocked by genistein (Tilly, Billig, Kowalski, & Hsueh, 1992). Quercetin has the ability to induce apoptosis by

inhibiting the synthesis of heat shock protein (Wei et al., 1994). Quercetin and luteolin significantly induce apoptosis in MiaPaCa-2 cells by blocking the EGFR-signaling pathway (Kanadaswami et al., 2005). Huang et al. (1999) showed that quercetin and luteolin induced apoptosis in tumor cells such as A431, MiaPaCa-2, Hep G2, and MCF 7. At low concentrations, 15-25 μM, luteolin was able to slow the growth of A431 and MiaPaCa-2 by oligonucleosomal DNA cleavage, cytoplasmic blebbing, and PARP degradation.

The worst danger of cancer disease is that it has the ability to metastasize, spreading of cancer from one organ to another (Liotta, Steeg, & Stetler-Stevenson, 1991). The invasion of cancer cells correlates with the production of certain matrix metalloproteinases (MMPs) (Liabakk, Talbot, Smith, Wilkinson, & Balkwill, 1996). MMPs degrade the components of the extracellular matrix. Cancer cells require MMPs to degrade extracellular enzymes to cross the tissue barriers they encounter. Flavonoids have been studied as agents that down-regulate the biosynthesis of MMPs (Kim, 2003). Through the PKC pathway, quercetin was able to decrease pro-MMP-9 in a dose-dependent manner by blocking phorbol-12,13-diutyrate, a PKC activator (Zhang, Huang, & Xu, 2004). In A431 cells, both quercetin and luteolin were able to decrease the secretion of MMP-2 and MMP-9 (Huang et al., 1999). *In vitro*, MDA-MB-231 MMP-9 activity was down-regulated by genistein; therefore, the invasion of MDA-MB-231 was inhibited (Magee, McGlynn, & Rowland, 2004). Apigenin, quercetin, and luteolin have shown the ability to inhibit MMP-2 and MMP-9. Moreover, flavonoids with more

hydroxyl groups or glucoside groups inhibited the effect of MMP-2 and MMP-9 at a higher rate (Ende & Gebhardt, 2004).

Cellular Uptake of Flavonoids

The uptake of flavonoids into cells may help determine their biological action. The rate of flavonoid uptake *in vivo* is dependent on cell type, intracellular metabolism, and rate of export (Spencer, Mohsen, & Rice-Evans, 2004). A precise measurement of flavonoid uptake by cells is very hard to determine. Flavonoids can either enter a cell or interact with extracellular receptors. Current extraction protocols cannot precisely measure the amount of flavonoid within the cytosol or on the cell membrane because the protocols cannot differentiate between these two possibilities (Spencer et al., 2004).

Various flavonoids were observed for the degree of cellular uptake in Caco-2 cell monolayers in HBSS and DMEM. Cells were treated with 50 μM flavonoids including myricitrin, rutin, isoquercitrin, quercetin, myricetin, kaempferol, luteolin, and apigenin for 2 hr. Viable cells were extracted by methanol, and the flavonoids in cellular extracts were quantified by HPLC. The degree of flavonoid uptake was different between media used for cell incubation. In HBSS, the order of uptake was quercetin = kaempferol = luteolin > apigenin. In DMEM, the order of uptake was kaempferol > luteolin = apigenin > quercetin. Myricitrin, rutin, isoquercitrin, and myricetin were not detected in cell extracts. Cells did not absorb glycosides such as myricitrin, rutin, and isoquercitrin. The authors suggest that glycosides must be metabolized to be sufficiently absorbed by cells. Myricetin, an aglycone, was not detected in cell extracts; this might have been because of its instability as it rapidly degraded in both media. The degree of flavonoid uptake was

dependent on both incubation time and concentration in both media. Flavonoids were incorporated into cells rapidly in the first 30 min after incubation; the incorporating rate was then reduced. The degree of flavonoid uptake in HBSS was approximately four times higher than in DMEM (Yokomizo & Moriwaki, 2006).

In addition, Caco-2 cells were able to uptake epicatechin, epigallocatechin gallate, and quercetin-3-glucoside after 24 hr. Studies with epicatechin showed that 24 hr after incubation, 0.43 ng/10^6 cells was detected, and the uptake increased to 3.5 ng/10^6 cells at 72 hr. The uptake of epicatechin increased over incubation time; however, epigallocatechin reached the maximum level at 48 hr. Meanwhile, quercetin-3-glucoside was not detected after a 72-hr incubation. The authors proposed that cells did not take up quercetin-3-glucoside because it was more polar (Salucci, Stivala, Maiani, Bugianesi, & Vannini, 2002).

Another case of quercetin uptake was investigated in the human hepatocarcinoma cell line, HepG2. These cells accumulated quercetin 9.6-fold and exhibited the formation of a 3'-O-methylated metabolite. However, after 8 hr, quercetin was no longer detected. The authors suggest that quercetin was extensively metabolized (Watanabe et al., 2001).

The rate of flavonoid cell uptake *in vitro* may not be similar to uptake *in vivo*. *In vitro*, cells grow in a monolayer form; as such, they can be exposed more to the flavonoids in the cell culture medium. *In vivo*, the amount of flavonoid exposed to cells might not be as high as *in vitro* because of tight growing surface in the cellular environment (Spencer et al., 2004).

Flavonoid Oxidation

In plant tissue, quercetin can undergo oxidation by oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase (POD) to form three major oxidative products (Gulsen et al., 2007). The same oxidation can be generated *in vitro* with potassium ferricyanide under alkaline conditions (Makris & Rossiter, 2002). Under these conditions, a high amount of oxidation product was generated, forming a quercetin dimer. This oxidation product was analyzed under positive ion mode LC-MS and has an m/z of 603 $[M + H]^+$. A possible mechanism proposed for quercetin oxidation is shown in Figure 3. The quercetin oxidation by potassium ferricyanide generated the same set of products compared with the oxidation of quercetin by mushroom PPO and horseradish POD. During the oxidation process of several flavonoids, only the B ring was affected. If the hydroxyl groups on the B ring were methylated, there was no reaction (Fig. 3). *In vitro*, flavonoids readily undergo oxidation in cell culture medium. However, *in vivo*, oxidation of flavonoids may be limited because of low oxygen tension in the cellular environment (Pelter, Bradshaw, & Warren, 1971).

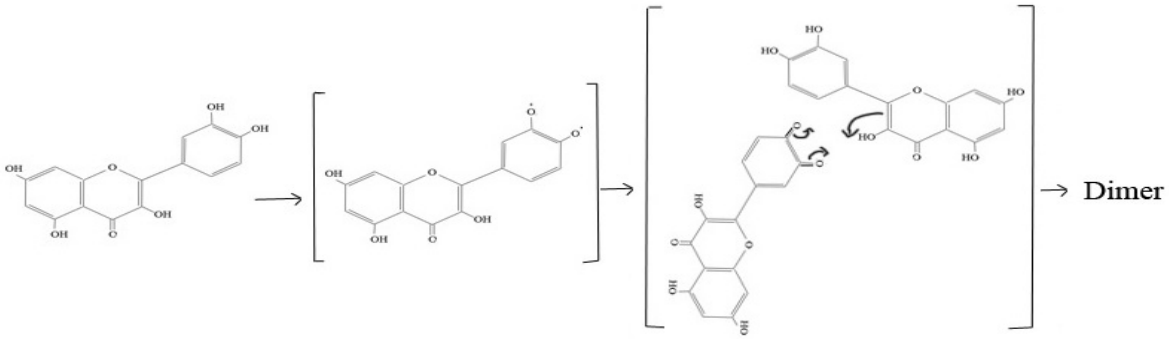


Fig. 3 Proposed mechanism of quercetin oxidation. During the oxidation process, only the hydroxyl groups on B ring are involved. If the hydroxyl groups are methylated, oxidation cannot proceed (Krishnamachari, Levine, & Pare, 2002).

Liquid Chromatography and Mass Spectrometry in Flavonoid Studies

Mass spectrometry is useful in the detection of compounds in a solution. It is a very powerful tool that has high sensitivity and provides accurate molecular mass (Cuyckens & Claeys, 2003). A German physicist, Wilhelm Wien, developed the foundation of mass spectrometry in 1899. He constructed an instrument that could separate compounds based on their charge-to-mass ratio using electric and magnetic fields (de Hoffmann & Stroobant, 2004). In 1912, a British physicist, Sir Joseph John Thomson, modified Wien's foundation and created one of the first mass spectrometry instruments to separate neon isotopes by their masses. Recently, mass spectrometry has become popular in biological applications. More mass spectrometry invention and improvements have been acknowledged in the science field. In 2002, John Bennett Fenn received the Nobel Prize in Chemistry for his development of electrospray ionization (ESI). In the same year, Koichi Tanaka received the Nobel Prize in Chemistry for the development of soft laser desorption.

In this thesis, the data were acquired from liquid chromatography-mass spectrometry (LC-MS). Liquid chromatography coupled with mass spectrometry has been a popular method used to study these small molecules because of its sensitivity and accuracy (Plazonic et al., 2009). It is a process that involves compounds that are dissolved in solution (mobile phase) that are passed through a stationary phase which seperates the compounds based upon a particular property. The stationary phase we have used is known as a reverse-phase which separates compounds based upon polarity. Flavonoids have different affinities to reverse-phase columns based on their polarity.

More polar flavonoids elute earlier than less polar ones. Because most have different polarities, they can be separated for analysis by a mass spectrometer (Fig. 4). After chromatography separation, the molecules are introduced directly into a mass spectrometer, a tool that consists of an ion source, mass analyzer, and a detector.

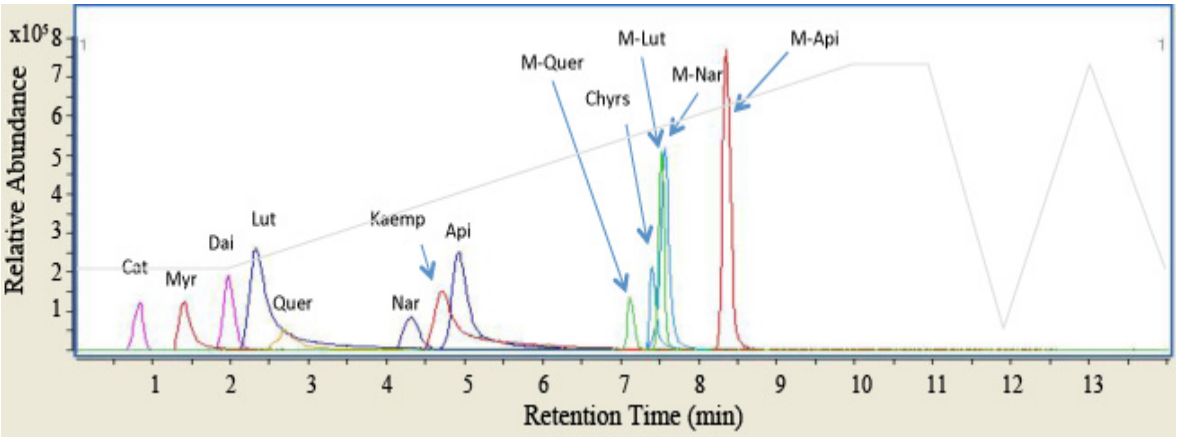


Fig. 4. Flavonoids separation by liquid chromatography. X axis: retention time (min), Y axis: relative abundance. See method section for mobile and stationary phase. Cat: catechin, Myr: myricetin, Dai: Daidzein, Lut: luteolin, Quer: quercetin, Nar: naringenin, Kaemp: kaempferol, Api: apigenin, M-Quer: methyl-quercetin, Chrys: chrysin, M-Lut: methyl-luteolin, M-Nar: methyl-naringenin, M-Api: methyl apigenin.

Electrospray ionization and matrix-assisted laser desorption/ionization are the two most common ion sources. In this project, electrospray ionization was the choice for the ion source. The sample from the liquid chromatography, which contains the molecule(s) of interest, is in liquid form. It is then sprayed through a small capillary into a strong electric field, which adds a charge to each droplet. The charged droplets are sampled into the vacuum stage of the instrument. The drying gas is used to evaporate the solvent from these droplets to create lone ions (Fenn, Mann, Meng, Wong, & Whitehouse, 1989). ESI can apply either a positive or negative charge on the droplet. For positive-ion mode,

formic acid is usually added to the solvent to enhance protonation. For negative-ion mode, ammonium formate is used to enhance deprotonation. Positive-ion mode was used in this project.

The mass analyzer is the second part of a mass spectrometer. Molecules will be measured by their mass-to-charge ratio. A good mass analyzer must be sensitive, possessing both mass resolution and mass accuracy (Aebersol & Mann, 2003). There are different types of mass analyzers such as time-of-flight (TOF), quadrupole, fourier transform ion cyclotron, and the orbitrap mass analyzer. In this project, we used TOF and quadrupole mass analyzers. The quadrupole is used as a filter to help sort different ions. It consists of four parallel rods to which voltage is applied. As charged molecules leave the ion source, they travel through these rods; only certain molecules will not collide with the rods and reach the other side. Depending on the voltage applied to the rods, molecules of a specific mass-to-charge ratio can be filtered. As the molecules leave the quadrupole mass analyzer, they enter the TOF mass analyzer. This analyzer uses an electrical field to accelerate these charged molecules through a path. It measures the mass of the molecules based on how long it takes for them to reach the detector. Molecules with larger mass reach the detector later than smaller ones.

As molecules leave the TOF mass analyzer they impact with a detector, a metal plate. Electrons on this plate will neutralize the charge of the molecules. The neutralization is detected and recorded for mass and abundance data.

The Agilent Technologies 6520 Q-TOF mass spectrometer can accurately detect masses of flavonoids up to four decimal places and has mass accuracy of two to five parts

per million. Most of the flavonoids tested have distinguishable masses (Table 1). Combining the specific retention time from the HPLC and the distinguished masses from the mass spectrometer, the amount of flavonoids can be detected and quantified in a solution. Mass spectrometry only gives a relative abundance of a flavonoid in a solution. It does not provide the exact concentration of the molecule. However, with a set of standards, flavonoids can be accurately quantified from a solution.

Table 1. Flavonoids with specific molecular formulas and masses.

Flavonoids	**Molecular Formula**	**Exact Mass (g/mol)**	**m/z [M+H]**
Apigenin	$C_{15}H_{10}O_5$	270.0528	271.0601
Methyl-Apigenin	$C_{18}H_{16}O_5$	312.0998	313.1071
Catechin	$C_{15}H_{14}O_6$	290.0790	291.0863
Chrysin	$C_{15}H_{10}O_4$	254.0579	255.0652
Daidzein	$C_{15}H_{10}O_4$	254.0579	255.0652
Genistein	$C_{15}H_{10}O_5$	270.0528	271.0601
Kaempferol	$C_{15}H_{10}O_6$	286.0477	287.0550
Luteolin	$C_{15}H_{10}O_6$	286.0477	287.0550
Methyl-Luteolin	$C_{19}H_{18}O_6$	324.1103	343.1176
Myricetin	$C_{15}H_{10}O_8$	318.0376	319.0448
Naringenin	$C_{15}H_{12}O_5$	272.0685	273.0757
Methyl-Naringenin	$C_{18}H_{18}O_5$	314.1154	315.1227
Quercetin	$C_{15}H_{10}O_7$	302.0427	303.0499
Methyl-Quercetin	$C_{20}H_{20}O_7$	372.1209	373.1282
Quer-3-O-GP	$C_{21}H_{20}O_{12}$	464.0954	465.1028

Flavonoids Induce MDA-MB-231 Cell Death

Our group has characterized the ability of a group of flavonoids to induce cytotoxicity in a variety of breast cancer cell lines. We determined the IC_{50} values for flavonoids after 72 hr of treatment and reported the results for the MDA-MB-231 cell line (Table 2). The results show that not all flavonoids are able to induce cytotoxicity. However, for those that are, the IC_{50} values vary (Yadegarynia et al., 2012).

Table 2. IC_{50} values for flavonoids after 72 hr incubation with MDA-MB-231 cells. Values are reported as μM. Numbers in parenthesis are the range for 95% confidence interval.

Flavonoid	IC50 (95% CI)
Apigenin	14 (11-19)
M-Apigenin	33 (27-40)
Catechin	>200
Chrysin	40 (35-45)
Daidzein	179 (144-222)
Genistein	51 (43-61)
Kaempferol	38 (31-47)
Luteolin	16 (14-20)
M-Luteolin	21 (12-38)
Myricetin	>200
Narigenin	>200
M-Narigenin	75 (59-96)
Quercetin	101 (82-124)
M-Quercetin	54 (39-75)

Method

Materials and Equipment

Flavonoids were purchased from Indofine Chemical Co. Stocks were prepared in 100% dimethyl sulfoxide (DMSO) at 50 mM and stored at -20^{o}C. MDA-MB-231 cells were purchased from American Type Culture Collection. Cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics, penicillin and streptomycin (referred to as complete medium), at 37^{o}C and 5% CO_2 in a humidified environment. DMEM with high glucose, L-glutamine, and sodium pyruvate were purchased from Cellgro. Antibodies and FBS were purchased from Hyclone.

HPLC-grade H_2O, acetonitrile (ACN), and methanol (MeOH) were purchased from Fisher Scientific. An Agilent Technologies 1100 HPLC was coupled to an Agilent Technologies Q-TOF LC-MS 6520 mass spectrometer. For data acquisition and analysis, the software packages used were Agilent Masshunter Workstation, Agilent Masshunter Qualitative, Agilent Masshunter Quantitative, and Agilent MassProfiler.

Cell Cytotoxicity of Flavonoids

MDA-MB-231 cells were plated at a density of 3 x 10^6 cells/plate in 10 cm dishes. After 24 hr, the medium was removed from the dishes. Cells were washed with 3 ml of phosphate buffered saline (PBS). New medium with 100 μM flavonoid was added to the dishes; DMSO was added as control. After 24 and 48 hr, the medium was removed from the dishes, and cells were washed with 3 ml of PBS. To remove the cells, 3 ml of trypsin was added, and cells were incubated for 2 min. Both trypsin and cells were

collected into 15 ml conical tubes. Another 7 ml of PBS was used to ensure the cells were completely washed from the plate. The washed cells were combined into conical tubes, which were then centrifuged at 1500 rpm for 5 min. Supernatants were removed. Cells were resuspended and mixed in 5 ml of PBS. To determine the cell viability, 10 μl of cells were mixed with 10 μl of Trypan Blue. Cell viability was determined using a Bio-Rad TC10 Automated Cell Counter. The viability percentages were normalized to the DMSO treatment.

Cell viability of fresh quercetin solution and 24 hr-old quercetin solution were compared. Cells were plated at 5 x 10^6 cells/dish in complete medium. For one set, cells were treated with new medium with fresh 100 μM quercetin. For the other set, cells were treated with 24 hr-old 100 μM quercetin medium. Cell viability was determined 24 hr after incubation. The viable percentages were normalized to the DMSO treatment. For control purposes, this experiment was repeated with kaempferol.

Cell viability of single-dose treatment and multiple-dose treatment of quercetin were compared. Cells were plated at 5 x 10^5 cells per well in six-well dishes in complete medium. For single-dose treatment, cells were treated once with 100 μM quercetin medium. For multiple-dose treatment, cells were treated three times with 100 μM quercetin medium. At hour 0, 6, and 12, old medium was removed, and new 100 μM quercetin medium was added. The cytotoxicity was determined at 72 hr after the initial incubation. The viable percentages were normalized to the DMSO treatment. For control purposes, this experiment was repeated with kaempferol.

Cellular Uptake of Flavonoids

After performing cell viability counts, the remaining cells were centrifuged at 1500 rpm for 5 min. Supernatants were removed. To extract flavonoids, 5 ml of 40% MeOH with 0.1% (v/v) formic acid was added to cells, which were mixed and placed on ice for 15 min. Cells were then centrifuged at 1500 rpm for 5 min. Supernatants were collected for LC-MS analysis. To ensure all flavonoids were extracted from cells, another 1 ml of 40% MeOH with 0.1% (v/v) formic acid was added to the cell pellet and resuspended, transferred into microcentrifuge tubes, and placed on ice for 15 min. Cells were centrifuged at 14,000 rpm for 5 min, and supernatants were collected for LC-MS analysis.

The extraction volume of 5 ml and 1 ml of 40% MeOH was enough to extract 99% of flavonoids from cells (Table 3). The first 5 ml of 40% MeOH extracted out 94.8% of the total flavonoids. The second 1 ml of 40% MeOH extracted out 4.4% of the total flavonoids. The third 1 ml of 40% MeOH extracted out 0.8% of the total flavonoids, which was determined to be negligible. Therefore, we chose the 5 ml and 1 ml of 40% MeOH for flavonoid extraction from viable cells.

Table 3. Methanol extraction volume. The first 5 ml of 40% MeOH extracted out 94.8%, and the next 1 ml of 40% MeOH extracted out 4.4% of total flavonoid extracted from viable cells.

5 ml + 1 ml of 40 % MeOH extraction

	Picomole per injection	picomole total	Percent
1st: 5 ml	1.914	9570	94.8%
2nd: 1 ml	0.44	440	4.4%
3rd: 1 ml	0.085	85	0.8%
Total	2.439	10095	100%

LC-MS Parameters

An Agilent Technologies 1100 HPLC was coupled to an Agilent Technologies Q-TOF LC-MS 6520 for data acquisition. Flavonoids were separated with a Cogent Bidentate C18 column, a reverse stationary phase with spherical shaped thermally treated Type C Silica (150 mm x 2.1 mm I.D., 4 μm particle size). The column was held at 30°C. A binary mobile phase system of solvent A (water and 0.1% (v/v) formic acid) and solvent B (acetonitrile with 0.1% (v/v) formic acid) with a flow rate of 0.5 ml/min provided the best separation using the following gradient: 30% B was held for 2 min; a linear ramp increased B to 95% from 2 to 10 min; 95% B was held until 11 min; a linear ramp decreased B to 5% from 11 to 12 min; from 12 to 13 min, B was increased to 95% then decreased to 30% at minute 14 (Fig. 5). The sample injection volume was 1 μl.

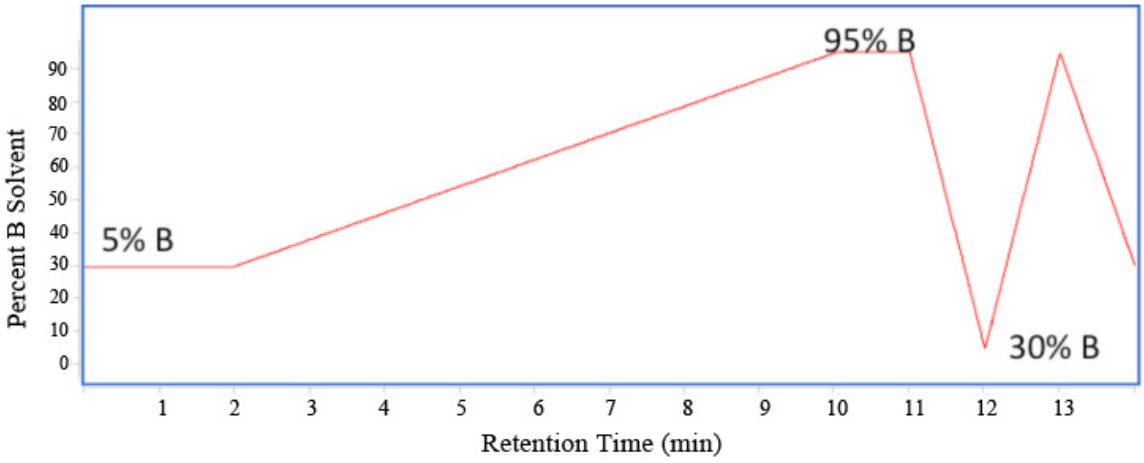


Fig. 5. The HPLC gradient of sample separation using Cogent Bidentate C18 column. A: Water and 0.1% formic acid, B: acetonitrile and 0.1% formic acid. 0-2 min: 30% B, 2-10 min: increased to 95% B, 11-12 min: decreased to 5% B, 12-13 min: increased to 95% B, 13-14 min: decreased to 30% B.

After separation, the compounds were ionized for mass spectrometry detection by positive ion electrospray ionization. The mass spectrometer was set with the following conditions: gas temperature, 350^{o}C; drying gas rate, 8 liters of liquid nitrogen/minute; nebulizer, 350 psig; fragmentor voltage, 150 V; skimmer voltage, 65 V; acquisition rate, 1.03 spectra/s.

Flavonoid Standard Curve Preparation

The mass spectrometer provided only the relative abundance of the flavonoid detected in the extracts, not the exact concentration. To determine the concentration of flavonoid detected from the extracts, we created standard curves for each individual flavonoid.

Each purified flavonoid was prepared in a series of dilutions: 75 μM, 50 μM, 10 μM, 1 μM, and 0.1 μM in 40% MeOH. To make the 75 μM standard, 7.5 μl was drawn from a 50 mM stock flavonoid solution and mixed with 5000 μl of 40% MeOH. From the 75 μM standard, 1000 μl was drawn and mixed with 500 μl of 40% MeOH to make a 50 μM standard. Next, 200 μl was drawn from the 50 μM standard and mixed with 800 μl of 40% MeOH to make a 10 μM standard. Next, 100 μl was drawn from the 10 μM standard and mixed with 900 μl 40% MeOH to make a 1 μM standard. Lastly, 100 μl was drawn from the 1 μM standard and mixed with 900 μl 40% MeOH to make a 0.1 μM standard. From each standard, 1 μl was injected onto the LC-MS to obtain the abundances detected for 75, 50, 10, 1, and 0.1 pmol of flavonoid. An equation in a form of $y = mx + b$ was obtained using Agilent Masshunter Quantitative software, where the y

axis represented the number of responses from the abundances of the standards, and the x axis represented the pmol in the standards (Fig. 6).

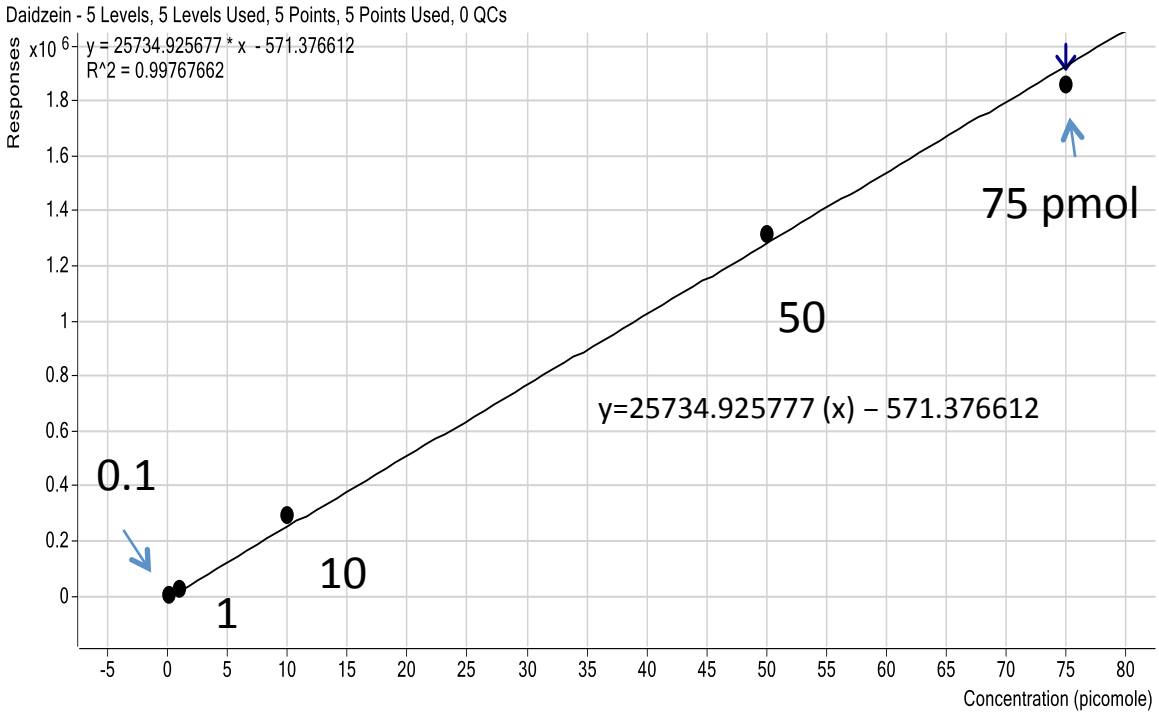


Fig. 6. An example of flavonoid standard curve. Different concentrations of daidzein were used to generate the standard curve; y axis: responses from the abundance of the standards; x axis: pmol of the standards. Y = 25734.925777 x – 571.376612.

Flavonoid Concentration Calculation From Cell Extract

After injecting the cell extracts onto the LC-MS, the data obtained were the number of responses detected from the abundance of the flavonoid within cells. The Agilent Masshunter Quanttative software used the y = mx + b from the standard curve to determine the exact concentration of flavonoid in the cell extract (Table 4).

Table 4. An example of data collected from daidzein treatment at 48 hr. The first column represents the names of the sample. The RT column represents the retention times of the flavonoid. The Resp. column represents the abundances detected. The Calc. conc. column represents the final concentrations of the flavonoids detected.

				Daidzein Method	Daidzein Results			
Name	Data File	Type	Level	Exp. Conc.	RT	Resp.	Calc. Conc.	Accuracy
Dai 0.1	Daid 0.1uM C18 062411.d	Cal	1	0.1	2.047	1399.0627	0.09968589	99.68589
Dai 1	Daid 1uM C18 062411.d	Cal	2	1	1.962	28092.613	0.99992733	99.99273
Dai 10	Daid 10uM C18 062411.d	Cal	3	10	1.994	290503.92	10.048271	100.4827
Dai 50	Daid 50uM C18 062411.d	Cal	4	50	1.984	1317608.7	49.8477416	99.69548
Dai 75	Daid 75uM C18 062411.d	Cal	5	75	1.973	1862062.2	75.1090788	100.1454
DMSO	DMSO 48hr 062311.d	Sample			1.428	209.04212	0.05963543	
3a2	D100 3A2 48hr 062311.d	Sample			2.102	2136.0161	0.1244918	
3b2	D100 3B2 48hr 062311.d	Sample			2.075	3172.6149	0.15938838	
3c2	D100 3C2 48hr 062311.d	Sample			2.169	2401.1256	0.10341607	
3a1	D100 3A1 48hr 062311.d	Sample			1.951	7875.2618	0.317768	
3b1	D100 3B1 48hr 062311.d	Sample			1.969	9925.052	0.38683713	
3c1	D100 3C1 48hr 062311.d	Sample			2.035	8167.2828	0.3395642	

Table 4 is an example of how the flavonoid concentration was calculated. The first five rows are the data for the standard samples. The next seven rows are the data for the cell extracts. Data were obtained in triplicate. Column 1 shows the names of the samples. The data from samples 3a1, 3b1, and 3c1 are from the first extraction (5 ml of 40% MeOH) from three different plates. The data from samples 3a2, 3b2, and 3c2 are from the second extraction (1 ml of 40% MeOH). This was described in the cell extract methodology above.

In the first extraction from sample 3c, after 1 μl of sample was injected, 0.3395642 pmol of daidzein was detected. The total extraction volume for the first extraction of 3c sample was 5000 μl. Therefore, the total pmol extracted from the first extraction was 0.3395642 pmol x 5000 μl = 1697.821 pmol. In the second extraction

from sample 3c, after 1 μl of sample was injected, 0.1034607 pmol of daidzein was detected. The total extraction volume for the second extraction of 3c was 1000 μl. Therefore, the total pmol extracted from the second extraction was 0.1034607 pmol x 1000 μl = 103.41607 pmol. After two extractions, the total pmol extracted from viable cells were 1697.821 + 103.41607 = 1801.23707 pmol. In this experiment, the total number of viable cells at 48 hr after treatment with daidzein 100 μM was 7,100,000 cells. Therefore, the amount of daidzein extracted from one viable cell was 1801.23707 pmol/7,100,000 cells = 2.537×10^{-4} pmol/cell.

Similar calculations were performed for all other flavonoids in this study. The average and standard deviations were taken from the triplicate.

Flavonoid Stability in Complete Medium

A 100 μM flavonoid solution was prepared in 5 ml of complete medium. The medium with flavonoid was incubated at 37°C and 5% CO_2 in a humidified environment. At both time points, 0 and 24 hr, the medium was mixed and 600 μl was drawn into a microcentrifuge tube. The medium was then mixed with 400 μl of 100% MeOH. The sample was mixed and placed on ice for 15 min, then centrifuged at 14,000 rpm for 10 min to pellet precipitated salt and protein. Supernatant was collected and 1 μl was injected into LC-MS for analysis.

Detection of Quercetin Dimer using Agilent MassProfiler Software

Quercetin was prepared in complete medium at the concentration of 100 μM and incubated in the cell-tissue-culture incubator. Quercetin was extracted in 40% MeOH at 0, 6, and 24 hr. At each time point, the sample was mixed and placed on ice for 15 min.

The sample was centrifuged at 14,000 rpm for 10 min to remove precipitated salts and proteins. Supernatant was collected and 1 μl was injected into LC-MS for analysis. Agilent MassProfiler software was used to detect any changes in mass and abundance from different time points.

Detection of Quercetin Dimer in the Presence of Fetal Bovine Serum

Two solutions were used to monitor quercetin dimerization, PBS with 10% (v/v) normal FBS and PBS with 10% (v/v) boiled FBS. For PBS with 10% normal FBS, 1 ml of FBS was added to 9 ml of PBS, and quercetin was added to 100 μM. For PBS with 10% boiled FBS, 2 ml of FBS were first boiled for 10 min, then, 1 ml of boiled FBS was added to 9 ml of PBS and quercetin was added to 100 μM. Both solutions were incubated at 37^{o}C, 5% CO_2 in a humidified environment. Samples were extracted at 0 and 24 hr in 40% MeOH after incubation. The samples were mixed and placed on ice for 15 min. They were then centrifuged at 14,000 rpm for 10 min to pellet precipitated salts and proteins. Supernatants were collected and 1 μl of each sample was injected into LC-MS for analysis. Quercetin and quercetin dimer, M+H 303.0499 m/z and 603.0769 m/z, respectively, were measured for analysis at both 0 and 24 hr.

Detection of Quercetin Dimer in the Presence of BSA and Actin

Quercetin was mixed in PBS with two different proteins, BSA and actin. For negative control, quercetin was mixed in 1000 μl of PBS to 100 μM. For PBS with BSA, 2.5 mg of BSA were mixed with 1000 μl PBS and quercetin was added to 100 μM. For PBS with actin, 2.5 mg of actin were mixed with 1000 μl PBS and quercetin was added to 100 μM. Three different sets of tubes were prepared; each was incubated at a different

temperature: 22°C, 4°C, and 37°C. At each time point, hour 0, 6, and 24, 200 μL of sample were drawn into microcentrifuge tubes and 150 μl of 100% MeOH were added to achieve 42% MeOH in the final solution. The tubes were mixed and placed on ice for 15 min. The tubes were centrifuged at 14,000 rpm for 10 min. Supernatants were collected and injected into LC-MS for analysis.

Quercetin Dimer Synthesis and Isolation

Quercetin dimer can be synthesized from quercetin oxidation with potassium ferricyanide (Gulsen et al., 2006). Three hundred forty milligrams of quercetin were mixed with 80 ml of acetonitrile. At room temperature, a 20 ml solution of 50 mM potassium ferricyanide and 50 mM sodium carbonate was added dropwise over 30 min under continuous stirring. The potassium ferricyanide, sodium carbonate, and quercetin final concentration was 10 mM. The solution was stirred for another 3 hr in the dark. Concentrated hydrochloric acid was used to adjust the pH to 2. The solution was concentrated under vacuum until all of the acetonitrile was removed. Forty milliliters of ethyl acetate:diethyl either (8:2 v/v) solution were added to extract the solution. The solution was extracted four times. Water was removed by adding magnesium sulphate; the solution was then filtered and evaporated by rotary evaporation. The final product was verified using silica thin layer chromatography (TLC) and mass spectrometry to detect the presence of quercetin dimer.

The solvent for TLC was methanol:dichloromethane (1:9 v/v). The TLC's zone with quercetin dimer product was isolated and eluted out with 5 ml of

methanol:dichloromethane (2:8 v/v). The solution was concentrated under vacuum. NMR and mass spectrometry verified the final quercetin dimer solution.

Statistical Analysis

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 $p<0.05$ was regarded as statistically significant.

Results

Flavonoids Cytotoxicity on MDA-MB-231 Cells after 24 and 48 hr

Fourteen different flavonoids were used at 100 μM (luteolin was used at 50 μM) on MDA-MB-231 cells, and cell viability was determined after 24 and 48 hr. Cell viability percentages were normalized to the DMSO treatment. This viability decreased with time for all flavonoids except for catechin, myricetin, and m-naringenin. Kaempferol and luteolin had the strongest effect in decreasing cell viability (Fig. 7). These results are consistent with those of Sina Yadegarynia (2012), which reported cell viability for 72 hr.

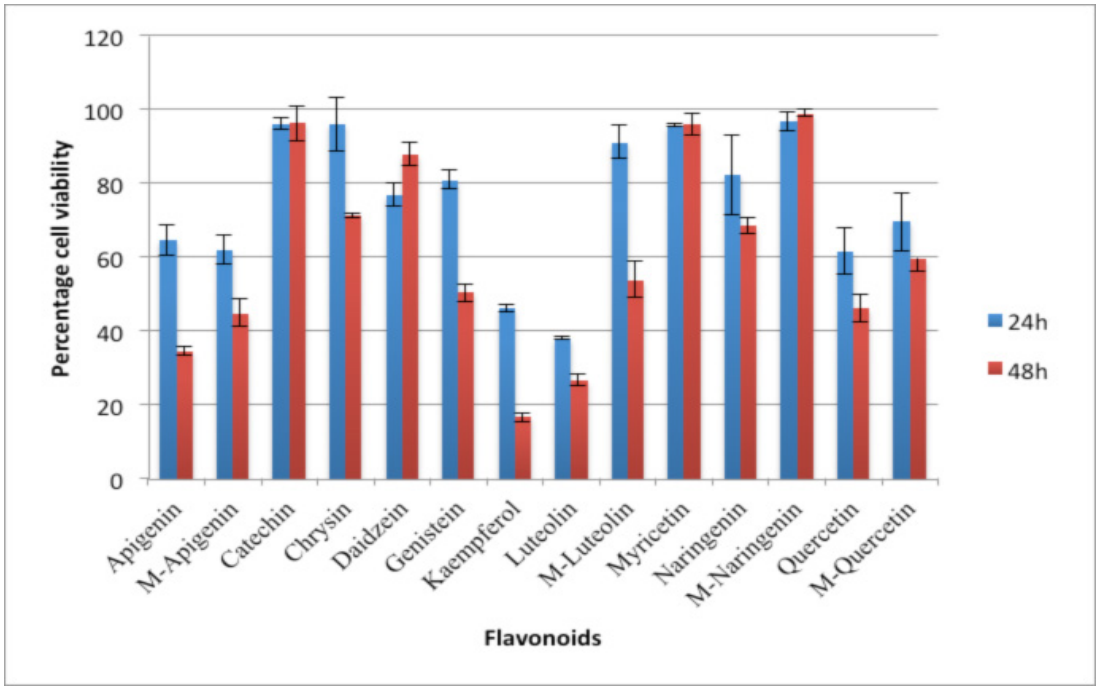


Fig. 7. Cell viabilities at 24 and 48 hr after treated with flavonoids at a concentration of 100 μM. Except for luteolin, the concentration was 50 μM.

We grouped the flavonoids based on their ability to decrease cell viability into three categories. Cell viability that was less than 50% was considered a strong killer; cell

viability ranging from 50% to 75% was considered a moderate killer; and cell viability that was more than 75% was considered a weak killer. Kaempferol and luteolin were most effective at inducing cell death. At 24 hr, cell viabilities were 46.1% and 37.9% for kaempferol and luteolin, respectively. At 48 hr, cell viable percentages were 16.6% and 26.4% for kaempferol and luteolin, respectively. Apigenin, methyl-apigenin, and quercetin were moderate killers at 24 hr, but strong killers at 48 hr. At 24 hr, the cell viabilities were 64.6%, 61.8%, and 61.3% for apigenin, methyl-apigenin, and quercetin, respectively. At 48 hr, the cell viabilities were 34.3%, 44.6%, and 46.1% for apigenin, methyl-apigenin, and quercetin, respectively. Methyl-quercetin was a moderate killer at both 24 and 48 hr. The cell viabilities for methyl-quercetin were 69.5% and 59.6% at 24 and 48 hr, respectively. Chrysin, genistein, methyl-luteolin, and naringenin were weak killers at 24 hr and moderate killers at 48 hr. At 24 hr, the cell viabilities were 95.8%, 80.8%, 90.89%, and 82.2% for chrysin, genistein, methyl-luteolin, and naringenin, respectively. At 48 hr, the cell viabilities were 71.3% 50.3%, 53.75%, and 68.52% for chrysin, genistein, methyl-luteolin, and naringenin, respectively. Daidzein was a weak killer at both 24 and 48 hr. The cell viabilities were 76.9% and 87.7%, at 24 and 48 hr, respectively. Catechin, myricetin, and methyl-naringenin did not induce cell death at either 24 or 48 hr.

Intracellular Concentration of Flavonoids after 24 and 48 hr Incubation

Based on the previous results, the next step was to determine the intracellular concentration of flavonoids. After incubating MDA-MB-231 cells with different flavonoids, viable cells were counted and extracted with 40% MeOH. The extracts were

injected into the LC-MS for analysis. For most flavonoids, the amounts of compounds detected within cytosol were higher at 48 hr than at 24 hr (Fig. 8).

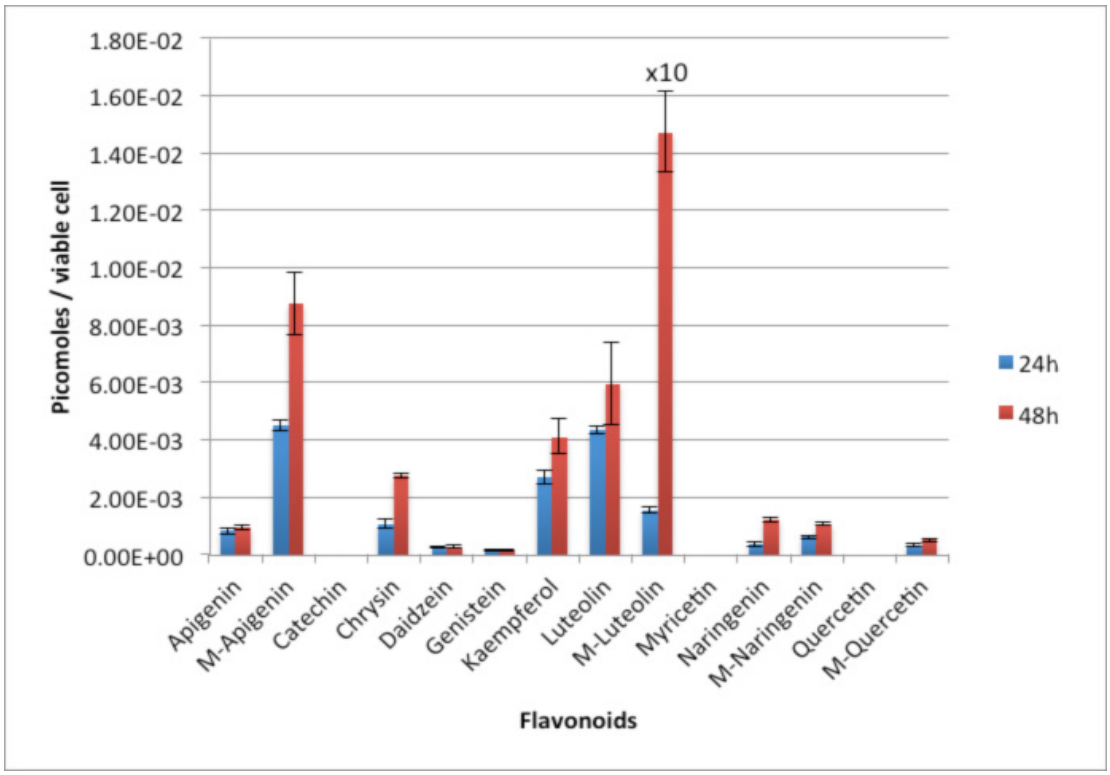


Fig. 8. The intracellular amount of flavonoid detected from cell extracts after 24 and 48 hr. Reported values are in pmol per viable cell.

We grouped the amount of flavonoid within viable cell into different categories. The amount that was considered high was more than 4 x 10^{-3} pmol per cell; moderate, pmol per cell ranged from 1.5 x 10^{-3} to 4 x 10^{-3}; low, pmol per cell were less than 1 x 10^{-3}. The amount of flavonoid within viable cells was high for methyl-apigenin, luteolin, and methyl-luteolin at both 24 and 48 hr. For methyl-apigenin, 4.47 x 10^{-3} and 8.73 x 10^{-3} pmol per cell were detected at 24 and 48 hr, respectively. For luteolin, 4.22 x 10^{-3} and 5.93 x 10^{-3} pmol per cell were detected at 24 and 48 hr, respectively. For methyl-luteolin,

the amount was extremely high; 1.52 x 10^{-2} and 1.47 x 10^{-1} pmol per cell were detected at 24 and 48 hr, respectively. For kaempferol, the amount of flavonoid within cells was moderate at 24 hr and high at 48 hr; 2.66 x 10^{-3} and 4.07 x 10^{-3} pmol per cells were detected at 24 and 48 hr, respectively. The amount of flavonoid within cells was low at 24 hr and moderate at 48 hr for chrysin; 1.04 x 10^{-3} and 2.74 x 10^{-3} pmol per cell were detected at 24 and 48 hr, respectively. The amount of flavonoid within cells was low for apigenin, daidzein, naringenin, methyl-naringenin, methyl-quercetin, and genistein at 24 and 48 hr. At 24 hr, the amount of flavonoid detected was 8.02 x 10^{-4}, 1.98 x 10^{-4}, 3.50 x10^{-4}, 5.86 x 10^{-4}, 3.02 x 10^{-4}, and 1.18 x 10^{-4} pmol per cell for apigenin, daidzein, naringenin, methyl-naringenin, methyl-quercetin, and genistein, respectively. At 48 hr, the amount of flavonoid detected was 9.21 x10^{-4}, 2.54 x 10^{-4}, 1.20 x 10^{-3}, 1.06 x 10^{-3}, 4.95 x 10^{-4}, and 1.14 x 10^{-4} pmol per cell for apigenin, daidzein, naringenin, methyl-naringenin, methyl-quercetin, and genistein, respectively. Catechin, myricetin, and quercetin were not detected within viable cells at either 24 or 48 hr.

Flavonoid Stability in Cell Culture Medium

Because we did not detect catechin, myricetin, and quercetin in our cell extracts, it prompted us to look at flavonoid stability in the medium over time. We prepared flavonoids in the medium (no cells present) at 100 μM and monitored the levels at 0 and 24 hr after incubation using LC-MS. Apigenin, methyl-apigenin, chrysin, daidzein, kaempferol, luteolin, methyl-luteolin, naringenin, methyl-naringenin, and methyl-quercetin were stable in cell culture medium over time (Fig. 9). The abundance of these flavonoids was stable after 24 hr. Except for quercetin, catechin, and myricetin, these

flavonoids were not stable in cell culture medium over time (Fig. 10). Catechin and quercetin could be detected at 0 hr but decreased over time. Myricetin could not be detected in medium at any time point.

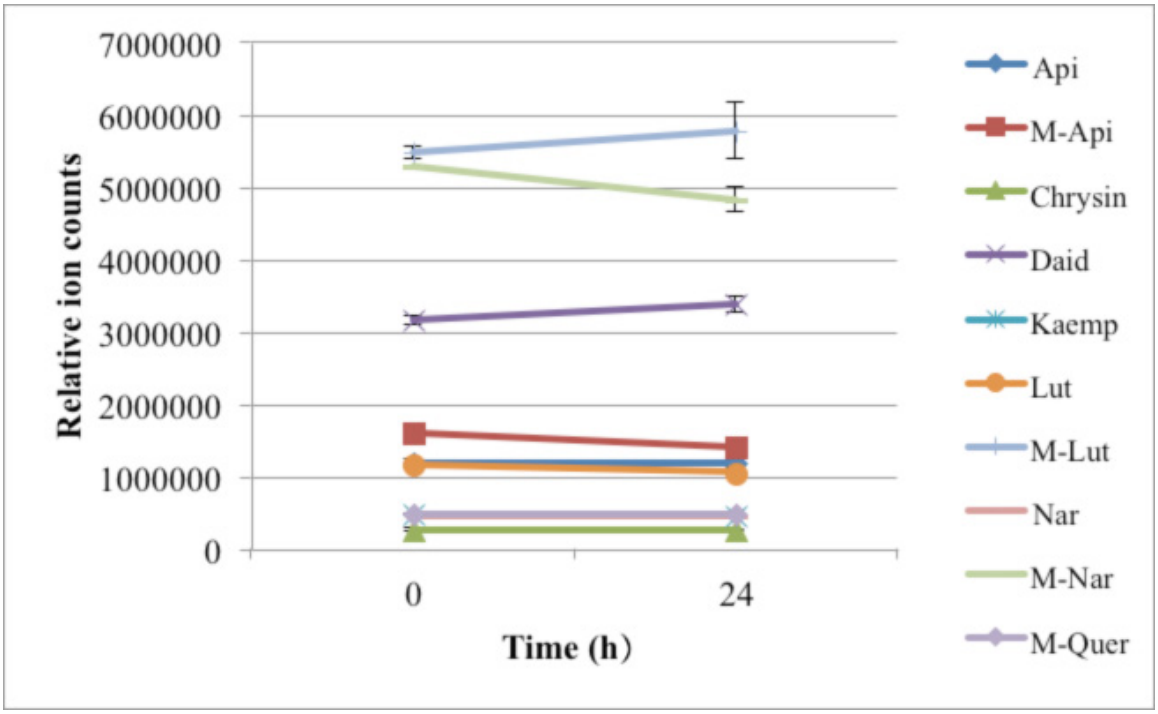


Fig. 9. Flavonoid relative ion counts at 0 and 24 hr in cell culture medium.

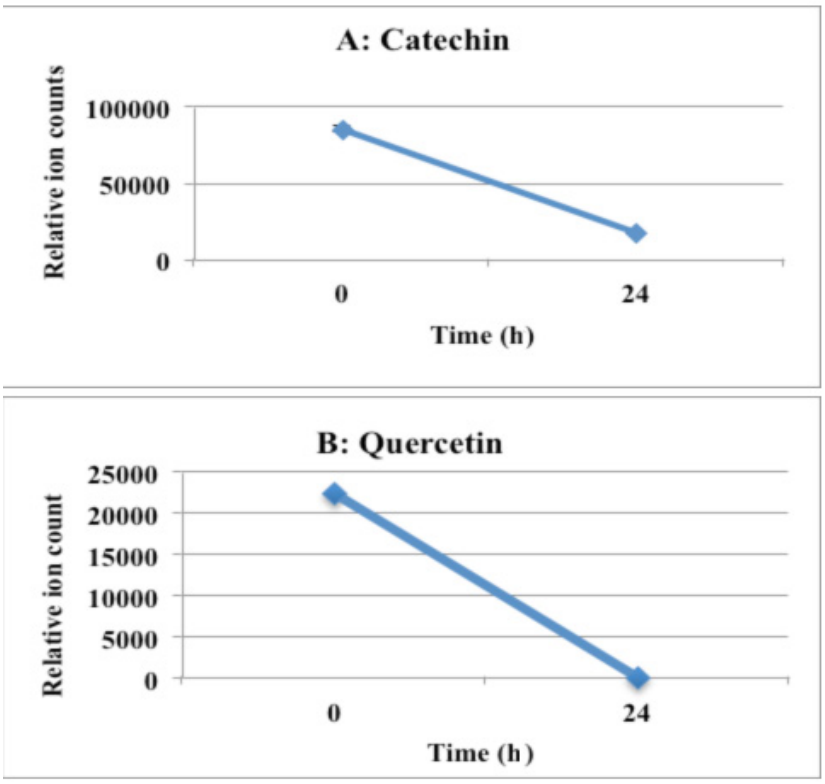


Fig. 10. Non-stable flavonoids in cell culture medium. Catechin (A), quercetin (B).

Quercetin Dimerized in Cell Culture Medium

The amount of quercetin rapidly decreased after 24 hr in cell culture medium. It was either degraded or modified. Therefore, samples were analyzed using Agilent Masshunter Qualitative and MassProfiler software. The MassProfiler identified all of the masses in the samples. It compared the differences in masses between the 0 hr and 24 hr samples. The MassProfiler identified a mass of 302.0427 unique to the 0 hr sample, and a mass of 602.0697 was unique to the 24 hr sample (Fig. 11). A careful review of the literature reveals that a dimer of quercetin with the mass of 602.0797 is naturally present in onion (Ly et al., 2005) (Fig. 12).

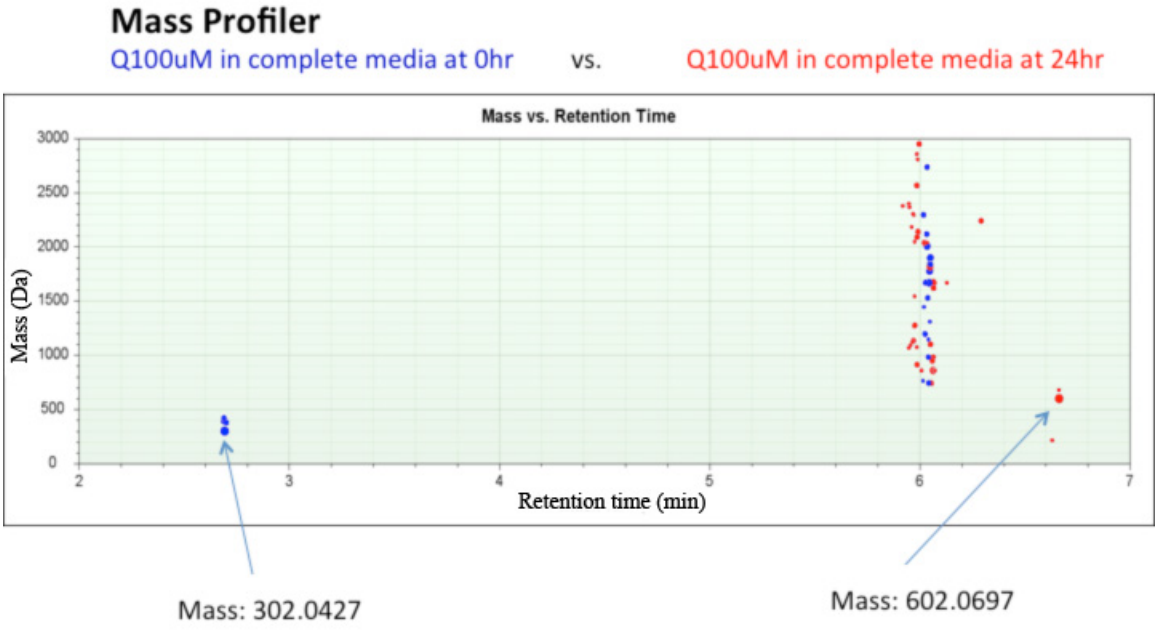
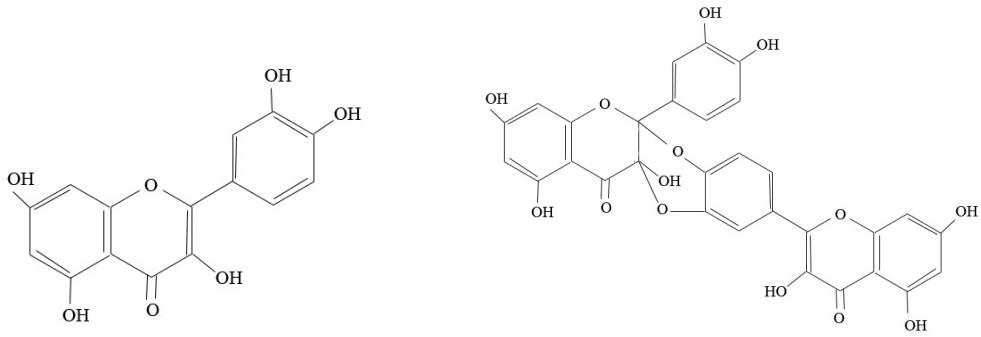


Fig. 11. Detection of unique masses from 0 and 24 hr samples using Agilent MassProfiler software. Blue: compound unique to 0-hour sample. Red: unique to 24 hr sample. At the retention time of 2.7 min, the mass 302.0427, quercetin, is only present in the 0 hr sample. At the retention time of 6.7 min, the mass 602.0697, quercetin dimer, is only present in the 24 hr sample.



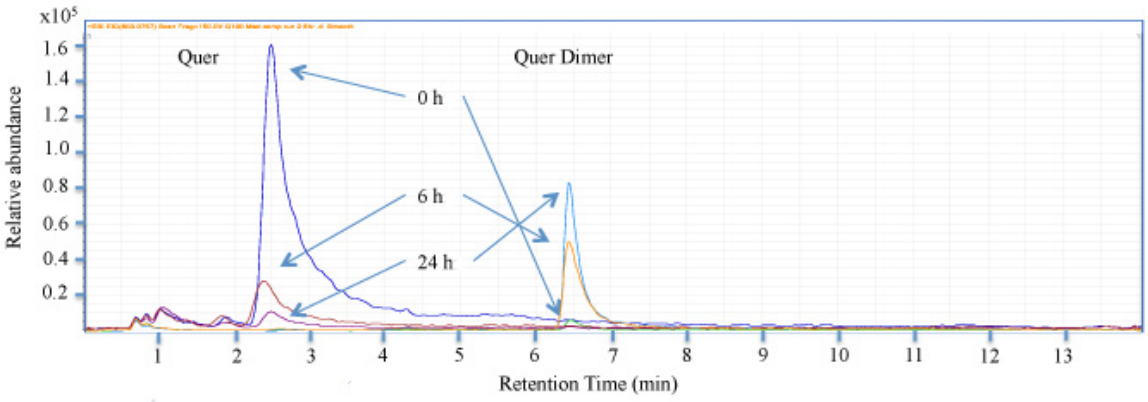
A B

Fig. 12. Molecular structure of quercetin (A) and a quercetin dimer (B) (Ly et al., 2005). Quercetin has a structure of $C_{15}H_{10}O_7$ and a mass of 302.0427. Quercetin dimer has a structure of $C_{30}H_{18}O_{14}$ and a mass of 602.0697.

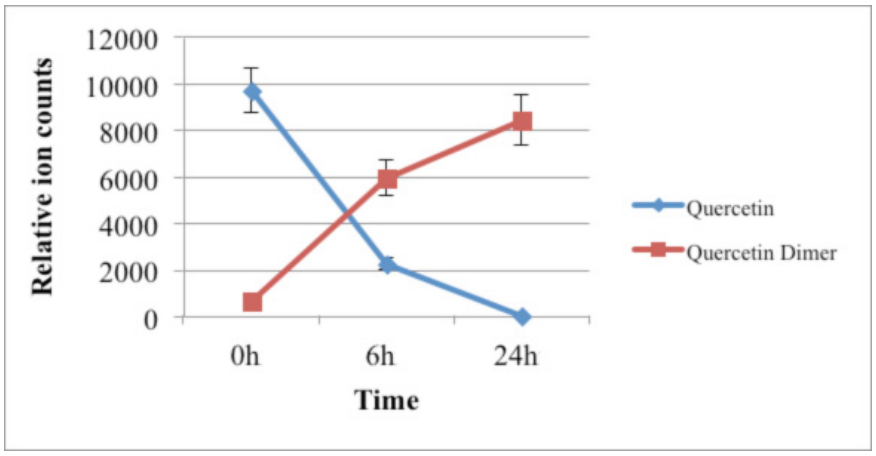
Quercetin and quercetin dimer were adequately separated by the HPLC column.

Quercetin had a retention time of 2.6 min, and quercetin dimer had a retention time of 6.5

min. The abundance of each compound was analyzed at 0, 6, and 24 hr. The results showed that the amount of quercetin dimer increased over the incubation period and the amount of quercetin decreased (Fig. 13).



A



B

Fig. 13. Chromatogram of quercetin and quercetin dimer after 0, 6, and 24 hr(A). Quercetin and quercetin dimer ion counts after 0, 6, and 24 hr (B).

Quercetin Dimerized in the Presence of Fetal Bovine Serum

Quercetin dimerized in complete medium, and we suspected this was due to protein(s) found in FBS. An experiment was conducted to confirm this. We prepared

two tubes that contained 100 μM quercetin in PBS supplemented with 10% (v/v) FBS. The difference between the tubes was that the second tube contained boiled FBS, denaturing the proteins. Samples were collected at 0 and 24 hr, extracted, and analyzed by LC-MS. Quercetin rapidly decreased, and the amount of quercetin dimer rapidly increased in the presence of normal FBS. In the presence of boiled FBS, the amount of quercetin also decreased but at a slower rate, and the amount of quercetin dimer could be detected at 24 hr but with a much smaller amount than the sample in normal FBS (Fig. 14).

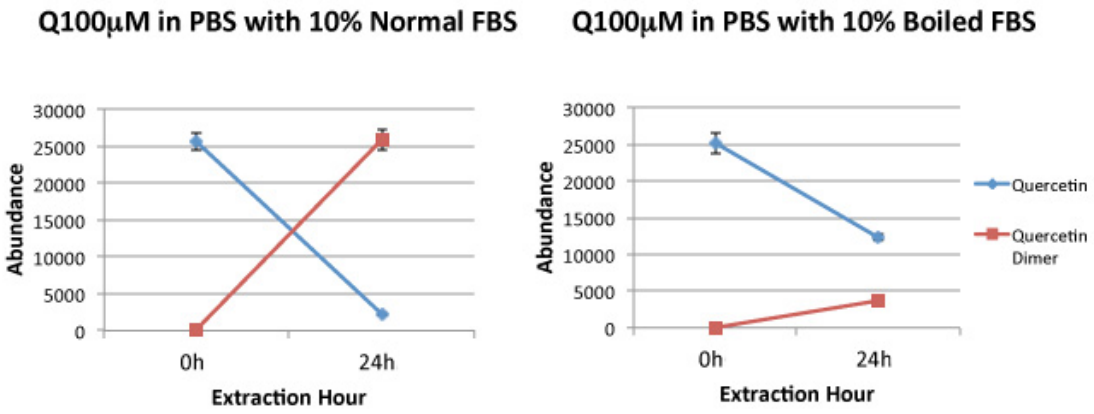


Fig. 14. Quercetin and quercetin dimer after 24 hr in PBS with 10% normal FBS and PBS with 10% boiled FBS.

Quercetin Dimerized in the Presence of Albumin

The most abundant protein in FBS is albumin. Therefore, we tested purified albumin to determine if it caused the dimerization of quercetin. Tube 1 contained 100 μM quercetin in PBS. Tube 2 contained 100 μM quercetin in PBS with 2.5 mg/ml BSA. Tube 3 contained 100 μM quercetin in PBS with 2.5 mg/ml actin. Samples were collected, extracted, and analyzed by LC-MS at 0, 6 and 24 hr after incubation. This

experiment was done at three different temperatures, 22°C (room temp), 4°C, and 37°C. In PBS without proteins, the amount of quercetin was temperature dependent. Quercetin decreased with a high rate at 37°C and with a low rate at 4°C. Quercetin dimer was not detected from the PBS-only sample. In the presence of BSA, quercetin rapidly decreased with temperature dependence, and the amount of quercetin dimer rapidly increased with temperature dependence. With BSA, quercetin decreased with a high rate at 37°C and with a low rate at 4°C; quercetin dimer increased with a high rate at 37°C and low rate at 4°C. In the presence of actin, quercetin decreased over time; however, the decrease was at a lower rate than with the other two samples. The quercetin dimer was not detected in the presence of actin (Fig. 15).

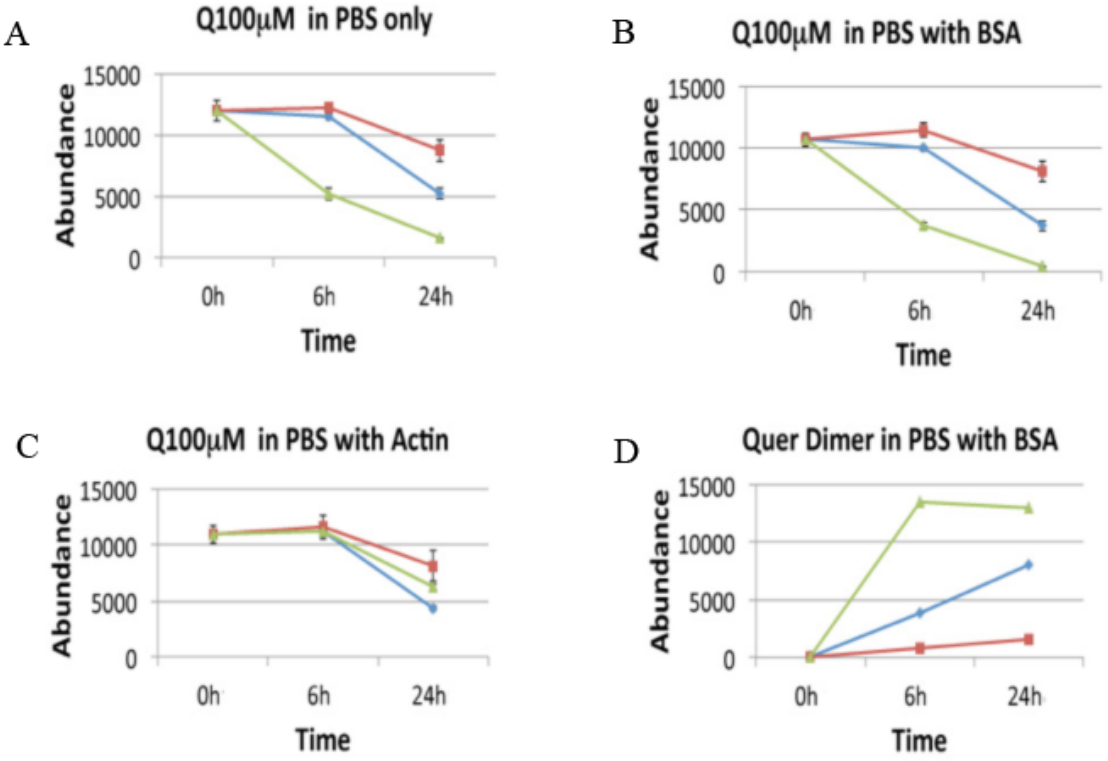
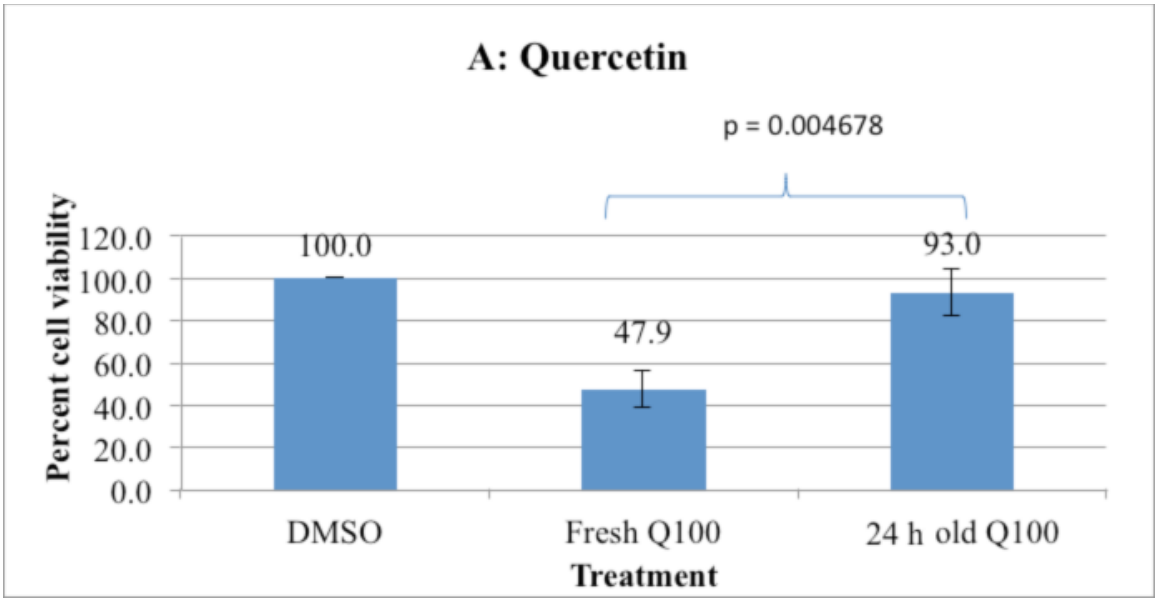


Fig. 15. Quercetin in PBS only (A), quercetin in PBS with BSA (B), quercetin in PBS with actin (C), and quercetin dimer in PBS with BSA (D).

Cell Viability is Based on the Status of Quercetin in Medium

MDA-MB-231 cells were treated with two different quercetin media, fresh 100 μM quercetin medium and 24 hr 100 μM quercetin medium. The results were normalized to the DMSO treatment. After 24 hr, the cell viabilities were 47.9% for cells treated with fresh 100 μM quercetin medium and 93.0% for cells treated with 24 hr 100 μM quercetin medium (Fig. 16 A). In the 24 hr 100 μM quercetin medium, quercetin modified into dimer; therefore, it could not induce cell death. For control purposes, this experiment was repeated with kaempferol. After 24 hr, the cell viabilities were 58.0% for cells treated with fresh 100 μM kaempferol medium, and 60.4% for cells treated with 24 hr 100 μM kaempferol medium (Fig. 16 B).



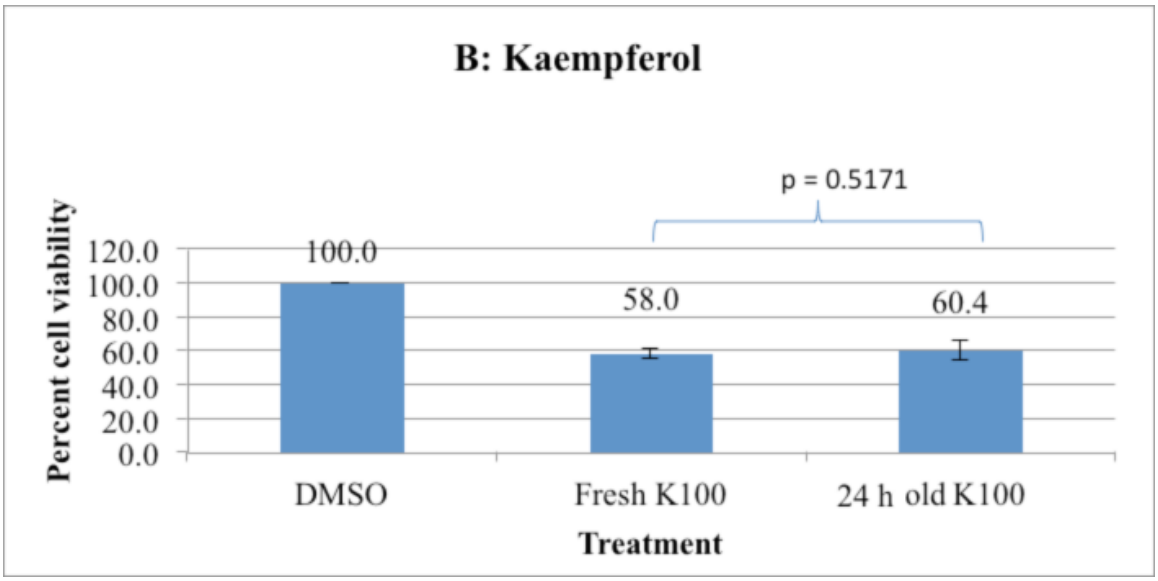


Fig. 16. Cell viability of fresh flavonoids and 24 h-old flavonoids after 24 hr of treatment. Quercetin (A) and kaempferol (B).

MDA-MB-231 cells were treated with 100 μM quercetin medium at 0, 6, and 12 hr. Each time, cells were treated with freshly prepared 100 μM quercetin medium. The cell viability was determined at 24 and 72 hr. A single dose of quercetin was used for control. The results were normalized to the DMSO treatment. At 24 hr, the viable percentages were significantly different between a single-dose and multiple-dose treatment (p=0.01852). The cell viabilities were 63.1% and 47.6% for a single-dose and multiple-dose treatment, respectively. At 72 hr, the viable percentages were significantly different (p=0.000318), 20.9% and 8.1% for a single-dose and multiple-dose treatment, respectively (Fig. 17 A). This suggests that quercetin from a single-dose treatment dimerized over time and did not induce cell death to the same degree as the multiple-dose treatment. In the multiple-dose treatment, with the addition of fresh 100 μM quercetin media, the cytotoxicity was higher. For control purposes, this experiment was repeated

with kaempferol. At 24 hr, the viable percentages were significantly different between a single-dose and multiple-dose treatment. The viable percentages were 46.1% and 28.2% for a single-dose and multiple-dose, respectively. At 72 hr, the viable percentages were significantly different. The viable percentages were 24.6% and 16.6% for a single-dose and multiple-dose, respectively (Fig. 17 B).

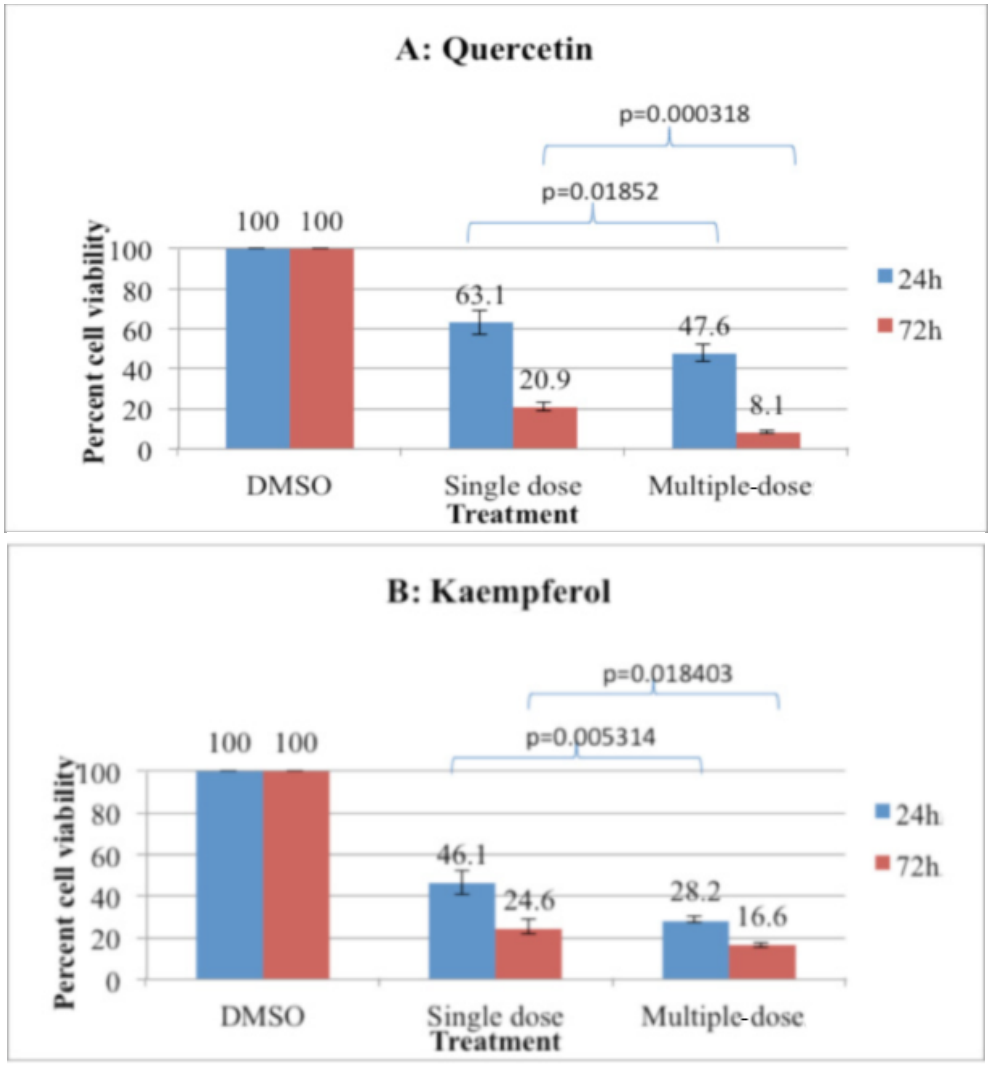


Fig. 17. Percent cell viability compared between a single-dose treatment and multiple-dose treatment. Cells were treated with 100 μM quercetin (A) or kaempferol (B).

Quercetin Dimer did not Induce Cell Death

We sought to determine if the quercetin dimer was able to kill MDA-MB-231 cells. We synthesized quercetin dimer by performing quercetin oxidation with potassium ferricyanide (Gulsen, Makris, & Kefalas, 2007). We used preparatory TLC to isolate quercetin dimer. The isolated dimer was analyzed by LC-MS, and the most abundant mass in the isolated sample was $[M+H]^+$ 603.0769 m/z, which was the mass of quercetin dimer. In addition, the isolated dimer was analyzed by proton NMR (CD_3OD): 5.93, 6.18, 6.40, 6.70, 7.09, 7.18, 7.23, 7.87, and 7.96 ppm. These results were similar to the published data that proposed the structure of quercetin dimer, 1,3,11a-trihydroxy-9-(3,5,7-trihydroxy-4H-1-benzopyran-4-on-2-yl)-5a-(3,4-dihydroxyphenyl)-5,6,11-hexahydo-5,6,11-trioxanaphthacene-12-one (Ly et al., 2005). This isolated quercetin dimer was dissolved in DMSO and treated to cells at 100 μM. After 72 hr, the isolated quercetin dimer did not induce cell death (Fig. 18).

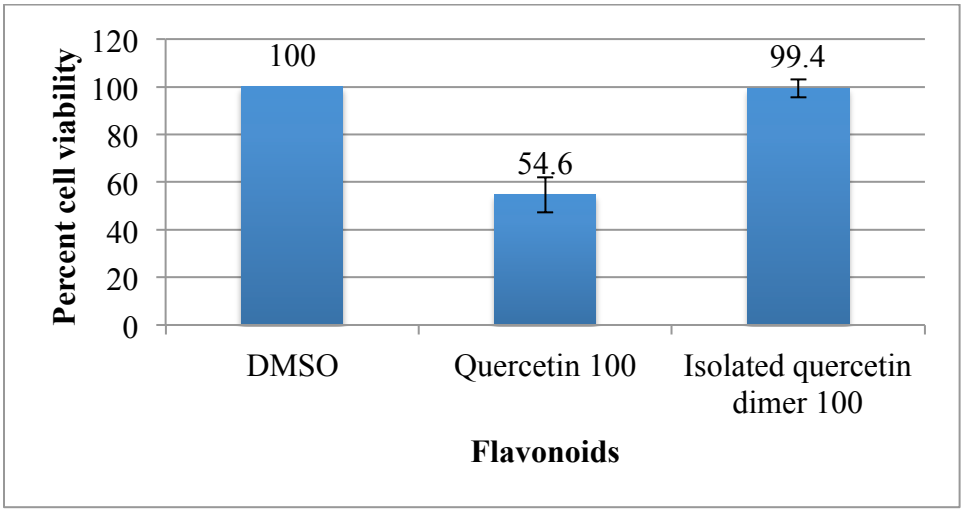


Fig. 18. Isolated quercetin dimer cytotoxicity on MDA-MB-231 cells after 72 hr. Quercetin killed and isolated quercetin dimer did not. Quercetin 100 μM viability was 54.6%, and isolated quercetin dimer 100 μM viability was 99.4%.

Discussion

Flavonoid Cytotoxicity and Intracellular Concentration on MDA-MB-231 Cells

Cell cytotoxicity and the amount of flavonoid found in MDA-MB-231 cell extracts varied between tested flavonoids. For most of the tested flavonoids, the amount of the flavonoid found in cell extracts was higher after 48 hr of incubation. The highest levels of flavonoid in cell extracts were seen with methyl-apigenin, kaempferol, and luteolin. The lowest detectable levels were seen with daidzein, naringenin, and methyl-naringenin. The amount of flavonoid found within cells correlated with the amount of cell death at 24 and 48 hr. For kaempferol, luteolin, and methyl-apigenin, the levels of flavonoids found within cells were high; therefore, the cell death percentages were also high at both 24 and 48 hr. For daidzein, naringenin, and methyl-naringenin, the levels of flavonoids found within cells were low; therefore, the cell death percentages were low for these flavonoids. For apigenin and methyl-quercetin, the levels of flavonoid found within cells were low; however, the cell death percentages were moderate. Further investigation is needed for these flavonoids.

Catechin, myricetin, and quercetin were not detected in cell extracts at either 24 or 48 hr and, as expected, did not induce cell death. Catechin and myricetin were not detected in cell culture medium, without cells, after 24 hr. This suggests that these two flavonoids were rapidly degraded. Yokomizo and Moriwaki (2005) reported that myricetin degraded immediately after addition in DMEM, and our results are in accord with this. However, catechin degradation in DMEM should be investigated further. Quercetin was not detected intracellular and cell culture medium at 24 and 48 hr.

Interestingly, quercetin was able to induce cell death, 61.3% viable cells at 24 hr, and 46.1% viable cells at 48 hr. This suggests that quercetin was modified in cell culture medium.

The flavonol class is the most abundant in nature as well as the most interesting. Each one of the tested flavonoids behaved differently. Kaempferol's ability to enter cells was high, methyl-quercetin's was low, quercetin was modified over the incubation period, and myricetin rapidly degraded after addition to medium. Kaempferol's structure shows that flavonols' structure has the ability to travel across MDA-MB-231 cell membrane. The flavonol group has a double bond between carbons 2 and 3, which makes flavonoids planar, giving them a greater ability to enter cells. This also suggests that myricetin, quercetin, and methyl quercetin should be able to enter cells well. However, the results from this thesis do not support this idea.

Methyl-apigenin, chrysin, luteolin, and methyl-luteolin are in the subclass of flavones. Flavonoids in this class were able to enter cells at a higher rate than others tested; methyl-luteolin had the highest concentration in cells. The double bond between carbons 2 and 3 in the flavones group makes the structure planar, increasing the permeability of the cell. Methyl-luteolin entered cell at a much higher rate than luteolin. The methyl groups enhanced the cell membrane permeability for methyl-luteolin. However, the cytotoxicity of luteolin was much higher than methyl-luteolin. This suggests that once a flavonoid enters cells, it must bind to the appropriate proteins to induce cell death.

Flavonoids from the flavanones class, naringenin and methyl-naringenin, entered cells at approximately the same rate. This suggests that methylation does not enhance the cell membrane permeability for methyl-naringenin. The structure of flavanones is very similar to that of flavones, except for a single bond instead of a double bond between carbons 2 and 3. This result shows that a flavonoid with a double bond between carbons 2 and 3 has a better ability to travel across cell membranes than a flavonoid with a single bond.

Daidzein and genistein, which are both in the isoflavones class, had the lowest concentration in cells of all flavonoids tested. The structures of flavonoids in this class are different from the others. Unlike other classes, the B ring is connected to carbon number 3 instead of carbon number 2. This result shows that flavonoids with the B ring at carbon number 3 have lower membrane permeability than flavonoids with the B ring at carbon number 2.

Some flavonoids with methyl ether groups, methyl-apigenin and methyl-luteolin, had a greater ability to enter a cell; this was not observed for methyl-naringenin and methyl-quercetin. After 24 and 48 hr, the level of methyl-apigenin and methyl-luteolin found within cells was much higher than the level of apigenin and luteolin, respectively. Similar results have been reported in other studies (Walle et al., 2007; Wen et al., 2006). This suggests that the methyl groups enhance cell membrane permeability. However, methyl-naringenin did not enter cells as well as the other methylated flavonoids. Even though this flavonoid has methyl ether groups, it has a single bond between carbons 2 and 3 indicating that this flavonoid is not planar. Methyl-quercetin was also unable to

adequately enter cells. According to the literature, with five additional methyl ether groups attached, methyl-quercetin should be able to enter cells easily. This needs to be investigated further.

Flavonoid Stability in Cell Culture Medium

Most of the flavonoids, with the exception of catechin, myricetin, and quercetin, were stable in cell culture medium. This suggests that there was no loss of flavonoids during the period of incubation with cells. After 24 hr in cell culture media, 37^{o}C, and 5% CO_2, the amount of flavonoids remained the same; therefore, the activity remained the same as hour 0.

Catechin is most different of the tested flavonoids. This structure does not have a ketone group on the three-carbon bridge. In addition, this flavonoid has two chiral carbons. These are the probable reasons catechin is unstable in cell culture medium.

The stability of flavonoids in the flavonols class is not uniform. Kaempferol and methyl-quercetin were stable within cell culture medium, myricetin rapidly degraded after addition to the medium, and quercetin dimerized in cell medium. These flavonoids' structures are very similar, yet dimerization only occurs in quercetin. Quercetin has two hydroxyl groups on the B ring, where kaempferol and myricetin have one and three, respectively. The dimerization is likely specific to flavonols with two hydroxyl groups on the B ring. Alternatively, methyl-quercetin did not undergo dimerization. The methyl groups probably block the dimerization process at the three-carbon bridge.

Quercetin-3-O-glucopyranoside has a glucopyranoside group at carbon number 3 instead of a hydroxyl group compared to quercetin (Fig. 19). Quercetin-3-O-

glucopyranoside did not dimerize in cell culture medium (data not shown). This suggests that the dimerization of quercetin involves the three-carbon bridge. In addition, quercetin-3-O-glucopyranosdie was not able to enter cells. The glucopyranoside attached makes the structure bulky and polar, thus reducing the permeability into the cell membrane.

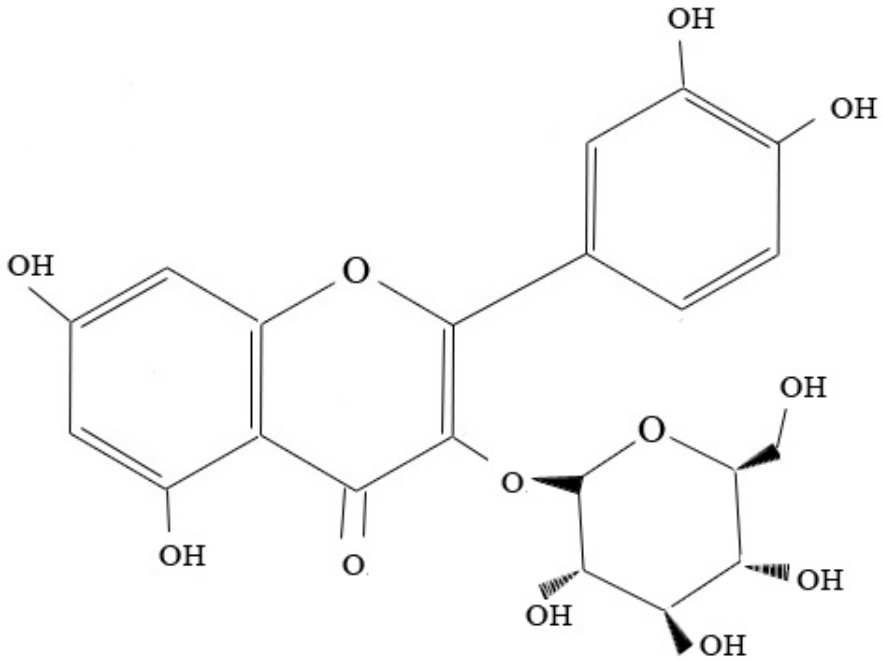


Fig. 19. Quercetin-3-O-glucopyranoside structure.

Quercetin Dimerized in Cell Culture Medium

Quercetin was not detected by our method in cell extracts at any of the time points tested. However, quercetin was able to induce cell death. This suggests that quercetin was degraded or modified. The stability of quercetin in cell culture medium was tested. The result showed that quercetin abundance decreased during incubation. A new mass, 602.0697, was only detected in the sample at hour 24. That mass was almost double the

mass of quercetin, 302.0427. In addition, quercetin was only detected in the hour 0 sample. Because of this, we suspected that dimerization of quercetin occurred during incubation with cell culture medium. Quercetin was mixed with DMEM complete media and incubated for 24 hr. Three different time points were collected from the solution, 0, 6, and 24 hr, for LC-MS analysis. The results showed that dimerization was time dependent. The lowest level of quercetin dimer detected was at 0 hr, and the highest level was at 24 hr.

As suggested in the literature, quercetin dimerizes at the hydroxyl group on carbon number 3. To confirm this structure, quercetin-3-O-glucopyranoside was incubated in cell culture medium. After 24 hr, no dimer was detected (data not shown). The glucopyranoside group was replaced with the hydroxyl group at carbon 3 on quercetin. This blocked the oxidation process at this location; therefore, quercetin was not able to dimerize in cell culture media. This suggests that dimerization of quercetin occurs at carbon number 3.

Quercetin Dimerization

Quercetin only dimerized in medium with FBS; it did not dimerize in medium without FBS. Because globular protein, bovine serum albumin (BSA), is a major component of FBS, it is logical to assume that albumin is the cause for quercetin dimerization. Results show that in the presence of albumin, quercetin dimerized over time. Quercetin is oxidized to products that can covalently bind to albumin (Kaldas et al., 2005). This dimerization is unique to quercetin and no other flavonoid in that same class. This suggests that dimerization requires hydroxyl groups at carbon numbers 3, 3' and 4'.

Kaempferol cannot undergo dimerization because it is missing a hydroxyl group at carbon 3'; myricetin cannot undergo dimerization because it has an extra hydroxyl group at carbon 5'; methyl-quercetin cannot undergo dimerization because it has the methyl groups.

Quercetin Dimer did not Induce Cell Death

Results from the experiment using 24 hr-old quercetin medium showed that quercetin left in the media could no longer induce cell death. This suggested to us that the dimerized form of quercetin could not kill cells. In addition, the result from the single-dose versus multiple-dose treatment showed that when fresh quercetin was added, cells died at a higher rate. The quercetin added from later doses replaced the original that had already dimerized in cell culture media. This shows that fresh quercetin was able to induce cell death until it dimerized.

We do not know whether quercetin has the ability to enter cells. We were not able to detect any quercetin or quercetin dimer in cells after 24 hr. However, the results from the synthesized quercetin dimer showed that it did not have the ability to enter cells and did not induce cell death.

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