THESIS

SYNTHETIC LETHALITY OF FLAVONOIDS TOWARDS HOMOLOGOUS RECOMBINATION DEFICIENT CELLS THROUGH PARP INHIBITION

Submitted by

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ABSTRACT

SYNTHETIC LETHALITY OF FLAVONOIDS TOWARDS HOMOLOGOUS RECOMBINATION DEFICIENT CELLS THROUGH PARP INHIBITION

Flavonoids can be isolated from many different sources such as plants, fruits, and beverages and they have long been associated with various health benefits. Both *in vitro* and *in vivo* studies have shown potential anti-inflammatory, anti-allergic, anti-viral, and antioxidant activities associated with these compounds. Previously published research has shown that the anti-cancer effects of flavonoids on *BRCA2* deficient cells can be attributed to a PARP inhibitory mechanism. Therefore, thirteen structurally similar flavonoids were screened and identified as PARP inhibitory flavonoids. Seven different cell lines: Chinese hamster lung V79 cells, its *BRCA2* deficient derivative V-C8 cells, gene corrected V-C8 cells, Chinese hamster ovary (CHO) wild type cells, rad51D deficient CHO cells (51D1), Human colorectal adenocarcinoma cells (DLD-1), and their *BRCA2* knockout cells (DLD1 *BRCA2*$^{-/-}$) were used to assess the degree of synthetic lethality due to PARP inhibition. Colony formation and doubling time assays identified selective toxicity in DNA repair deficient cells for the flavonoids Kaempferol, Myricetin, Quercetin, Theaflavin and Epigallocatechin gallate. A Sister Chromatid Exchange (SCE) assay indicated Kaempferol, Myricetin, Quercetin Theaflavin and Epigallocatechin gallate exhibited a marked increase in SCE rate, which is indicative of PARP inhibition. These results were confirmed via an *in vitro* PARP inhibition assay. This study identified Kaempferol as a natural PARP inhibitor leading to potential lethality to *BRCA2* cancers. All flavonoids identified as effective PARP inhibitors had similar structural components: hydroxyl groups on the 5 and 7 position of the A-

ring, another hydroxyl on the B ring in the 4 position, and a C-2,3 double bond (a 4-ketone function).

ACKNOWLEDGEMENTS

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CHAPTER ONE:

Background

1.1 Introduction

Cancer is a major health problem, causing mortality and morbidity in all age groups across the world and is the second leading cause of death in the USA [1]. Around 1.5 million people are expected to be diagnosed with cancer in the USA in 2019, and 606,880 Americans are expected to pass away from cancer by 2019 [2]. Breast cancer is the second deadliest type of cancer in the USA, with an estimated 268,600 women and 2,670 men diagnosed in 2019 [2]. It is also estimated that around 42,260 (41,760 women, 500 men) patients will die from breast cancer in 2019 [2]. The rate of mortality in breast cancer is still relatively high even though it declined from 40% in the 1980s to 20% by 2016. This decrease could be attributed to increased awareness of the symptoms, better early screening procedures, and the development of new treatments [3]. There are many factors that can play a role in the development of breast cancer: 1) old age, 2) inherited genetic factors such as mutations in *BRCA1* and *BRCA2* tumor suppressor genes; and 3) lifestyle factors that affect breast cancer development are the use of birth control containing hormones, the over consumption of alcohol, which can increase the risk by 20%, choosing not to breast feed or have children, and obesity (Figure 1.1) [3-5].

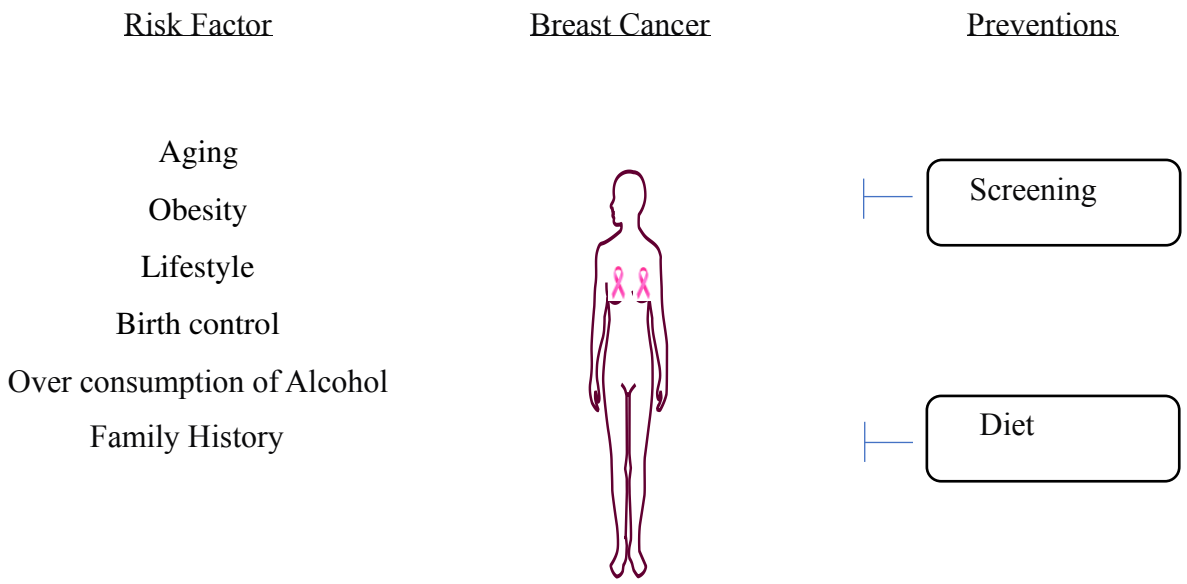


Figure 1.1: Factors playing a role in developing breast cancer and how to prevent it[5].

While genetic factors are not considered a primary influence on breast cancer occurrence or proclivity, only causing major issues in 5-10% of all patient cases, the genetics of various cells open up several avenues of treatment that cannot be avoided or controlled by patients [6]. Current research has shown that the inhibition of an enzyme related to single stranded break repair, poly (ADP ribose) polymerase (PARP), in *BRCA1and BRCA2* homozygous mutated cells results in selective killing of mutated cells [7, 8]. This mechanism, called synthetic lethality, is known to take place in BRCA1/2 homozygous mutated cancer cells but not in *BRCA1and BRCA2* heterozygous non-cancerous tissues making this a favorable target for potential cancer therapies or use as a preventative measure [6]. The enzyme PARP is involved in many important cellular functions including nucleic acid metabolism, modulation of chromatin structure, deoxyribonucleic acid (DNA) synthesis, DNA repair, and base excision repair (BER). BER is a complex of proteins consisting of DNA Ligase III, DNA polymerase beta, and the XRCC1 protein and functions as a repair mechanism for single-stranded DNA breaks (SSBs).

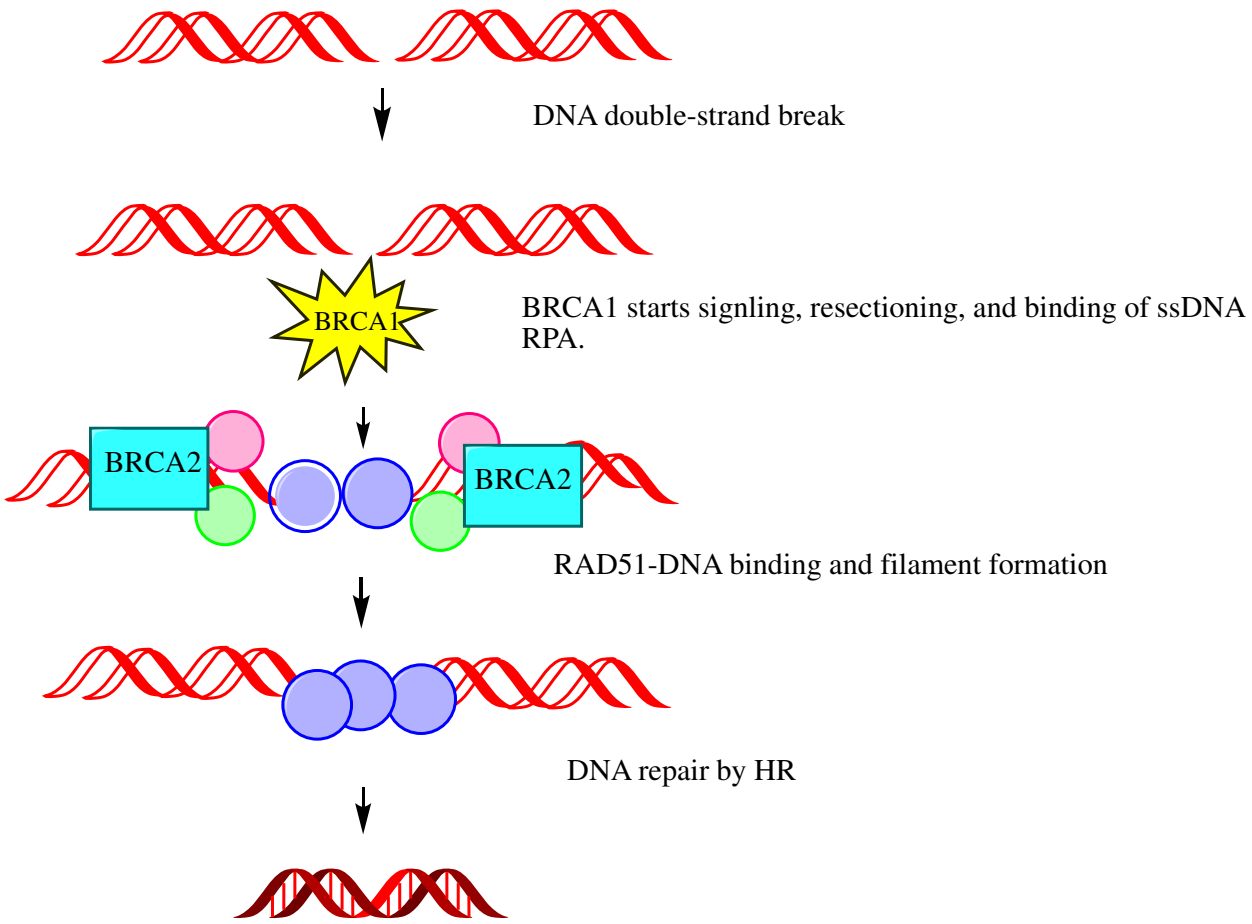


Figure 1.2: Mechanism of action for homologous recombination[9].

Homologous recombination (HR) is responsible for DNA repair and prevents the demise of damaged DNA replication forks, orchestrates the segregation of homologous chromosomes in meiosis I, and plays a role in telomere maintenance [10] (Figure 1.2). It is known that the breast cancer–associated genes *BRCA1* and *BRCA2* are involved in the repair of double-stranded (DS) DNA breaks via HR. Cells that are deficient in *BRCA1* display impaired HR function and are unable to effectively repair damaged chromosomes [11]. *BRCA2* also interacts with the DNA repair protein RAD51, a protein known to be important to HR because RAD51 deficient cells are hypersensitive to DNA cross-linking and chromosomal instability [11]. Defects in HR repair

mechanisms, arising from deficiencies in key repair proteins such as RAD51; or other heavily involved proteins like DSS1, RPA1, or CHK1; cause the effected cells to become more dependent on the repair activity of PARP and thus become sensitive to its inhibition [11, 12]

A common hypothesis is that PARP inhibition compromises SSB repair and BER in cells lacking intact HR mechanisms (*BRCA1* and *BRCA2* mutants). These lesions are converted into DS breaks, resulting in cell lethality. Further research in this field has shown that *BRCA1* or *BRCA2* deficient cells are 57-133-fold more sensitive to PARP inhibition than wild type cells [13]. These results imply that tumor cells with defective HR mechanisms can be potentially treated with PARP inhibitors, which would heavily impair DNA repair mechanisms and result in tumor cell death via synthetic lethality. This approach is very efficient and beneficial because it is highly selective towards tumors over normal, wild type cells [13]. There are many synthetic and naturally occurring PARP inhibitors, but these studies focuses on naturally occurring flavonoids found in the human diet. Flavonoids are known for both their health benefits and bioactivity [14]. In addition, if these chemicals are approved in animal studies as a potential treatment for cancer via PARP inhibition, they will prove cheaper and more easily accessible.

1.2 Statement of Project:

In *in vitro* and animal studies, Flavonoids have shown to have potential health benefit and therapeutic value. Increased consumption of these dietary flavonoids can protect against various diseases, particularly cancer. This thesis is an examination of the cytotoxicity and genotoxicity responses induced by different subclasses of flavonoids. In particular flavonols (Galangin, Quercetin, Kaempferol, Myricetin and Morin), other subclasses flavo-3-ols (Epigallocatechin gallate and Theaflavin), Flavanones (Pinocembrin), Dihydroflavanones (Pinobanksin), Flavone (Luteolin) and Dihydroflavonols (Dihydrokaempferol, Taxifolin and Dihydromyricetin**).** To

determine cytotoxicity and genotoxicity responses, we used the colony formation assay, cell growth inhibition and cell cycle analysis, sister chromatid exchange, micronuclei, and PARP inhibition assay. These procedures were completed using Chinese hamster ovary (CHO) cells, CHO DNA repair deficient (rad51D deficient) cells, Chinese hamster lung origin V79 and its DNA repair deficient V-C8 (BRCA2 deficient) and their gene complemented V-C8 cells. In addition, to compare effects seen to human cells, we used human colorectal adenocarcinoma cells (DLD-1), and their BRCA2 knockout cells (DLD1 BRCA2$^{-/-}$).

1.3 Flavonoids:

Flavonoids are a group of polyphenolic compounds that are highly distributed throughout the plant kingdom, with over 4,000 kinds of flavonoids known to science [15]. In the USA, the daily consumption of mixed flavonoids is estimated to be in the range of 500–1000 mg [15, 16]. All flavonoids have the same basic chemical structure: a fifteen-carbon skeleton consisting of two benzene rings (A and B as shown in Figure 1.3) linked via a heterocyclic pyran ring (C). They can be divided into six main groups which are listed in Table 1.1 along with their major dietary sources.

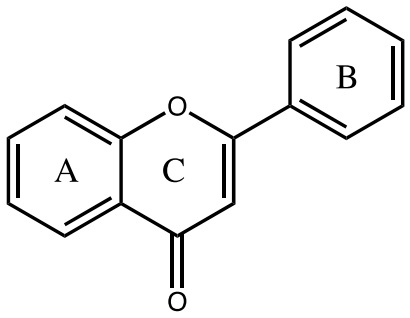
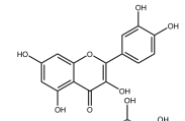
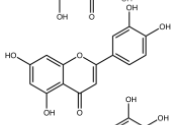
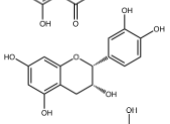
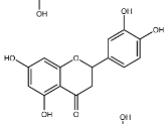
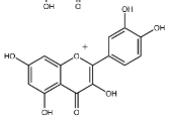
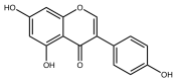


Figure 1.3: Basic chemical structure for flavonoids.

Table 1.1: The main flavonoid subclasses, structures, food source[15, 17]

Flavonoids Subclasses	Example of Compounds	Basic Chemical Structure	Major Dietary Sources	References
Flavonol	Quercetin, Kaempferol, Galangin, Fisetin, Myricetin, Morin and other		Broccoli, Onion, and various fruit.	[14]
Flavones	Apigenin, Diosmin, Luteolin		Herbs, especially parsley & celery.	[14]
Favan-3-ol	(+) Catechin,(-) Epicatechin, (-) Epigallocatechin		Green & Black teas.	[13,14]
Flavanones	Narrogin, Hesperetin , Pinobanksin Pinocembrin		Citrus fruit, such as orange and grapefruit.	[14]
Anthocyanidin	Cyanidin, Delphinidin		Colored berries and other fruit.	[14]
Isoflavones	Daidzein Genistein Glycitein.		Soy products.	[14]

1.4 Human Health and Therapeutic Benefits

Research into historical diets showed that those high in saturated fat were correlated with low occurrence of cardiovascular disease, a discovery that enhanced scientific interest in Flavonoids and their effects on human health [18]. This correlation was thought to be the result of ingesting large amounts of fruits and vegetables along with the high saturated fat [19]. Further investigation into flavonoid-rich diets and supplements led to an accumulation of *in vitro*, *in vivo*, and epidemiological evidence that suggest a large number of health benefits (Figure 1.4).

There is evidence that flavonoids can slow down the development of atherosclerosis by inhibiting oxidation of low-density lipoprotein (LDL) [20]. Catechins, a flavonoid found in tea, may delay the formation of atheromatous lesions by inhibiting arterial invasion of smooth muscle cells [21].Resveratrol, flavonoids located in fruits, reduces platelet aggregation via cyclooxygenase I inhibition [22]. Quercetin, the most abundant type of flavonoid, suppresses metalloproteinase 1, which breaks up atherosclerotic plaques [23]. Many different flavonoid types

have also demonstrated marked chemo preventive effects also anticancer in numerous studies [24, 25].

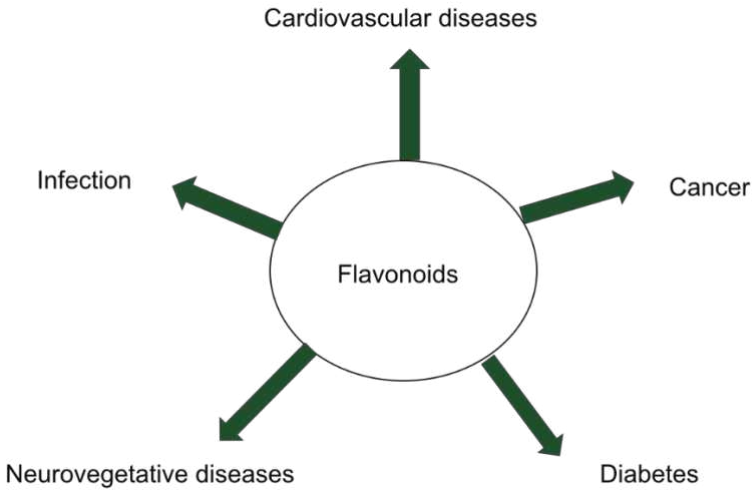


Figure 1.4: Protective health benefits associated with the consumption of flavonoids[13].

Carcinogenesis develops in three different phases: initiation, promotion, and progression [26]. Initiation involves a genetic stimulus while promotion requires a non-genetic stimulus to trigger cell proliferation. The sequence of these two events can lead to progression or malignancy. Flavonoids demonstrate several chemo preventive effects that inhibit various phases of carcinogenesis through several diverse mechanisms: cell cycle arrest and apoptosis, induction of detoxification enzymes, alteration of cellular signaling, and immune system regulation [27]. Theaflavin, a catechin found in black tea, actively interferes with metastasis in human breast cancer cells by downregulating p53 metastatic proteins [28]. Treatment of human breast cancer cells by the isoflavone genistein, found in soy products, induces cell cycle arrest at relevant concentrations [29]. In mice, Quercetin protects against benzo(a)pyrene induced lung cancer via radical scavenging [29]. Additionally, resveratrol activates apoptosis by triggering the caspase cascade in human pancreatic cancer stem cells [30].

Flavonoids also show potential in treating neurodegenerative diseases such as Alzheimer's and Parkinson's. In rodent studies, both Rutin and Nobiletin, flavonoids from buckwheat and citrus peel respectively, were shown to have a measurable effect on brain activity. Rutin treatments in rats improved memory retrieval and protected against β-amyloid toxicity, a major proponent in Alzheimer's disease [31]. Meanwhile, Nobiletin enhanced dopamine release which improved general motor functions in rodent models of Parkinson's disease [31].

Epigallocatechin gallate (EGCG), a flavan-3-ol flavonoid, has demonstrative anti-diabetic properties in both *in vitro* and animal studies [32]. Under glucotoxicity conditions, EGCG promotes insulin release by upregulating insulin receptors and modulating apoptosis through inhibition of pro-apoptotic proteins [32]. The flavanol Kaempferol promotes cell survival under cytotoxic conditions by rescuing cells from lipid peroxidation-induced apoptosis [33].

There is now a plethora of evidence available that supports the use of dietary flavonoids as a form of disease prevention in concert with other, approved therapies and dietary changes. However, most flavonoids are not easily solubilized in water due to their crystalline structure. This, in turn, reduces their efficacy as a valid cancer therapy as it reduces bioavailability [34]. Bioavailability of flavonoids varies between subclasses and even flavonoid sources, possibly due to the methods and locations of absorption into the body and the subsequent enzymatic modifications made to flavonoid structure during metabolism. An example of this variability can be seen with Quercetin, when isolated from apple there is a 30% higher bioavailability than when the same compound is isolated from onion [34]. Therefore, it can be concluded that the metabolism of flavonoids has an unmistakably strong influence on the efficacy of flavonoids.

1.5 Metabolism of Flavonoids in Humans

The reports on the therapeutic value of flavonoids are diverse, but the bioavailability of flavonoids compound must be addressed in order to optimize the health benefits. Flavonoid physicochemical properties determine absorption in either the small intestine or the colon [35]. Flavonoids that undergo glycosylation are called glycosylated flavonoids, and flavonoids without glycosylation are called aglycone flavonoids. Small intestine usually absorbed aglycone flavonoids, where glycosylated requires enzymatic transformation into aglycones before absorption. Preferential flavonoid glucoside hydrolysis occurs in the lumen of the small intestine through β- glucosidases. Glycosides are also hydrolyzed by microflora in the colon without β-glucosidases specific substrates, but absorption capacity is much more limited, and the free aglycone flavonoids are degraded [35, 36]. Once absorbed, aglycone flavonoids are metabolized into phenolic acids by the liver and are combined with glucuronides, sulfates or methylated compounds [37]. These chemicals are mostly eliminated in feces, with only a small fraction eliminated in the urine [37]. There are many factors that can affect the absorption and bioavailability results of flavonoids such as dose, gender differences, colonic microflora population, delivery vessel, and diet [38]. Figure 1.5 summarizes flavonoid metabolism in humans.

Regardless of the method of metabolism, flavonoid metabolites, on average, show a marked decrease in bioactivity [34].There are several different methods to increase bioavailability of flavonoid that are currently being investigated by researchers [34, 39]. These methods are focused primarily on improving intestinal absorption, changing the site of absorption, increasing metabolic stability, and food supplementation [34].

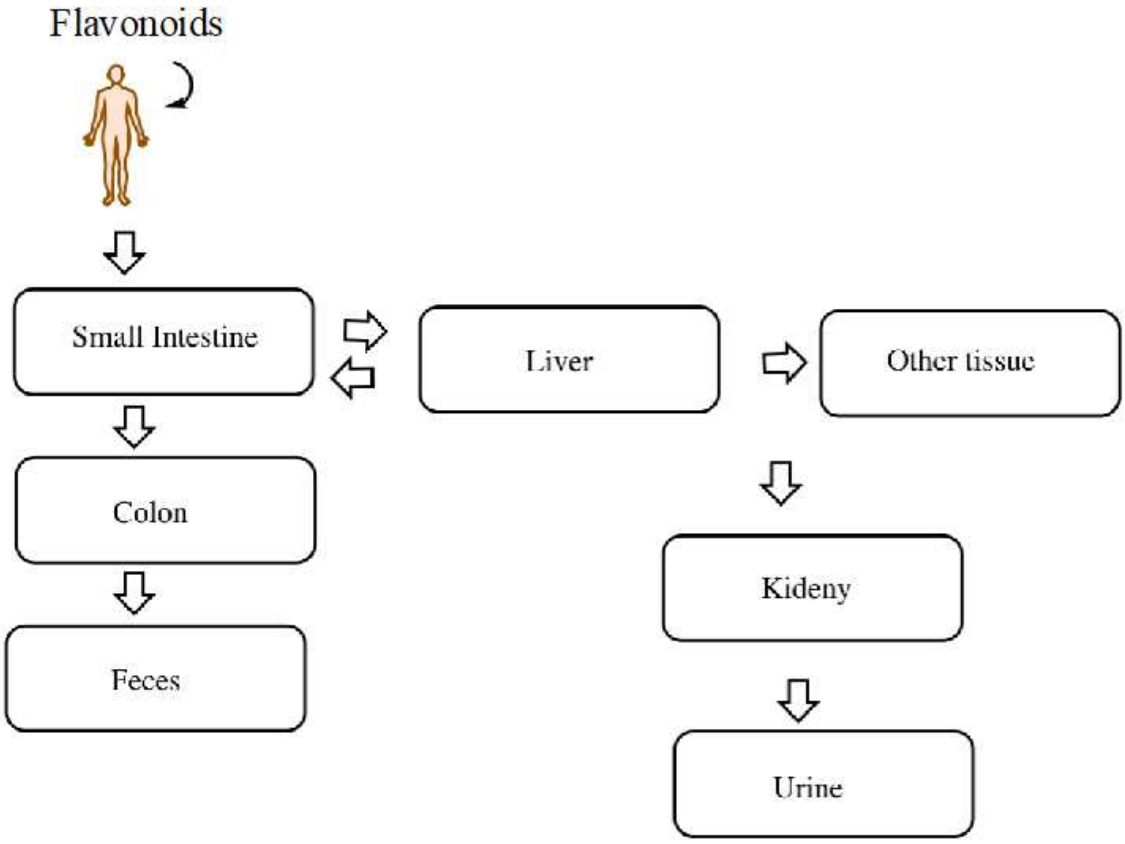


Figure 1.5: Metabolism of flavonoids in humans [16].

1.6 Properties of Flavonoids

Flavonoids can prevent oxidative damage in cells that have the potential to develop into cancer [37]. Oxidation happens when an electron is transferred and uncoupled from one atom to another. This will produce reactive oxygen species (ROS) -- like superoxide (O2•-), peroxyl (ROO•), alkoxy (RO•), hydroxyl (HO•), or nitric oxide (NO•) -- that can severely damage the cells. Accumulating ROS will go on to attack lipids, proteins, carbohydrates, and DNA, wreaking havoc on cellular processes, damaging and mutating DNA, and disrupting membrane and enzyme activity and function. Dying cells will release lipid peroxidation products that also promote the development of cancer. This oxidative damage could be reduced or abated by antioxidant treatment [40].

Flavonoids are composed of conjugated ring structures with hydroxyl groups and have the potential to function as antioxidants [37]. Flavonoids use a variety of mechanisms to combat oxidation: scavenge free radicals, delay lipid peroxidation, or chelate transition metals. Flavonoids inhibit oxidases, which naturally promote the production of superoxide radicals, via their chemical structure and hydroxylation. Another common antioxidant behavior of flavonoids is the reduction of alpha- tocopherol radicals by hydrogen donation. These protective antioxidant effects, while normally demonstrated in plants, can be potentially beneficial in the study of human health and are the major point of interest in flavonoid research [27].

1.7. Flavonoids and subclasses in our study

We have shown that natural plant and synthetic flavonoids chemicals in previous studies, including various flavonoids such as flavanols (Quercetin), flavanones (Hesperidin and Naringenin), the rosemary and tea extract PARP inhibitor effect [6, 13, 41, 42]. These studies suggest changes in PARP inhibitory effect and selective cytotoxicity to *BRCA2* deficient cells due to the specific chemical structure. For example, Flavonol-like Quercetin is a better inhibitor of PARP than Hesperidin and Naringenin [42]. gallic acid and Carnosic acid also showed PARP inhibitor better than Carnosol, and Rosmarinic acid [6]. In our research, we are investigating thirteen different flavonoids as potential PARP inhibitors. In our study, we analyzed five flavanols (Galangin, Kaempferol, Quercetin, Myricetin, and Morin), Dihydroflavonols (Dihydrokaempferol, Taxifolin and Dihydromyricetin), flavanones (Pinocembrin), Dihydroflavanones (pinobanksin) and flavone (Luteolin). The basic structure of a flavonoid allows for a multitude of substitution patterns in the A, B and C rings, resulting in various subgroups. The flavonoids are divided into subclasses according to their oxidation level on the C ring, which

include anthocyanidins, flavanols (catechins), flavones, flavanols, flavanones and iso- flavonoids [24].

1.7.1 Flavanols:

Galangin

Galangin (4H-1-benzopyran-4-one,3,5,7-trihydroxy-2-phenylor3,5,7-trihydroxyflavone), pictured in Figure 1.6, is primarily derived from various medicinal herbs such as *Alpinia officinarum*, *Alnus pendula*, *Matsum* and *Plantago major*. It is used in Asian cultures as a treatment for many ailments such as cough, cold, gastrointestinal diseases, and diarrhea [43]. It is also responsible for antimicrobial activity of propolis, which is found in mouthwash and cosmetic products because of their anti-inflammatory, astringent, and antioxidant effects [44, 45]. Galangin is a derivate of flavanol, and it is the most lipophilic compound among the flavonoids [46, 47]. Several studies illustrate the multiple health benefits of Galangin: anti-cancer, antimicrobial, antioxidant, and anti-inflammatory properties; effects on bone, and hepatoprotective tendencies due to chemical structure[48]. Additionally, Galangin given to male and female rats at a high dose of 5g/kg and at lower doses of 500, 1000, 2000 mg/kg did not show any toxic effect for either gender [49]. Galangin also did not show any acute toxicity or mortality when given orally to female rats at a dose of 2000mg/kg for 14 days [48]. However, a different study reported that some propolis containing Galangin, caused allergic reactions [48].

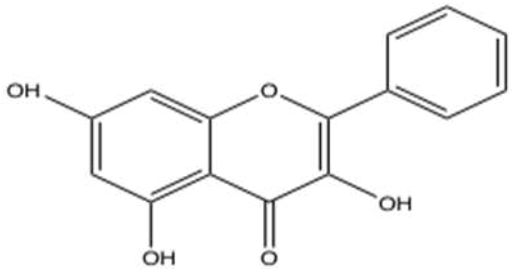


Figure 1.6: Chemical structure of Galangin.

Kaempferol

Kaempferol (3,5,7-trihydroxy-2-(4- hydroxyphenyl)-4H-1-benzopyran-4-one) pictured in Figure 1.7, is a yellow compound with molecular weight (286.2 g/mol) it found in fruit and vegetables such as broccoli, tomatoes, tea and grapes [50]. It has been estimated that the human dietary intake of this polyphenol may be up to approximately 10 mg/day [51]. It has also been estimated that the human dietary intake of this polyphenol may be up to approximately 10 mg/day [38, 50, 52]. Kaempferol may induce anti-mutagenic activity and genotoxic effects, so adequate safety procedures must be followed while working with this chemical [52]. Although flavonoids are known as antioxidants, these compounds can also act as pro-oxidants and can play important roles in genotoxic effects. Kaempferol at high concentration may generate ROS and increase the activity and toxicity of anti-cancer agents with pro-oxidant mechanisms [51, 52]. This biological effect induced by Kaempferol *in vitro* may not occur *in vivo* when Kaempferol is taken orally because the low oral bioavailability and high metabolism of Kaempferol in humans may be overcome by using different route [52].

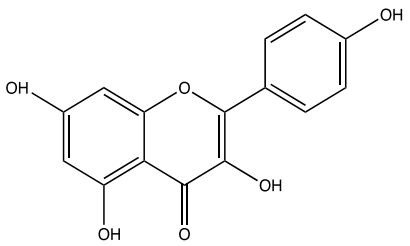


Figure 1.7: Chemical structure of Kaempferol.

Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxyflavone), pictured in Figure 1.8, is a plant-derived flavonol that is usually found in various foods such as onions, tomatoes, capers, peppers, cranberries, apples, and grapes. The name Quercetin stems from the Latin word "Quercetum" (oak woodland) and has been used since 1857 [51]. Quercetin received great attention because of previous health benefits attributed to Quercetin such as anti-cancer, potential antioxidant, anti-allergic, anti-inflammatory, anti-diabetic, anti-microbial, and cardioprotective activities [51]. Quercetin has exhibited cytotoxicity and genomic instability through inhibition of the growth and arrest of the cell cycle and induces sister chromated exchange and micronuclei in various type of cancer cells. In addition, it impairs DNA repair capability through PARP [13, 53, 54]. The average, daily intake of Quercetin is estimated to be between 25mg to 500mg per day [26] .

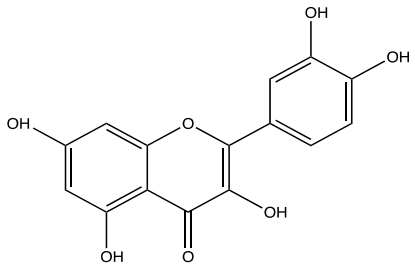


Figure 1.8: Chemical structure of Quercetin.

Myricetin

Myricetin (3,5,7, 3′,4′,5′- hexahydroxy flavone), pictured in Figure 1.9, is a naturally occurring flavonol. Myricetin is one of the main constituents of numerous human foods and drinks, including vegetables, teas and fruits [55]. Extensive studies show a biological benefit of myricetin is its anticancer properties [55]. Myricetin induces cytotoxic toward a number of human cancer cell lines such as hepatic, skin, pancreatic and colon cancer cells [55]. Myricetin also inhibits key enzymes involved in the initiation and progression of cancer. Research into the mode of action disclosed that cytotoxicity may be caused by the C2-C3-double bond, aromatic ring-B at C-2, and hydroxy groups in ring-B [43]. Beside its anti-cancer properties, Myricetin is widely recognized for its iron-chelating, antioxidant, and anti-inflammatory properties [56]. Myricetin was found to be non-toxic in several *in vivo* models; however, the results of these and several other studies also suggest that more toxicity studies should be conducted before Myricetin is included in cosmetic preparations and nutraceuticals [55, 57].

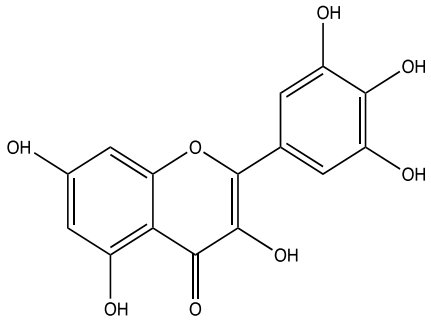


Figure 1.9: Chemical structure of Myricetin.

Morin

Morin (3,5,7,2040, -pentahydroxy flavone), shown in Figure 1.10, is a type of flavonoid found in old fustic, Osage orange, almonds, mill, onion and apples [58]. Morin is used in herbal medicines and is known to have certain biological characteristics, including antioxidant,

antiallergic, anti-inflammatory, antimutagenic, anticarcinogenic properties. Morin has been shown to be a power antioxidant with chelating capabilities of metal ions [58]. Morin exhibits anti-cancer activities by regulating cell proliferation and apoptosis in lung cancer, colorectal cancer, breast cancer and leukemia [47, 48]. Morin hydrate is toxicologically safe even in elevated doses for experimental animals and for extended administration [59].

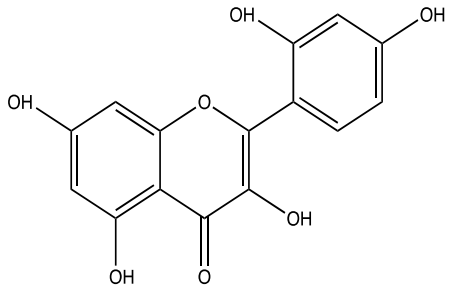


Figure 1.10: Chemical structure of Morin.

1.6.2 Dihydroflavonols or Flavanonols:

Dihydrokaempferol

Dihydrokaempferol is a member of the class of compounds known as flavanonols, also known as aromadendrin, show in Figure 1.11. Flavanonols are compounds comprising a flavan-3-one moiety with a structure characterized by a hydroxyl group bearing 2-phenyl-3,4-dihydro-2H-1-benzopyrene and a carbon C2 and C3 ketone respectively. Dihydrokaempferol can be found in European plums and tea, which makes dihydrokaempferol a potential biomarker for the consumption of these food products. Dihydrokaempferol is practically insoluble (in water) and a very weakly acidic compound (based on its pKa) [60].

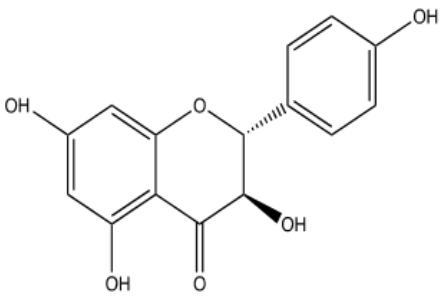


Figure 1.11: Chemical structure of Dihydrokaempferol.

Taxifolin

Taxifolin, also known as Dihydroquercetin, is a common fairly bioactive component found in food and herbs, which has shown to have a wide range of pharmacological and biochemical activity such as hepatoprotective, antidiabetic, cardioprotective, antitumor, neuroprotective and anti-inflammatory impacts, and has played a great part in preventing Alzheimer's disease (Figure 1.12). Taxifolin also has an efficient antioxidant activity that boots apoptosis promoted by a variety of anti-cancer and is accessible on the market as Venerations (semisynthetic form) [61].

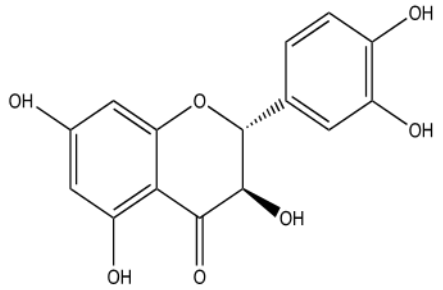


Figure 1.12: Chemical structure of Taxifolin or Dihydroquercetin.

Dihydromyricetin

Dihydromyricetin referred to *Ampelopsis grossedentata,* has a comparable structure to Myricetin (3,5,7- trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone), shown in figure 1.13. It is found in Chinese herb that is commonly distributed in south China, is used to treat cold. It

includes a wealthy resource of ampelosion which is the main flavonoid. A naturally occurring flavonoids found in grapes, berries, fruits, vegetables, herbs and other anti-cancer. As significant bioactive component of Ampelopsis grossedentata, it has been shown that Dihydromyricetin is primarily accountable for the reported biological activities, including these that are hypoglycemic, antioxidant and hepatoprotective effects. Dihydromyricetin has also improved the impacts of neutrophilic granulocytes and monocytes by enhancing chemokinesis and chemotaxis. Moreover, it has been demonstrated that Dihydromyricetin has certain anti-cancer properties [62].

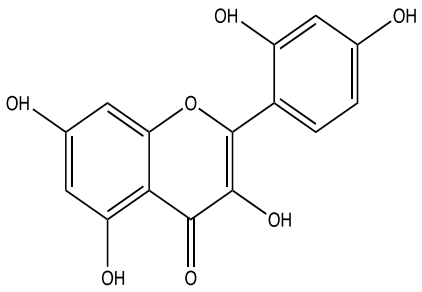


Figure 1.13: Chemical structure of Dihydromyricetin.

1.6.3 Flavanones:

Pinocembrin

Pinocembrin (5,7- dihydroxyflavanone, Pino), pictured in Figure 1.14, is one of the most abundant flavonoids, found in high concentration in propolis. Although pinocembrin is widely found in honey and various plants, there is still insufficient yield from natural extraction. Pinocembrin is used mainly to treat ischemic stroke [60]. Recent studies, however, have shown that pinocembrin can have therapeutic effects on Parkinson's disease (PD) and Alzheimer's disease (AD) [60]. There is also anti-pulmonary fibrosis and vasodilating effect in Pinocembrin. Pinocembrin can also reduce blood brain barrier damage and neurological injury by reducing level of reactive oxygen species (ROS) and inflammatory factors [60]. Furthermore, Pinocembrin can

maintain mitochondrial integrity by activating the pathway2-related factor 2(Erk1/2-Nrf2) extracellular signal- regulated kinase/ nuclear factor. Pinocembrin also inhabit apoptosis by disrupt the p53 pathway, thereby affecting the Bax-Bacl2 ratio and cytochrome C output. Antioxidant and anti-inflammatory activities are the basic for specific Pinocembrin pharmacological [60].

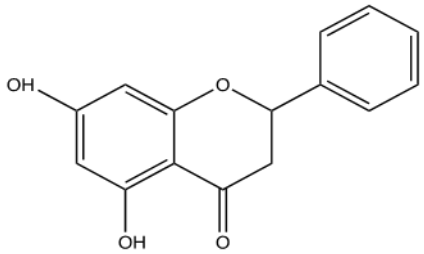


Figure 1.14: Chemical structure of Pinocembrin.

1.7.3Dihydroflavanones

Pinobanksin

Pinobanksin(3,5,7-trihydroxy-2-phenyl-chroman-4-one), shown in Figure 1.15, widely distributed in honey and propolis. The key propolis and honey flavonoids, pinocembrin, and pinobanksin are effective antioxidants. Pinobanksin is reported to induce apoptosis in a B-cell lymphoma cell line [59, 63].

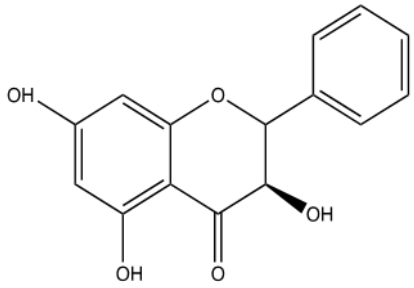


Figure 1.15: Chemical structure of Pinobanksin.

1.7.4 Flavones:

Luteolin

Luteolin (3′,4′,5,7-tetrahydroxy flavone), is shown in Figure 1.16 has a composition of C6-C3-C6 comprising two rings of benzene and one ring containing oxygen with a double bond of carbon C2-C3 (Figure 1.1) basic chemical structure for flavonoids. Studies of structure-activity have shown that the existence of hydroxyl molecules at carbon 5, 7, 3′ and 4′ luteolin structure positions and the presence of the 2–3 double bond are responsible for the compound's multiple pharmacological impacts. Luteolin is one of the most commonly found flavonoids in edible plants. It was identified in celery, carrots, peppers, olive oil, lettuce, chocolate, tea, rosemary, peppermint, thyme and cucumber [64]. Luteolin's pharmacological behaviors may be functionally interrelated. For example, luteolin's anti-inflammatory effect may also be associated with its anticancer role. Luteolin's anticancer property is correlated with apoptosis inducing redox control, DNA damage, and protein kinases inhibiting cancer cell proliferation and suppressing metastasis. Luteolin is especially permeable to the blood-brain barrier, making it relevant to the treatment of diseases of the central nervous system, including brain cancer [65] .

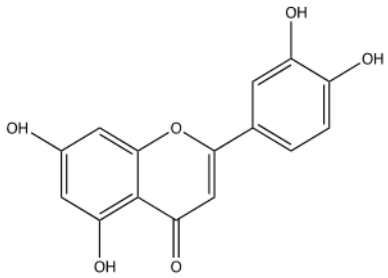


Figure 1.16: Chemical structure of Luteolin.

1.7.5 Flavan-3-ols:

Epigallocatechin gallate

One of the most common and effective types of catechin in green tea is Epigallocatechin gallate (EGCG), shown in Figure 1.17. Green tea is high in catechins relative to other drinks. 100 mL of green tea (1 g of dry tea leaves brewed for 5 min in 100 mL of hot water) contains 67 ± 11 mg of total catechins, including about 30 mg of Epigallocatechin gallate (EGCG), while black tea contains 15.4 mg of catechins. Many beneficial effects of EGCG on cognitive function and oxidative damage have been documented [66]. There are other health benefits of green tea which include cancer prevention, anti-obesity, anti-diabetes, and neuroprotective effects. Catechins antioxidant and metal chelating, anti-carcinogenic, anti-apoptotic pro-apoptotic, and anti-inflammatory activities are closely linked to their beneficial health effects, including the prevention of neurodegenerative diseases [66]. High oral doses of green tea polyphenol, EGCG, was found to be hepatotoxic in mice in this research [67]. This apparent toxicity is associated with oxidative stress activation in the liver. The levels at which toxicity was observed are much higher than those usually administered by ordinary tea intake, but in the sense of tea-based dietary supplements they are more readily achievable [67].

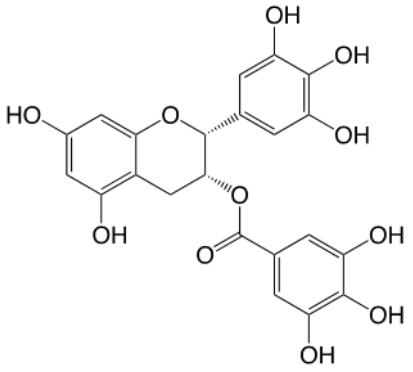


Figure 1.17: structure of Epigallocatechin gallate.

Theaflavin

A further class of polyphenol pigments found in black and oolong teas is Theaflavins in (figure 1.18). Theaflavins contributes to the distinctive bright orange-red color of black tea, reflecting 2 g/100 g of black tea's dried water extract. The major Theaflavins in black and oolong tea are Flavin (TF1), Theaflavin-3-gallate (TF2A), Theaflavin-3′-gallate (TF2B) and Theaflavin-3,3′-digallate (TF3). Both Catechins and TF have recently received a lot of attention as cardiovascular disease and cancer protection agents. They are also thought to have a wide range of other pharmaceutical advantages, including antihypertensive, antioxidant and hypolipidemic activities. It should have at least a 3-hydroxy group on an unsaturated C ring or a 2,3-double bond with the 3-OH group and 4-one in the C ring or an ortho-OH substitution pattern in the B ring where the OH groups are not glycated. The first and second structure complies with catechin and epicatechin molecules [67].

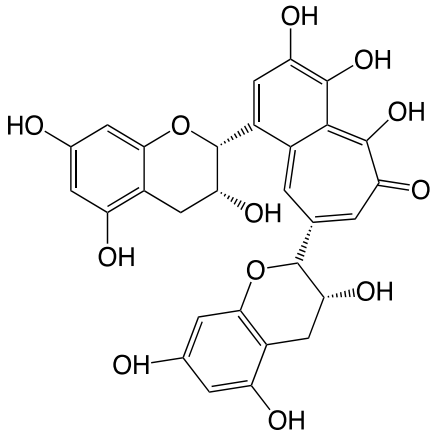


Figure 1.18: Chemical structure of Theaflavin.

In our study we used a cytotoxicity assay and genotoxicity assay and *in vitro* and in *vivo* PARP activity inhibition assays with different subclasses of flavonoids to determine which subclasses of flavonoids will be best PARP inhibitor.

1.8 What is cytotoxicity?

Cell viability levels and/or rates of cell proliferation are good methods to determine cellular health. Cell health and metabolism may be influenced by both physical and chemical agents. These agents may trigger cellular toxicity via various mechanisms such as cell membrane destruction, protein synthesis prevention, irreversible binding or receptors, polydeoxynucleotide elongation inhibition, and other important enzymatic reaction involved in cell death, cytotoxicity and genotoxicity [68]. Reliable, cheap, and reproducible short-term cytotoxicity and cell viability assays are need to determine the cell death caused by these mechanism [68]. There are some benefits to *in vitro* cytotoxicity and/or cell viability assays, such as speed, reduced cost, and automation potential and research with human cells may be more important than some *in vivo* animal testing. Nonetheless, there are some drawbacks in replacing animal tests simply because they are not yet technologically advanced enough [68] . In our study we used a colony formation assay, cell cycle analysis and cell growth inhibition to determine cellular health.

1.9 Assays:

1.9.1 Colony formation assay

The main goal of therapeutic radiotherapy and chemotherapy is to prevent tumor cell growth, which is why the endpoint of cell survival is widely used in *in-vitro* experiments [69]. The most important cell survival factor is cell viability, which is a cell's ability to maintain its physical and metabolic integrity and its clonogenicity, which is a cell's ability to undergo cell division [69-71]. It is only necessary to "kill" cells in the sense that they are rendered unable to divide and cause further growth and spread of malignancy in order to remove a tumor. The test utilized was an *in vitro* cell survival assay, known as the clonogenic assay or colony formation assay and it is based on a single cell's ability to grow into a colony following treatment. A surviving cell that has

retainedit's reproductive integrity and can proliferate indefinitely to create a colony, described as a group of at least 50 cells, is considered to be clonogenic [69-71]. Puck and Marcus described this technique for the first time in 1956 to test the ability of a single mammalian cell to form colonies. Their studies described the first x-ray irradiated radiation-dose survival curve for HeLa cells [70, 71]. Results from their experiments showed that these mammalian cells were much more radiosensitive than previously assumed for tissue cells and had an average lethal dose range of 1-2 Gy, paving the way for future studies to incorporate this approach [70, 71].

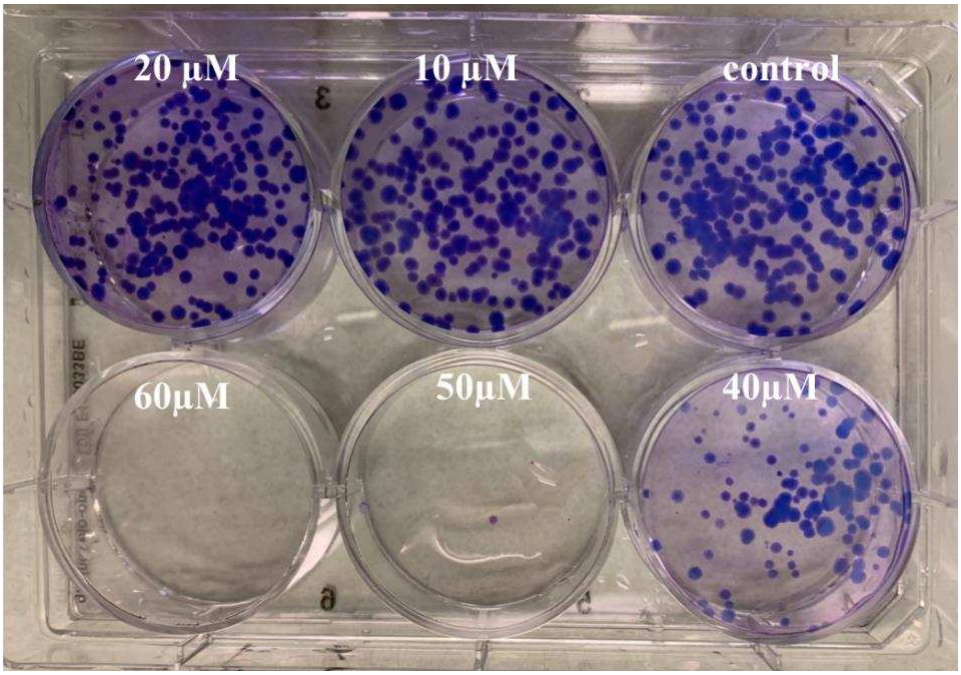


Figure 1.19: Effects of flavonoids on VC8 cell colony formation, counted 10 days post treatment

1.9.2 Cell cycle and flow cytometer

Two of the most popular cytometric flow applications are cell DNA content measurement and cell cycle analysis. A sample's cell cycle profile can be determined by immunostaining and the measurement of the brightness of DNA is done with fluorescent dye. The dye stains DNA

stoichiometrically, allowing cell differential in step G0/G1, S and G2/M as well as aneuploid populations to be identified. With differentiation sample forms, a number of staining procedures can be modified, but the basic method remains the same [72].

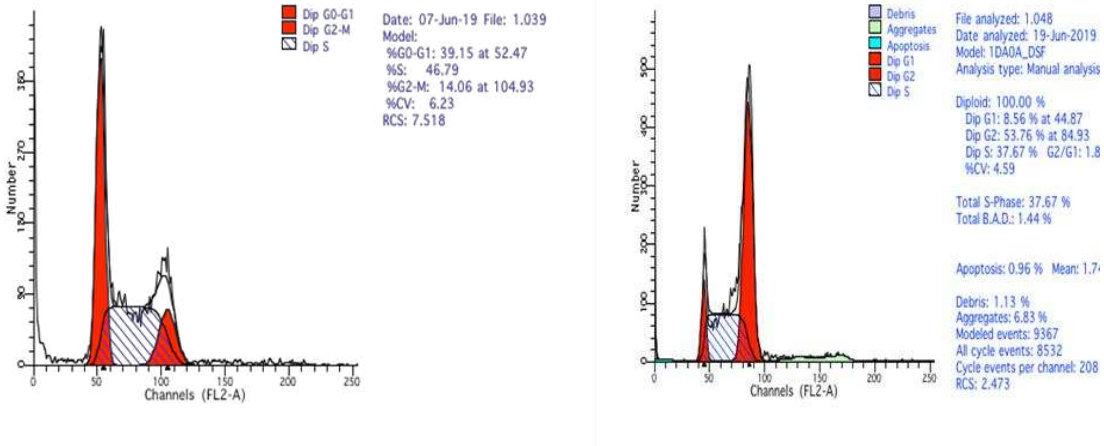


Figure 1.20: (**A**) Distribution of cells between cell cycle phases for control sample. (**B**) Distribution of cells between cell cycle phases for 51D1 (HR mutant) cells after treatment with 10µM of Kaempferol. Accumulation of 51D1 cells in the G2/ M phase as compared to control is observed.

1.9.3 Genotoxicity

Most genotoxicity experiments, both *in vitro* and *in vivo*, have been found to detect DNA damage or biological effects in prokaryotic (e.g. bacterial) and eukaryotic (e.g. mammalian, avian or yeast) cells with a variety of DNA damage. Such studies are used to determine the safety of industrial chemicals and consumer products and to examine the action mechanisms of known or suspected carcinogens [73]. A genotoxin is a chemical or agent that can cause DNA or chromosomal damage. This disruption if it occurs in germ cells has the potential to cause a heritable altered phenotype (germline mutation). DNA damage in a somatic cell can result in a mutation that can lead to cancer transformation. In our study, we used two genotoxicity assays:

the micronuclei assay and sister chromatid exchange to determine if flavonoids will be caused DNA damage.

1.9.4 Micronuclei assay

Micronuclei (MN) are small extranuclear bodies arising from chromosome breaks during anaphase (Figure 1.21). In the first interphase after cell division [74, 75]. Adding the cytoplasmic division blocker, cytochalasin B, during cell culture binucleated cells (BN) are often observed. Chromosome break or entire chromosomes that delay anaphases result in them not being integrated into the nuclei of the sister chromatid, resulting in the development of small extranuclear extranuclear bodies known as micronuclei [74]. Such MN have a distinctive nuclear structure composed of two membranes: the lamina and the nuclear pores. MN formation is an indicator of a genotoxic event or chromosomal instability. Mitotic cell death and mitotic catastrophe are both major causes of death in solid tumor cells [69, 75]. Initially in MN formation, the cycle is characterized by chromosome mis-segregation accompanied by aberrant mitosis or incomplete chromosome segregation, resulting in multinucleated cell formation. MN expression allows a cell to undergo a mitotic nuclear division and the preferred method for quantitative micronuclei evaluation is the cytokinesis block micronucleus (CBMN) assay developed by Fenech and Morley in 1985 [64,63]. The CBMN assay are developed to enable the evaluation of micronuclei in binucleated cells. CBMN assay cells were incubated with cytokinesis inhibitor Cytochalasin B in a concentration that allowed cytokinesis to be inhibited but still permitted karyokinesis. In contrast, Cytochalasin B did not produce MN on its own [74, 75]. It enables binucleated cells to be created that can be easily detected under a microscope, and MN expression occurs to the same extent as with normal cell division [69, 75, 76].

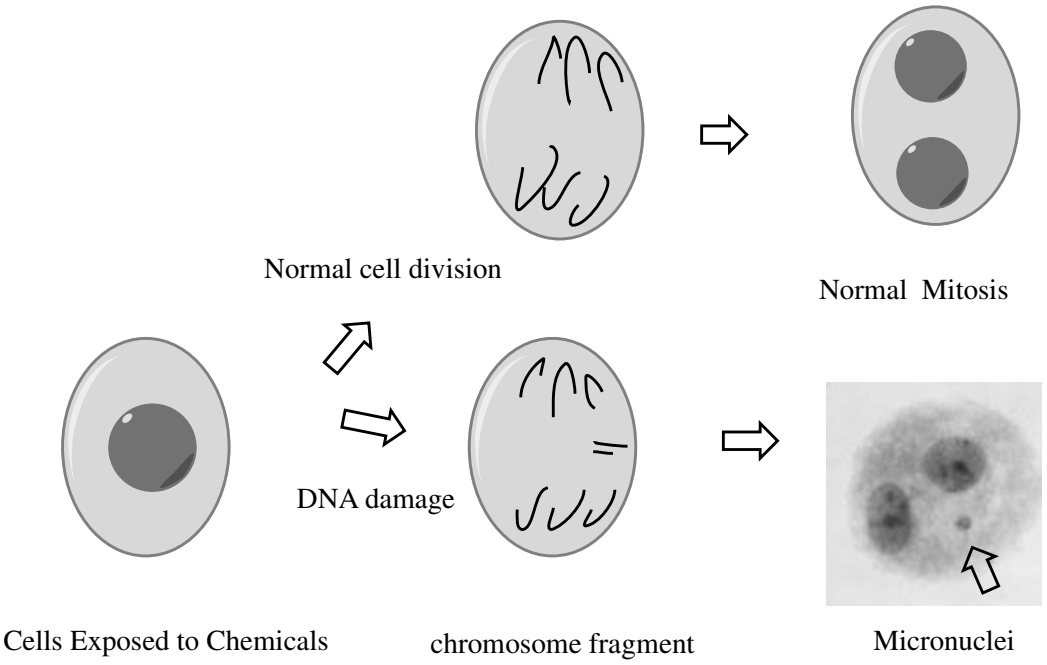


Figure 1.21: Shows how cells exposed to DNA damaging chemicals can result in chromosome fragmentation and micronuclei formation.

1.9.5 Sister Chromatid Exchange

The most developed means of detecting dysregulated homologous recombination, whether in cells with defective or deficient HR ability or in response to injury, is the sister chromatid exchange test (SCE). This method differentially stains sister chromatids, allowing for microscopic detection of the physical exchange of DNA which occurs with HR crossover [77]. The SCE experiment has been in use since the 1970s to identify potential chemical agents' "chromosomal mutagenicity." Chemicals that generate inter-strand cross-links, such as Mitomycin C, are powerful SCE inducers, as HR is necessary to repair the resulting blockage during replication [77]. Drugs and conditions that increase the number of SSBs also increase the number of SCEs, likely by overburdening the BER pathway to retain unrepaired SSBs, becoming DSBs during replication and triggering HR repair [77]. In the sister chromatid exchange protocol, we used fluorescence plus Giemsa (FPG) staining and 5-Bromo-2′-deoxyuridine (BrdU) incorporation to make exchange

between sister chromatids visible. BrdU is a thymidine-like nucleoside analog that is effectively integrated into DNA replication [77]. DNA replication is semiconservative and is introduced as the nascent strand is elongated after BrdU has been made available to cells. After two rounds of cell replication, during metaphase, the sister chromatids differ in the amount of BrdU incorporated into each strand [77]. One sister chromatid has one strand with BrdU substitution, while in the other sister chromatid both DNA strands have BrdU substitutions. The subsequent introduction of ultraviolet (UV) light-absorbing Hoechst 33258 dye into the DNA, accompanied by exposure to UV light, causes the DNA to be 'bleached' in proportion to the amount of BrdU incorporated into the double-stranded molecule, possibly due to free-radical damage. The subsequent staining with Giemsa of the UV-treated chromosomes renders this differential bleaching visible through a light microscope (Figure 1.22) [77]. BrdU resembles thymidine closely and is easily inserted during replication into elongating DNA strands [77].

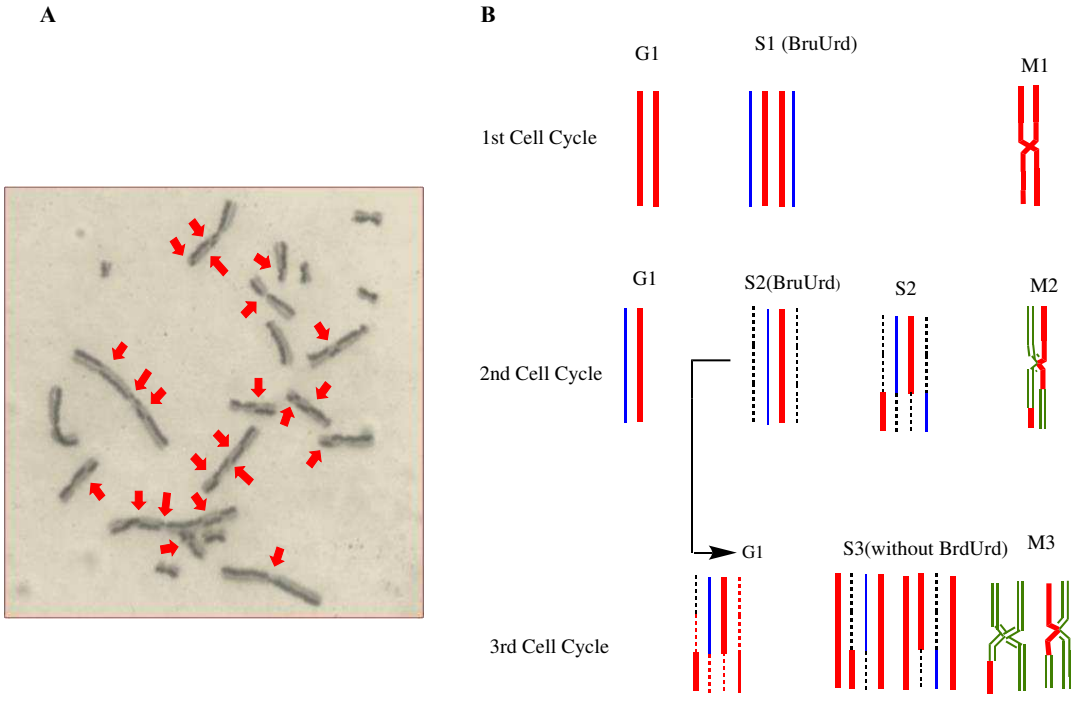


Figure 1.22: (**A**) Flavonoid-induced sister chromatid exchange indicated with red arrows. (**B**) Explains how sister chromatid exchange occurs within cellular DNA [78].

CHAPTER TWO:

The Effect of Green and Black Tea Polyphenols on BRCA2 Deficient Chinese Hamster Cells by Synthetic Lethality through PARP Inhibition

2.1 Introduction

Tea is the most widely consumed beverage in the world, after water, and it is sold in three major forms: green, oolong, and black. All three teas come from the same plant, *Camellia sinensis,* and the only difference between the three types of tea is the level of oxidation polyphenols, a major class of molecules in tea leaves, by fermentation [79]. Green tea is prevented from oxidizing at all, oolong is only partially oxidized, and black tea polyphenols are entirely oxidized in a process known as fermentation. Catechins, members of the flavanol family, are heavily prevalent in green teas and are excellent antioxidants that have been observed to scavenge free radicals and reduce oxidative stress [79]. The concentration of polyphenols in green tea are measured to be 200 to 300 mg/cup while concentrations found in black tea are ~16–24 mg/cup [80, 81]. Epigallocatechin gallate (Figure 2.1A) is an unoxidized polyphenol found in green tea and is considered one of the most potent dietary antioxidants within green tea [82]. When black tea is manufactured, the catechin undergoes oxidation into quinone, which is further condensed into several other chemicals, one of which is Theaflavin (Figure 2.1). Theaflavin is responsible for the characteristic color and taste of black tea, and theaflavin levels are directly correlated with the quality and taste of the tea [79]. The health effects of both types of tea have been previously studied; one study showed that daily consumption of green tea decreased the number of lymph node metastases and decreased the reoccurrence of stage I and stage II breast cancers [83]. A comprehensive meta-analysis confirmed that consumption of green tea significantly lowered the risk of breast cancer

while results for black tea consumption were mixed [84]. It is possible that some of the beneficial effects of green and black tea are due to the high concentrations of antioxidants in the form of flavonoids. Polyphenols have long been known to contain many health benefits. Polyphenols have a low molecular weight, are phenolic, and are found in most fruits, vegetables, nuts, herbs, and teas. The major benefits of polyphenols have been most documented in the treatment or prevention of adverse health effects and diseases such as diabetes, inflammation, and cardiovascular disease. They have also been shown to exhibit antiproliferation, apoptotic, and angiogenesis inhibiting effects in cancer cells, resulting in an observable level of antitumor capabilities [85, 86]. Moreover, tea polyphenols have also been reported for their protective action against ionizing radiation by their antioxidant capacity [87].

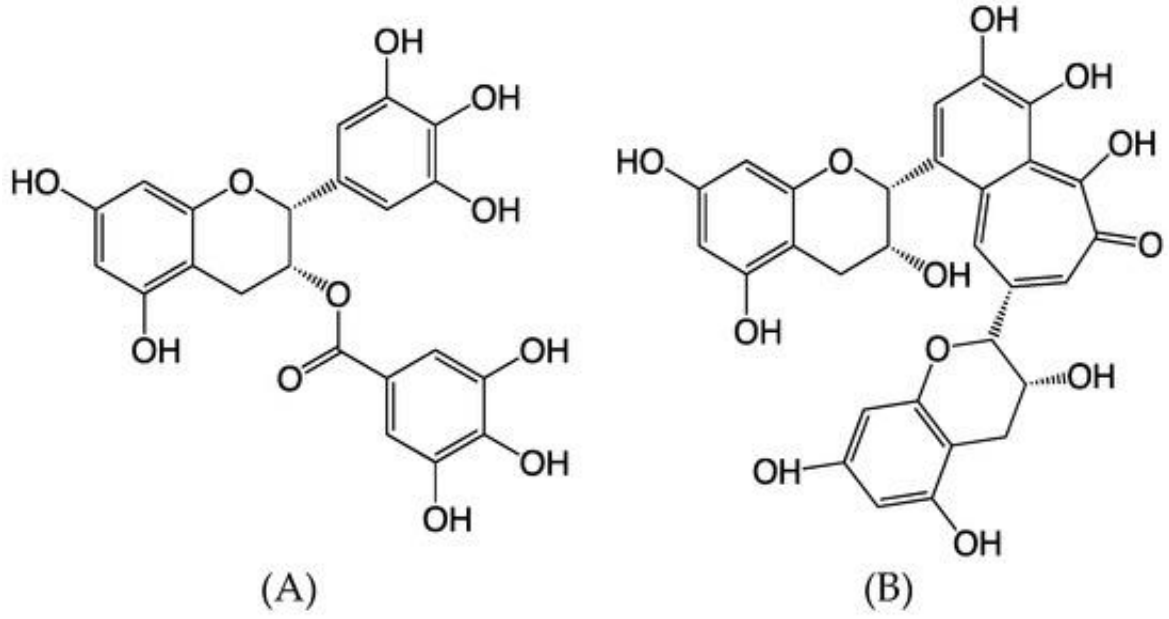


Figure 2.1: Chemical structures of tea polyphenols: (**A**) Epigallocatechin gallate. (**B**) Theaflavin.

It has been observed that many polyphenolic flavonoids can induce PARP inhibition [88, 89]. PARP proteins are essential for the DNA repair process known as base excision repair (BER)

and have strong downstream effects when inhibited [90]. One downstream effect of PARP inhibition is observed by selective cytotoxic effects. Specifically, it is observed that PARP inhibition is selectively lethal towards *BRCA2* deficient cell cultures and tumors. BRCA2, like PARP, is an important protein in DNA repair. However, unlike PARP, *BRCA2* is primarily involved in the repair of double stranded DNA lesions through a pathway known as HR repair. HR repair is mediated by many proteins including BRCA1, BRCA2, and rad51D. Inhibition or mutation of any of these proteins can result in the inaccessibility of the HR pathway by cells to repair double stranded damage. When this occurs, cells are forced to utilize other more error prone and dangerous pathways, such as non-homologous end joining (NHEJ) repair. Due to the essential functions of both PARP and BRCA2, the loss of activity of both simultaneously can result in cellular death through a process known as synthetic lethality [91]. Synthetic lethality is a result of an accumulation of single strand DNA breaks, which if not corrected through BER, can result in the subsequent formation of double stranded DNA breaks through replication machinery failure. Repair of double stranded DNA breaks through pathways like NHEJ can cause further mutation and can result in cell death. Cancers with *BRCA2* homozygous mutations have been proven to be very sensitive to treatment with PARP inhibitors like Olaparib [92].

The objective of this study was to determine which polyphenols in tea, Epigallocatechin gallate or Theaflavin, contained the highest level of selective cytotoxicity towards *BRCA2* deficient cells through inhibition of PARP. In order to test our hypothesis, Chinese hamster V79 cells, their *BRCA2* deficient mutant V-C8 cells, and V-C8 gene complimented cells were utilized along with Chinese hamster ovary (CHO) cells and rad51D mutated 51D1 cells. Both cell lines are isogenic.

2.2 Materials and Methods:

2.2.1 Cell Culture

Chinese hamster lung origin V79 and its DNA repair deficient V-C8 (*BRCA2* deficient) [93], and their gene complimented V-C8 cells, Chinese hamster ovary origin CHO10B2 cells were kindly supplied by Dr. Joel Bedford of Colorado State University (Fort Collins, CO, USA). CHO DNA repair deficient 51D1 (*rad51D* deficient) cells [94] were kindly supplied by Dr. Larry Thompson at the Lawrence Livermore National Laboratory (Livermore, CA, USA). Cells were maintained in Alpha MEM (Hyclone, ThermoFisher, Waltham, MA) with 10% heat inactivated Fetal Bovine Serum (Sigma, St. Louis, MO), antibiotics (Anti-Anti; Invitrogen, Grand Island, NY) and were cultured in 37°C incubators with 5% CO_2 and humidity.

2.2.2 Chemicals

Green extract Epigallocatechin gallate and black tea extract Theaflavin were purchased from Sigma Aldrich (St Louis, MO, USA). These slightly nonpolar polyphenols were dissolved in DMSO as 10 mM or 100 mM stock solution and stored at -20°C.

2.2.3. Clonogenic cell survival

Cells were plated in 6 well plates to form colonies. Various concentration of tea extract was added to cell culture media and cells were allowed to incubate for 1 week. Following incubation, colonies were fixed and stained using 100% ethanol followed by 0.1% crystal violet. Macroscopic colonies containing more than 50 cells were considered as a survivor [88]. Regression curves were drawn from cell survival fraction by Graphpad Prism 6 software (GraphPad, La Jolla, CA). IC_{50} values (drug concentration to achieve 50% cell survival) were obtained from regression curves.

2.2.4. Growth inhibition

For the capacity of the wells ten thousand cells were plated onto each well of 12 well plates with 10 μM of chemicals and cell number was counted by Coulter Counter Z1 (Beckman Coulter, Indianapolis, IN) every 24 hours for 4 days. Cell doubling time was calculated by using GraphPad Prism 6 with exponential growing equation from exponentially growing stage.

2.2.5 In vitro and *in vivo PARP activity inhibition assay*

PARP colorimetric assay kits (Trevigen, Gaithersburg, MD) were used to measure *PARP* activity as previously described [6]. PARP was incubated in a 96-well microplate with a reaction mixture containing 50 μM β-NAD+ (10% biotinylated β-NAD+), 90% unlabeled β-NAD+, 1 mM 1,4-dithiothreitol and 1.25 mg/ml nicked DNA. The formation of poly (ADP-ribose) polymers was detected with peroxidase-labeled streptavidin and 3,3',5,5'-tetramethylbenzidine (Invitrogen, Carlsbad, CA). PARP inhibition was assessed by the addition of flavonoids at various dosages to the reaction mixture. PARP activity directly proportional to absorbance at 450 nm and measured by a NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA). IC_{50} values of *PARP* inhibitory activity were derived by fitting dose-response curves using a sigmoidal dose response equation.

In vivo PARP activity inhibition was carried out as previously described [6]. V79 were treated with polyphenols for 30 minutes before exposure to 2 mM hydrogen peroxide for minutes. After hydrogen peroxide treatment, cells were washed with PBS and fixed in 4% paraformaldehyde for 15 minutes, followed by 0.2% Triton X-100 in PBS for 10 minutes. After blocking with 10% goat serum in PBS, anti-poly (ADP-ribose) mouse monoclonal antibody diluted in 10% goat serum was added to the cells and incubated for 1 hour at 37°C. Alexa 488 conjugated anti-mouse antibody was used for the secondary antibody for fluorescence signal detection. After

DNA staining with DAPI in slowfade (Thermo Fisher Scientific), fluorescence images were obtained by Zeiss Axiophot fluorescent microscope equipped with Q-imaging Exi Aqua cooled CCD monochrome camera with Q-capture Pro software (Q-imaging, Surrey, BC, Canada). Green pixels per cell was scored for a minimum of 30 cells.

2.2.6. Sister Chromatid Exchange

CHO cells were synchronized into the G1 phase using a mitotic shake-off procedure [95, 96]. Synchronized mitotic cells were subculture in 6 well plates and incubated for two hours at 37°C. Cells were then treated with various concentrations of testing chemicals and incubated with 10 μM of BrdU (Sigma) for two cell cycles. 0.2 μg/ml of colcemid (Gibco) was added to cells and allowed to incubate for an additional 6 hours. Cells were harvested during metaphase, trypsinized, and then suspended in 2 ml of 75 mM KCl solution warmed to 37°C and placed in a 37°C water bath for 20 min. A fixative solution of 3:1 methanol to acetic acid was added to the samples according to the standard protocol [97]. Fixed cells were dropped onto slides and allowed to dry at room temperature. Differential staining of metaphase chromosomes was completed using the fluorescence plus Giemsa technique with Hoechst 33258 dye [98]. Differentially stained metaphase chromosome images were scored under a Zeiss Axioskop microscope equipped with SPOT CCD camera RT 2.3.1 (Diagnostic Instrument, Inc.) and SPOT basic software. A minimum of 50 metaphase cells were scored for each treatment concentration. Data presented are the mean of SCE frequency per chromosome.

2.2.7. Micronuclei formation assay

Cells were synchronized into G1 phase by mitotic shake off method. Two hours after shake off, testing drugs were added to the cell culture with 4 μg/ml of Cytochalasin B (Sigma, St Louis, MO) for 22 hours [99]. Harvested cells were suspended in 2 ml of 75 mM KCl solution and fixed

in 1 ml of 3:1 methanol: acetic acid solution. Cells were dropped onto slides and allowed to air dry at room temperature. Slides were stained in 5% Giemsa solution in GURR solution (Invitrogen) for 5 min. 100 binucleated cells were scored per data points under Zeiss Axioskop microscope to obtain micronuclei frequency per binucleated cells.

2.2.8. Statistics

All experiments were carried out at least three times and error bars indicate standard error of the means. Data was analyzed using Prism 6 software for one-way *ANOVA* analysis. P-values <0.05 were categorized as significant differences.

2.3 Results:

2.3.1 Clonogenic Cell Survival

To determine if these polyphenols influence survival of BRCA2 deficient cells, Chinese hamster lung origin cells were treated with various concentrations of each polyphenol and were incubated until colonies were formed. Treatment of cells by epigallocatechin gallate strongly suppressed clonogenic activity for BRCA2 deficient V-C8 cells compared to wild type V79 cells and gene complimented cells (Figure 2.2 A,B). The IC_{50} values were 57.1, 55.6, and 29.9 µM for V79, gene complimented cells, and V-C8 cells, respectively. The survival fraction at 50 µM showed statistically significant difference for V-C8 cells compared to V79 and gene complimented cells ($p < 0.05$). Similarly, treatment of cells by theaflavin also strongly suppressed clonogenic activity for *BRCA2* deficient V-C8 cells compared to wild type V79 cells and gene complimented cells. The IC_{50} values were 79.7, 80.0, and 54.3 µM for V79, gene complimented cells, and V-C8 cells, respectively. The survival fraction at 100 µM showed statistically significant difference for V-C8 cells compared to V79 and gene corrected cells ($p < 0.05$). Therefore, both Epigallocatechin

gallate and theaflavin presented selective cytotoxicity toward to *BRCA2* deficient cells (Figure 2.2 C,D).

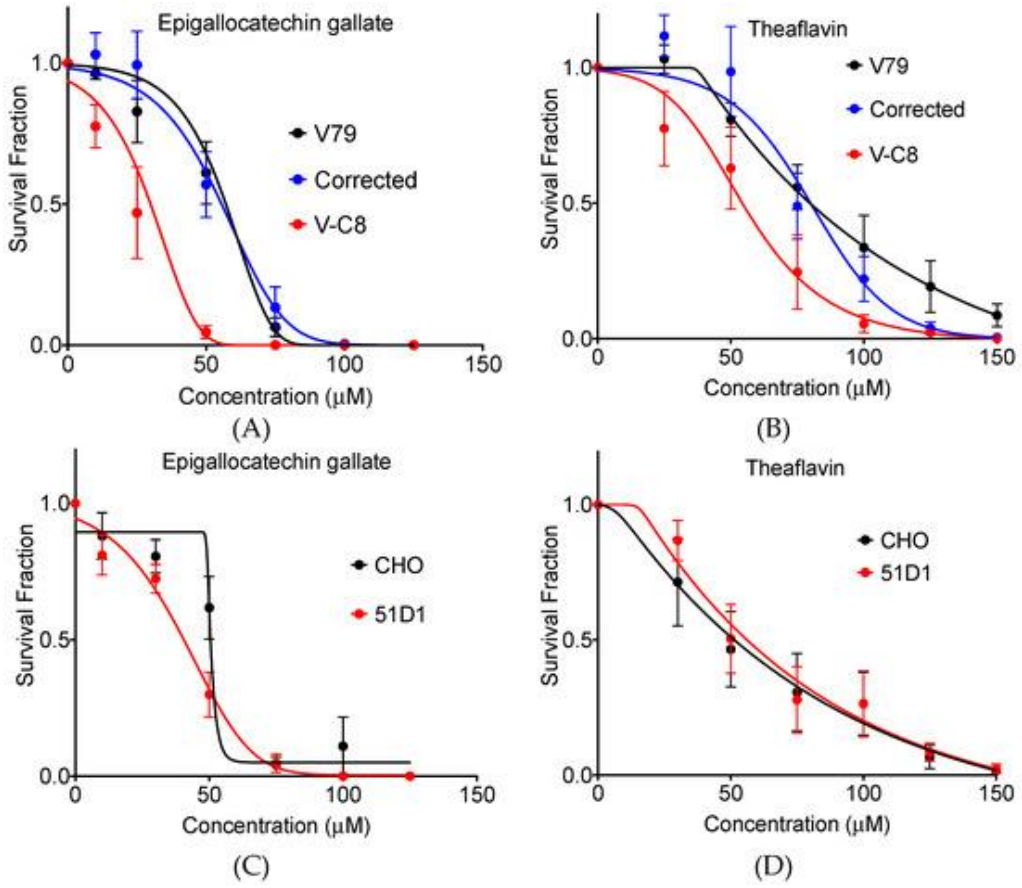


Figure 2.2: Clonogenic cell survival curves against tea polyphenol Epigallocatechin gallate and Theaflavin. (**A**) Epigallocatechin gallate toxicity to V79 cells, V-C8 cells, and V-C8 gene complimented cells. (**B**) Theaflavin toxicity to V79 cells, V-C8 cells, and V-C8 gene complimented cells. (**C**) Epigallocatechin gallate toxicity to Chinese hamster ovary (CHO) wild type cells and 51D1 cells. (**D**) Theaflavin toxicity to CHO wild type cells and 51D1 cells. Error bars represent standard error of the means. At least three independent experiments were carried out.

In order to expand this finding to other HR repair deficient cells, CHO wild type cells and rad51D mutated 51D1 cells were utilized. rad51 and BRCA2, as described earlier, are essential for HR repair function. Therefore, if both polyphenols showed selective cytotoxicity to 51D1 cells, the polyphenol effects can be expanded to all HR repair defective cells. Treatment of cells by

Epigallocatechin gallate suppressed clonogenic activity for *rad51d* deficient 51D1 cells compared to wild type CHO cells. The IC_{50} values were 50.2 and 40.1 μM for CHO and 51D1 cells, respectively. The survival fraction at 50 μM showed statistically significant difference for 51D1 cells compared to CHO10B2 cells ($p < 0.05$). However, treatment of cells by theaflavin did not suppress clonogenic activity for 51D1 cells compared to wild type CHO cells. The IC_{50} values were 50.0 and 54.2 for CHO and 51D1 cells, respectively. The survival fraction did not show a statistically significant difference between CHO wild type and 51D1 cells for theaflavin treatment. Therefore, tea polyphenol epigallocatechin gallate may present selective cytotoxicity to other HR deficient cells but theaflavin may be more limited to *BRCA2* deficient cells.

2.3.2 Growth Delay and Cell Cycle Analysis

Growth delay by tea polyphenols was tested by adding 10, 30, and 50 μM of the polyphenols to cell cultures. The number of cells was recorded daily and population doubling hours were obtained (Figure 2.3). For V79 cells, treatment of cells with both Epigallocatechin gallate and Theaflavin showed polyphenol concentration dependent growth delay. The basal doubling time was 12.9 h for V79 and 17.4 h for V-C8. No delay or a minor delay was observed for 10 μM of Epigallocatechin gallate treatment and 10 and 30 μM of theaflavin for V79 system cell lines. However, 50 μM of Epigallocatechin gallate or Theaflavin, particularly 50 μM of Epigallocatechin Gallate, strongly suppressed cellular growth. There was no clear cell line dependency in this aspect of the study. Statistically significant cell doubling time delay was observed for 50 μM of Epigallocatechin gallate treated V79 and V-C8 cells (both $p < 0.05$).

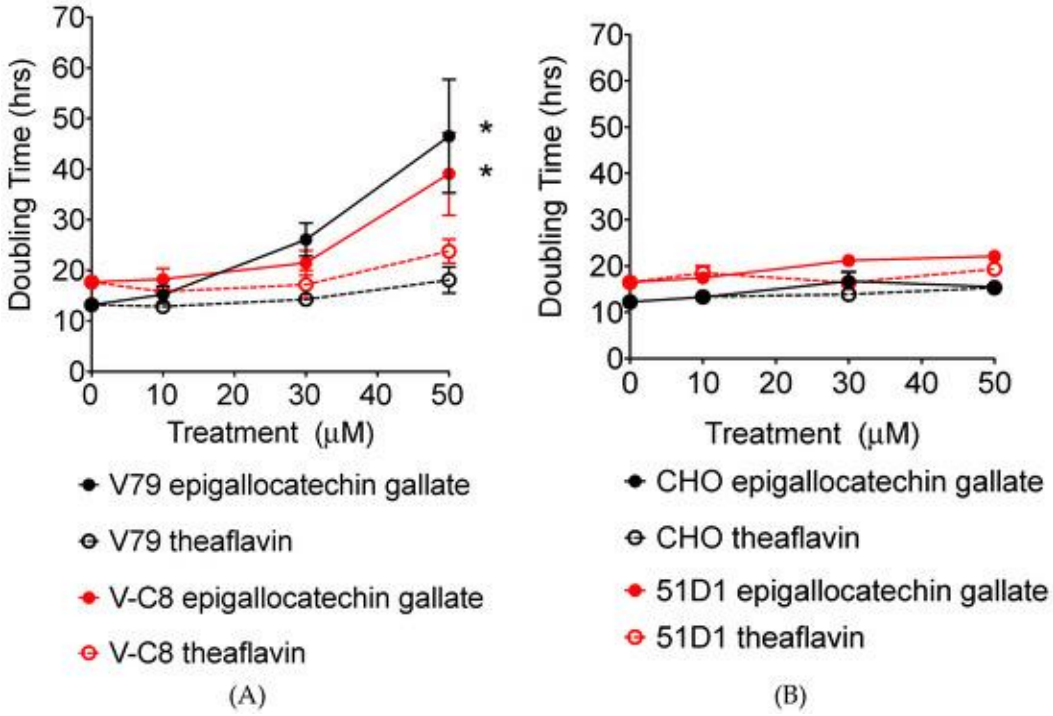


Figure 2.3: Cell population doubling time after polyphenol treatment: (**A**) V79 and V-C8 cells, (**B**) CHO and 51D1. Values are mean ± standard error of the means. (*) indicates statistically significant differences from control ($p < 0.05$).

For CHO and 51D1 cells, the effect of polyphenol for cell doubling time was much smaller than for the V79 cell system. A concentration dependent cellular growth delay was observed for both polyphenols and cell types. The basal doubling time was 12.1 h for CHO and 16.4 h for 51D1. The 50 μM of Epigallocatechin and Theaflavin increased CHO doubling time to 15.5 h and 15.2 h, respectively, and increased 51D1 doubling time to 22.1 h and 19.2 h, respectively. DNA repair deficiency specific growth delay was not observed for tested conditions.

2.3.3: In vitro and in vivo PARP Activity Inhibition

In vitro polyphenol effects for PARP activity were analyzed with *in vitro* PARP activity kits (Figure 2.4A). Quercetin was used as positive control. As predicted from their chemical structures, it was identified that Epigallocatechin gallate and theaflavin showed PARP inhibitory effects and significant inhibition at 10 μM ($p < 0.05$). Statistically, theaflavin showed the stronger

reduction of PARP activity (0.20, $p < 0.0001$) than Epigallocatechin gallate (0.45, $p < 0.0002$). PARP inhibition effects by tea polyphenols were comparable to Quercetin, which was formally reported for the natural flavonoids with strong PARP inhibition [10].

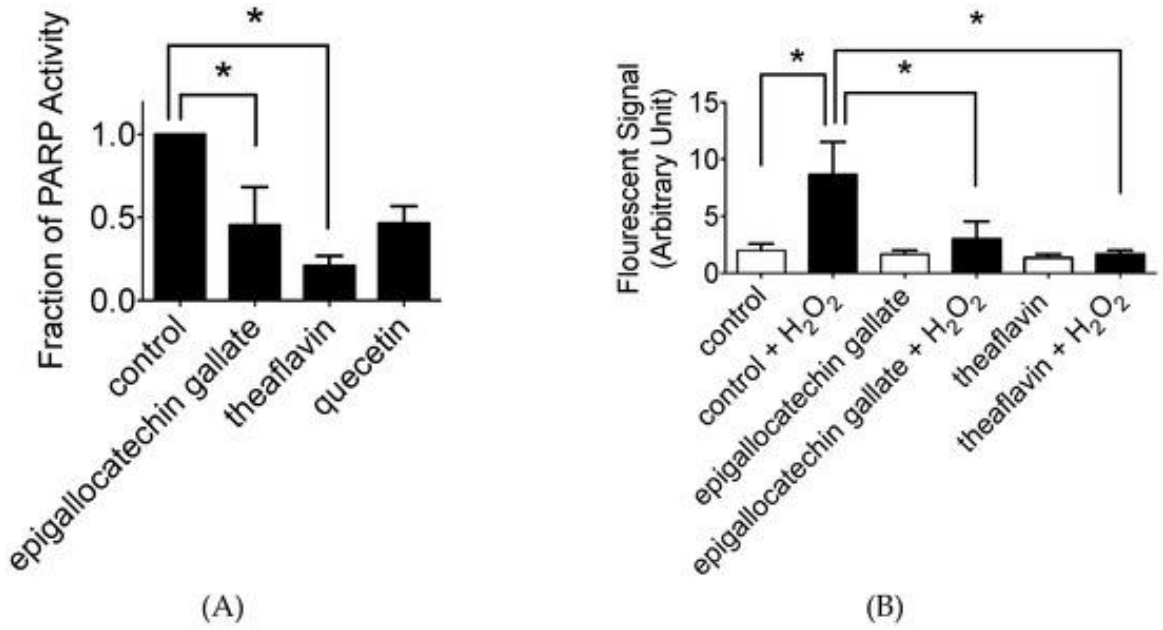


Figure 2.4: *PARP* inhibitory effect by tea polyphenols. (**A**) 10 µM of chemicals were tested for *in vitro* analysis. (**B**) 10 µM of chemicals were tested for *in vivo* analysis. V79 cells were pre-treated with polyphenol prior to H_2O_2 treatment. Values are mean ± standard error of the means. (*) indicates statistically significant differences from control ($p < 0.05$).

In vivo PARP inhibitory effect was tested with V79 cells. Prior to H_2O_2 treatment, 10 µM of Epigallocatechin gallate or Theaflavin were added to cell culture media, and their PARP inhibitory effects were assessed by measuring poly (ADP-ribose) formation in cells (Figure 2.4B). H_2O_2 treatment increased immunofluorescent cellular poly (ADP-ribose) signal from 1 to 9. Epigallocatechin gallate or theaflavin pretreatment suppressed H_2O_2 induced poly (ADP-ribose) formation (3 and 2, respectively).

2.3.3 Sister Chromatid Exchange

A sister chromatid exchange (SCE) assay was performed to determine if these polyphenols increase the genetic instability to the cells as a function of the PARP inhibitor or other mechanisms (Figure 2.5A). The background SCE frequency was 6.3 SCE per CHO metaphase cell. Treatment of 10, 30, and 50 μM of tea polyphenol increased SCE frequency in a dose dependent manner (Figure 2.5B). The regression lines showed SCE frequency increases 0.061 per μM of Epigallocatechin treatment and 0.038 per μM of Theaflavin treatment. The rate of increase was greater for Epigallocatechin gallate treatment. The statistically significant increase was observed for 30 and 50 μM of epigallocatechin and 50 μM of theaflavin treatment against control. Therefore, both Epigallocatechin gallate and Theaflavin increased SCE formation in CHO cells.

2.3.4 Micronuclei Formation

DNA damage during replication period can cause synthetic lethality. These DNA damages can be observed in the chromosome level as fragmented DNA (Figure 2.6A). Treatment of 10, 30, and 50 μM of tea polyphenol increased SCE frequency in a dose dependent manner for both V79 and CHO systems (Figure 2.6B,C). The regression lines showed micronuclei frequency increases of 0.0014 per μM of epigallocatechin treatment and 0.0009 per μM of theaflavin treatment for V79. The rate of increase was greater for V-C8 with Epigallocatechin gallate or Theaflavin treatment (0.039 and 0.006 per μM, respectively). The statistically significant difference of micronuclei formation was observed between V79 and V-C8 cells treated with 50 μM of Epigallocatechin gallate ($p < 0.05$). Compared to CHO cells, 51D1 also increased more MN formation with polyphenol treatment. Therefore, both Epigallocatechin gallate and Theaflavin increased micronuclei formation to HR deficient cells.

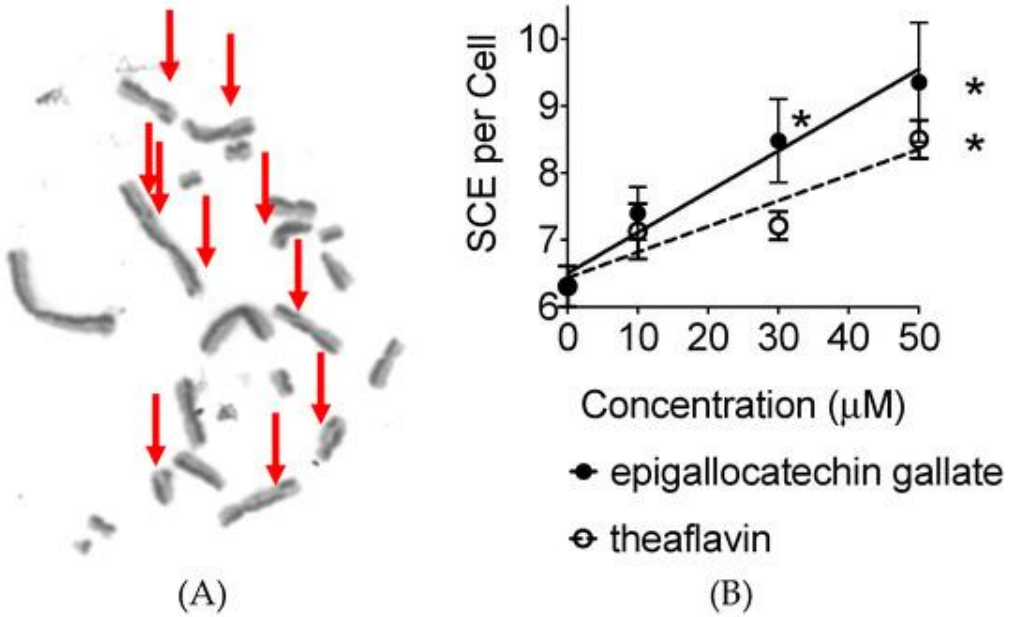


Figure 2.5: Sister chromatid exchange (SCE) induction by polyphenol treatment. (**A**) SCE after 50 μM of Epigallocatechin gallate treatment. Arrows indicate SCE formation. (**B**) The dose response increase in SCE frequency after treatment with polyphenols. Linear regression lines were drawn. Epigallocatechin gallate SCE = 0.061 × (Epigallocatechin gallate μM) + 6.5, Theaflavin SCE = 0.038 × (Theaflavin μM) + 6.4. Values are mean ± standard error of the means. (*) indicates statistically significant differences compared to 0 μM control ($p < 0.05$).

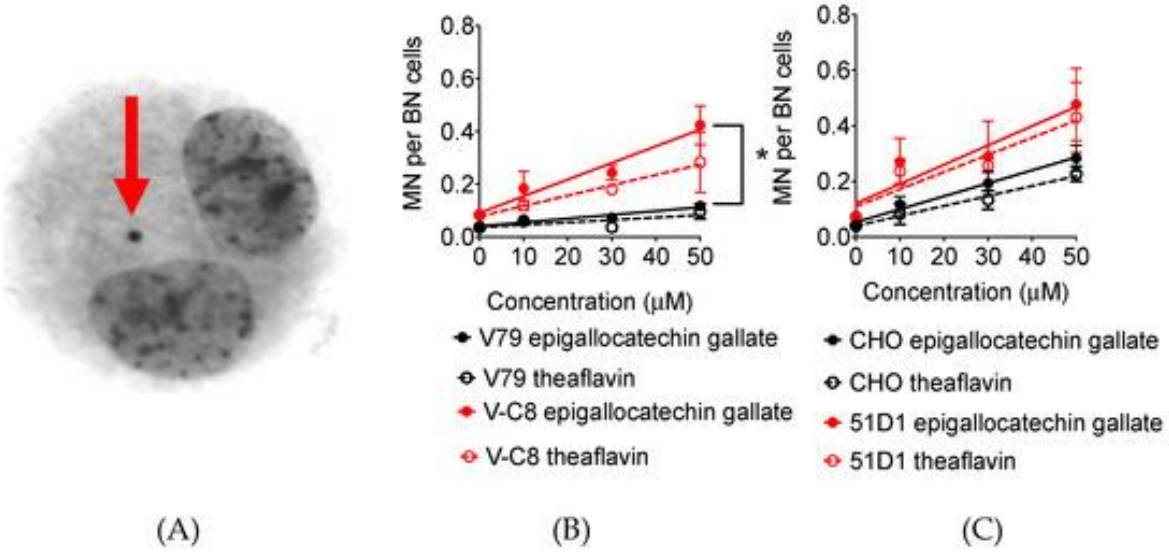


Figure 2.6: Micronuclei formation by tea polyphenol treatment. (**A**) Micronuclei formation in 51D1 cells after treatment by 50 μM of Epigallocatechin gallate. Arrow indicates micronuclei in binucleated cells. (**B**) Dose dependent micronuclei formation for V79 and V-C8 cells. (**C**) Dose dependent micronuclei formation for CHO and 51D1 cells. Values are mean ± standard error of the means. (*) indicates statistically significant differences from non-treated data ($p < 0.05$).

2.3.5 DPPH Radical Scavenging Capacity

Radical scavenging capacity of two polyphenols were compared for DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity (Figure 2.**7**). The DPPH radical scavenging capacity was much larger for Epigallocatechin gallate than Theaflavin. At the concentrations of 10 and 100 µM, statistically significant differences were observed ($p < 0.05$).

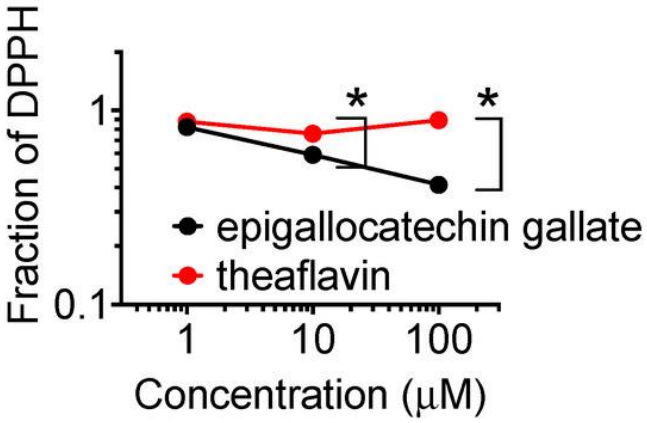


Figure 2.7: DPPH radical scavenging capacity of the two tested tea polyphenols. Data represents means and standard error of the means. (*) indicates statistically significant differences from non-treated data ($p < 0.05$).

2.4 Discussion:

In this study, we showed tea polyphenols can cause synthetic lethality in *BRCA2* deficient cells through PARP inhibition. In treated cells, cell doubling time was extended because tea polyphenol likely interferes with cell cycle progression and can postpone cell growth. This result is consistent with previous studies, which have shown tea polyphenols to induce perturbation of cell cycle progression in human prostate cancer cells [100]. Although in this study growth delay was not BRCA2 deficient dependent (Figure 2.3), clonogenic assay showed stronger cytotoxicity was induced by Epigallocatechin gallate and Theaflavin in *BRAC2* deficient cells (Figure 2.1). However, due to relative weak specificity to PARP inhibition, the differences of IC_{50} for tea polyphenols induced cytotoxicity in wild type *BRCA2* cells and *BRCA2* deficient cells were not as

large as known selective clinically used PARP inhibitors, such as olaparib [42]. In addition to PARP, another potential target of tea polyphenols is reported to be DNA topoisomerase II [101], histone deacetylases [102], and transcription factors [103]. This may explain why theaflavin displayed non-selective cytotoxicity to *rad51D* mutated cells. Sister chromatid exchange was used for replicative stress analysis induced by PARP inhibitory effect by polyphenols (Figure 2.7). However, any genotoxic effects directly or indirectly stress to cells and increase sister chromatid exchanges [104, 105]. Therefore, observed elevation of sister chromatid exchange rate by tea polyphenols may not be solely from PARP inhibition.

The PARP inhibition *in vitro* was stronger in Theaflavin compared to Epigallocatechin Gallate. This was clear contradiction with cell survival analysis and the two cytogenetics observations. Epigallocatechin gallate is more cytotoxic and genotoxic to CHO and V79 cells and their mutant cell lines. Epigallocatechin gallate is an ester of Epigallocatechin (gallocatechol) and gallic acid. We have previously reported gallic acid as a PARP inhibitor [6]. Identified PARP inhibitory effect of Epigallocatechin gallate supports that galloyl group structure may be important for PARP inhibitory effects and synthetic lethality. Theaflavin itself does not have this structure. But theaflavin-3-gallate is a Theaflavin derivative. This chemical is found in black tea alongside theaflavin. Although both green tea and black tea contains polyphenols with *PARP* inhibitory effects, the metabolism and bioavailability of these polyphenols are not fully understood. The other natural chemicals that contain galloyl structure are Epicatechin, Epigallocatechin, Catechin Gallate, Epicatechin Gallate, Gallocatechin, and Gallocatechin gallate. Ethyl gallate may be also possible to possess PARP inhibitory effect. Myricetin, a flavonoid with galloyl structure may also possess PARP inhibitory effect because Quercetin, a flavonoid with catechol structure shows PARP inhibitory effects [106].

Our study suggests that PARP inhibitory polyphenols in tea is not limited to Epigallocatechin gallate and theaflavin. Further research could yield interesting results in the testing and analysis of other polyphenols in tea to induce PARP inhibition. Moreover, the PARP inhibition induced synthetic lethality was only tested as individual chemicals in this study. It is reasonable to speculate that polyphenols may show a synergistic effect for PARP inhibition and improvement of bioavailability due to forming complexes. Further research is necessary to assess the true tea polyphenols effects to PARP inhibition induced synthetic lethality to BRCA2 deficient cells

2.5 Conclusions:

In conclusion, this chapter identified tea polyphenols, Epigallocatechin gallate and Theaflavin, to have PARP inhibitory activity and selective cell toxicity towards *BRCA2* deficient cells through synthetic lethality. This study also supported that the galloly group structure of Epigallocatechin gallate is important for PARP inhibition. Further research should be done to investigate *in vivo* synthetic lethality cancer preventative activities and the PARP inhibitory mechanisms of these polyphenols. A more concrete understanding of flavonoid structure and functional groups, along with their effects on therapeutic efficacy, is explored more thoroughly in chapter three.

CHAPTER THREE:

Synthetic Lethality of Kaempferol towards Homologous Recombination Repair Deficient Mammalian Cells through PARP inhibition

3.1 Introduction:

As previously stated, there are over 4,000 different flavonoids with many unique and different structural components [107, 108]. Flavonoids give color to plants and can be found in berries, vegetable, herbs, nuts, seeds, tea and spices[107, 109]. People regularly consume flavonoids in their diets, the highest concentrations being found in apples, green tea, blueberries, blackberries, broccoli, cabbage, chocolate, and strawberries [110]. Both *in vitro* and *in vivo* experiments have been performed to show the many beneficial properties of flavonoids, such as anti-inflammatory, anti-allergic, anti-viral, and antioxidant effects. In addition, flavonoids have also been proven to reduce the risk of developing cancer [111]. All flavonoids have a very similar basic chemical structure, consisting of a fifteen-carbon skeleton containing two benzene rings (A and B) linked via a heterocyclic pyrene ring (C) as shown in (Figure3.1). Based on their varying oxidation rates and the pattern of substitution on the C rings, flavonoids can be divided into different groups. Such forms are further subdivided into classes known as flavonoids, flavones (e.g., Apigenin, and Luteolin), flavonols (e.g., Quercetin, Kaempferol, Myricetin, Galangin and Morin), Flavanones (e.g., Flavanone, Hesperetin, Pinocembrin and Naringenin), and others. Nevertheless, the pattern of replacement of the A and B rings and the location of the hydroxyl group vary in each individual compound within each class [111]. The hydroxyl groups in Flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [110, 111].

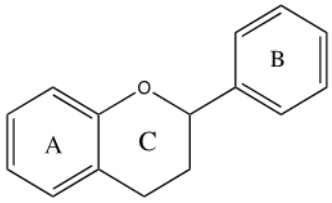


Figure 3.1: Basic flavonoid structure.

The second cause of death in females following lung cancer is breast cancer [111]. Both BRCA1 and BRCA2 proteins are essential for repairing double strand DNA breaks (DSBs) through homologous recombination, and in general, the Gene Conversion (GC) pathway. DNA damage occurs throughout the lifetime of a cell and these mechanisms of repair are necessary to maintain the genome's stability and integrity [112]. people with *BRCA1* or *BRCA2* heterozygous mutations have a higher risk of developing breast cancer [8, 9]. We hypothesized that the different sub-classes of flavonoids are selectively cytotoxic towards *BRCA2* deficient cell lines through *PARP* inhibition in a process known as synthetic lethality. To test this, we targeted the Base Excision Repair (BER) pathway in cells lacking wild-type *BRCA2* [113]. The BER pathway is important to repair certain kinds of DNA base damage such as DNA single-strand breaks (SSBs) and gaps [112]. To target BER, we inhibited Poly (ADP-Ribose) polymerase-1 (*PARP*-1) enzyme which plays a critical role in this pathway [114]. Deficiency of *PARP* causes failure to repair SSB lesions effectively but does not impede DSB repair [115]. As loss of function of either *BRCA1* or *BRCA2* impairs GC, it was thought that inhibiting *PARP* activity in a *BRCA2* defective cell lines might result in the generation of replication- associated DNA lesions normally repaired by sister chromatid exchange [53]. If so, this increase in un-repaired or mis-repaired DNA damage might lead to cell cycle arrest and/or cell death in a selective fashion.

In previous studies, we have shown natural plant chemicals [42], including various flavonoids from rosemary and tea extract, cause a PARP inhibiting effect. These studies suggest that the specific chemical structure of the flavonoids changes the degree of PARP inhibition effect and selective cytotoxicity towards to *BRCA2* deficient cells. For example, the Flavanol Quercetin was a better PARP inhibitor than Hesperidin and Naringenin, which are Flavanones [42, 116]. However, glucosyl modification on these flavonoids decreased PARP inhibitory effect and selective cytotoxicity [13]. This study was aimed at finding the most potent flavonoids and determining the ideal chemical structure needed to cause selective cytotoxicity. Various flavonoids are analyzed to determine their effects on cell cycle and their mechanism of action, thought primarily to be PARP inhibition, and their potential use in treating DNA repair deficient cancer cells.

3.2 Materials and Methods:

3.2.1 Cell culture:

Chinese hamster lung V79 cells, its mutant cell line V-C8 (*BRCA2* deficient) [26] and V-C8 cells that underwent gene correction, and CHO wild type and 51D1 (rad51D deficient) cell lines [117] were kindly provided by Dr. Joel Bedford (Colorado State University, Fort Collins, CO). The human colorectal adenocarcinoma cell line DLD-1 and its *BRCA2* $^{-/-}$ cells were purchased from Horizon Discovery (Waterbeach, United Kingdom). Cells were maintained in culture in minimum essential medium (Gibco, Grand Island, NY), and supplemented with 10% heat inactivated fetal bovine serum (FBS, Sigma, St. Louis, MO), and 1% antibiotics, and 0.1% antimycotics (Gibco, Grand Island, NY) in a humidified incubator at 37 °C and 5% CO_2.

3.2.2 Chemicals:

Chemicals were purchased from SIGMA-Aldrich (St Louis, MO, USA) and dissolved in DMSO (stock solutions of 100 mM and 10 mM stored in -20°C until use). Pinocembrin is a flavone. Galangin, Kaempferol, Quercetin, Myricetin, and Morin are flavonols. Dihydrokaempferol, Taxifolin, and Dihydromyricetin are Dihydroflavonols Dihydroflavanones Pinobanksin and Luteolin is a flavone. The chemical structures of the flavonoids used in this study are shown in Figure3.2.

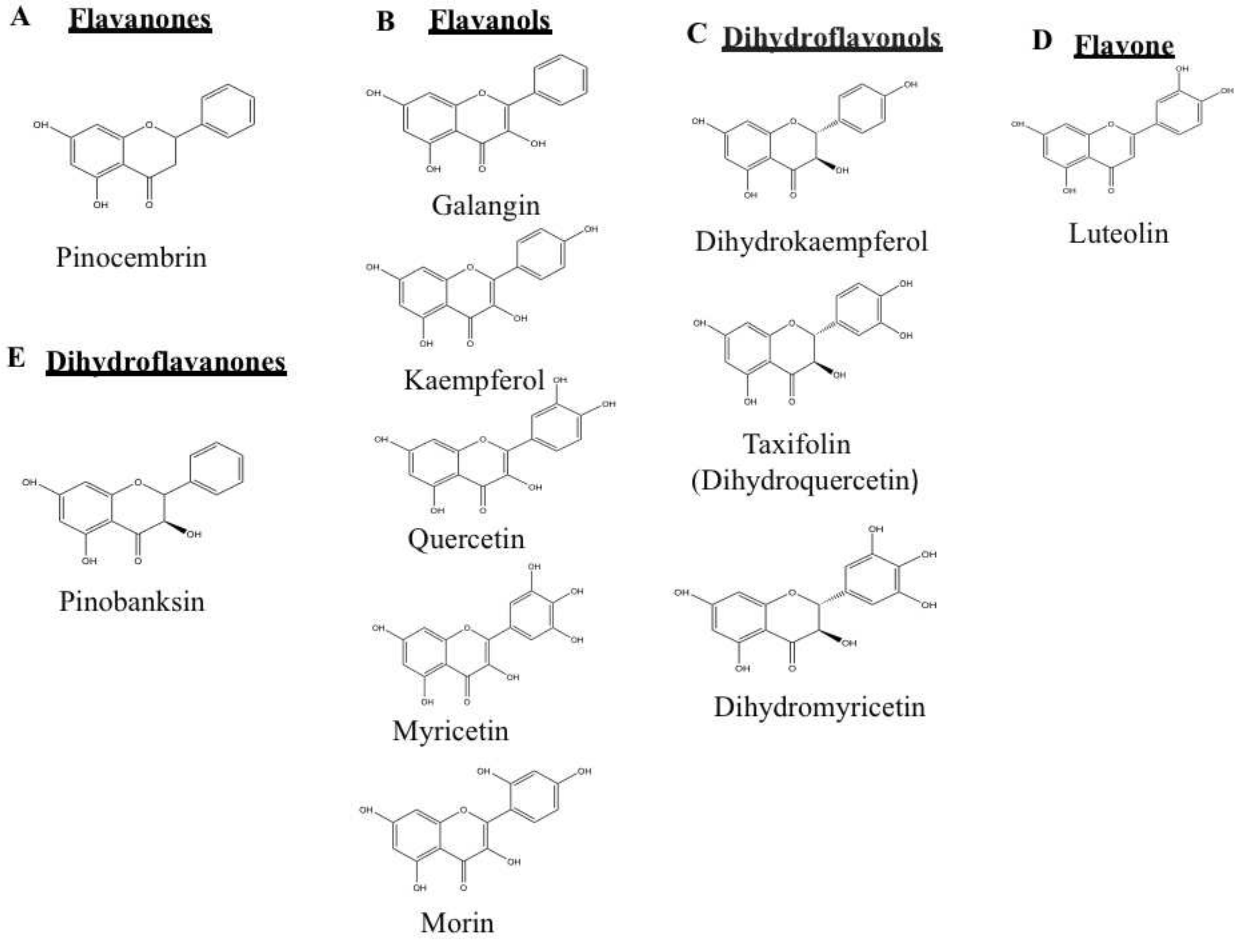


Figure 3.2: Chemical structure of the flavonoids used in the experiments.

3.2.3 Doubling Time Assay:

Ten thousand trypsinized cells were plated into 24-well cell culture plates. Cells were then treated with 10 μM of one of the selected flavonoids before being incubated. Cell numbers were counted by Coulter Counter Z1 (Beckman Coulter, Brea, CA) after 24, 48, 72, and 96 hours of incubation. Cell doubling time was calculated through GraphPad Prism 7.

3.2.4 Cell Survival Assay:

Exponentially growing cell cultures were trypsinized and approximately 300 cells were cultured before the chosen flavonoids were added to the plates at various concentrations. Cells were incubated for 7–10 days to form colonies. For the human colorectal adenocarcinoma cell line, DLD-1 and its *BRCA2* $^{-/-}$ cells, we plated 500 cells for the wild type and 1000 cells for DLD-1(*BRCA2* $^{-/-}$) and then treated them with various concentration of flavonols (Galangin, Quercetin, Kaempferol, Myricetin). Cells were in incubated for 14 days to form colonies. Afterwards, cells were fixed with 100% ethanol and stained with 0.1% crystal violet, then manually counted under microscope. Colonies having more than 50 cells were considered to be a survivor [28]. Regression curves were drawn from cell survival fraction by Graphpad Prism 6 software (GraphPad, La Jolla, CA, USA). IC_{50} values (drug concentration to achieve 50% cell survival) were obtained from regression curves.

3.2.5 Sister Chromatid Exchange Assay:

CHO cells were synchronized into G1 phase using a mitotic shake-off procedure [29,30] Synchronized mitotic cells were sub-cultured in T75 flasks and incubated for 2 hours at 37 °C. Cells were then treated with 10 μM of flavonoid and incubated with 10 μM of 5-bromo-2′-deoxyuridine (BrdU, Sigma, St. Louis, MO) for two cell cycles. Next, 0.2 μg/ml of colcemid (Gibco, Grand Island, NY) was added to the cells, which were then allowed to incubate for an

additional 6 hours. Cells were harvested during metaphase, trypsinized, and then suspended in 4 ml of 75 mM KCl solution, warmed to 37 °C, and then placed in a 37 °C water bath for 20 min. A fixative solution of 3:1 methanol to acetic acid was added to the samples according to the standard protocol. Fixed cells were dropped onto slides and allowed to dry at room temperature. Differential staining of metaphase chromosomes was completed using the fluorescence plus Giemsa (FPG) technique. Differentially stained metaphase chromosome images were taken using a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) equipped with SPOT camera. A minimum of 50 metaphase cells were scored for each treatment. The data presented is the mean of SCE frequency per chromosome

3.2.6 Micronuclei:

G1 synchronized CHO and 51D1 cells were incubated with 10 μM Flavonoid and 4 μg/ml of Cytochalasin B (Sigma, St. Louis, MO) for 20 hours. Cells were suspended in 5 ml of 75 mM KCl solution, centrifuged, and fixed in 3:1 methanol: acetic acid solution with formaldehyde (Fisher Scientific, NH, USA). Cells were dropped onto slides and allowed to air dry at room temperature. Slides were stained in filtered 10% Giemsa (Gibco, Grand Island, NY) in GURR (Gibco, Grand Island, NY) solution for 5 min. One hundred binucleated cells were scored per treatment dosage, and images were analyzed using a Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany) to obtain micronuclei frequency per binucleated cells.

3.2.7 Cells Cycle analysis:

In order to explain growth delay, cell cycle distribution analysis was carried out. 100,000 cells were cultured and then treated with 10 μM for 24 h. Cells were immediately trypsinized and fix ed with 70% ethanol. Following fixation, cells were stained with Propidium iodide and RNase A solution. The DNA profile was collected with an FACSCalibur (Beckton Dickinson, Franklin

Lakes, NJ). Modifit software (Verity software, Topsham, ME) determined the cell cycle distribution [118].

3.2.8 In Vitro PARP Activity Inhibition Assay:

PARP colorimetric assay kits (Trevigen, Gaithersburg, MD, USA) were used to measure *in vitro PARP* activity in test tubes as previously described [24]. *PARP* was incubated in a 96-well microplate with a reaction mixture containing 50 µM β-NAD+ (10% biotinylated β-NAD+), 90% unlabeled β-NAD+, 1 mM 1,4-dithiothreitol, and 1.25 mg/mL nicked DNA. The formation of poly (ADP-ribose) polymers was detected with peroxidase-labeled streptavidin and $3,3^{0},5,5^{0}$-tetramethylbenzidine (Invitrogen, Carlsbad, CA, USA). *PARP* inhibition was assessed by the addition of flavonoids at various dosages to the reaction mixture. *PARP* activity is directly proportional to absorbance at 450 nm and measured by a NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). IC_{50} values of *in vitro PARP* inhibitory activity were derived by fitting dose–response curves using a sigmoidal dose response equation [6]

3.2.9 Statistics

A minimum of three independent experiments were carried out, consequent data was analyzed using GraphPad Prism 6 software. The data is presented as the mean ± standard error of the means. IC_{50} values (50% inhibitory concentration doses of the specific endpoints) were derived by fitting dose response curves using a sigmoidal dose response equation obtained by GraphPad Prism. Differences with a $P<0.05$ were considered statistically significant, statistical comparison of mean values was performed using one-way ANOVA test.

3.3 Results:

3.3.1 Clonogenic Cell Survival:

Treatment of cells by flavonols; one of the subclasses of flavonoids which include Kaemperol, Quercetin and Myricetin; strongly suppressed clonogenic activity for *BRCA2* deficient V-C8 cells compared to wild type V79 cells and gene corrected V-C8 cells (Figure 3.3A, B, and C). The IC_{50} values for Kaempferol-treated V79 wild type cells was 29.74 μM, 19.32 μM for V-C8 BRCA2 deficient cells, and 31.35 μM for gene corrected V-C8 cells. These results indicate that a lower concetration of Kaempferol was required to negatively effect *BRCA2* deficient cell survival than wild type. However, when the deficiency was corrected in the gene complimented V-C8 cells, the concentration required to effect survival increased back up to wild type levels.

The remaining flavonols, Querctin and Myricetin showed comparable effects with the IC_{50} values for Quercetin-treated cells being 72.17 μM for V79 wild type, 29.86 μM for V-C8 cells, and 53.27 μM for gene corrected cells. Similary, with Myricetin - treated cells, the IC_{50} values were 63.08 μM, 32.83 μM, and 53.90 μM for V79, V-C8 cells, and V-C8 gene corrected cells respectively. The differences between survival fractions after exposure to 20 μM of flavonol are statistically significant between V-C8 cells compared to V79 wild type and gene corrected cells in both Kaempferol and Quercetin treated cells ($p < 0.05$). However, we could not see a statistically significant difference between V-C8, V79 and gene corrected cells for Myricetin even when the flavonol concentration was increased to 100 μM. The flavonols Galangin and Morin did not have any selective cytotoxic effect on V-C8 cell survival, indicating that their cytotoxic mechanism of action may be independent of the BRCA2 pathway (Figure 3.3D, E and F).

Of the dihydroflavonols tested, only dihydrokaempherol showed a similar effect on cell survival while Taxifolin and DihydroMyricetin showed no notable cytotoxic effect related to BRCA2 deficiency (Figure 3.3F, G, and H). The flavanone Pinocembrin and the Dihydroflavanone Pinobanksin also showed to statistically significant cytotoxic effect on cell survival across all V79,

V-C8, and gene corrected cell lines (Figure 3.3J, K). The lone flavone tested, Luteolin, actually showed a decrease in survival across all cell lines, regardless of concentration. This is showcased by the similar IC_{50} values of 5.79 µM and 4.95 µM for both VC8 and V79 respectively (Figure 3.3I).

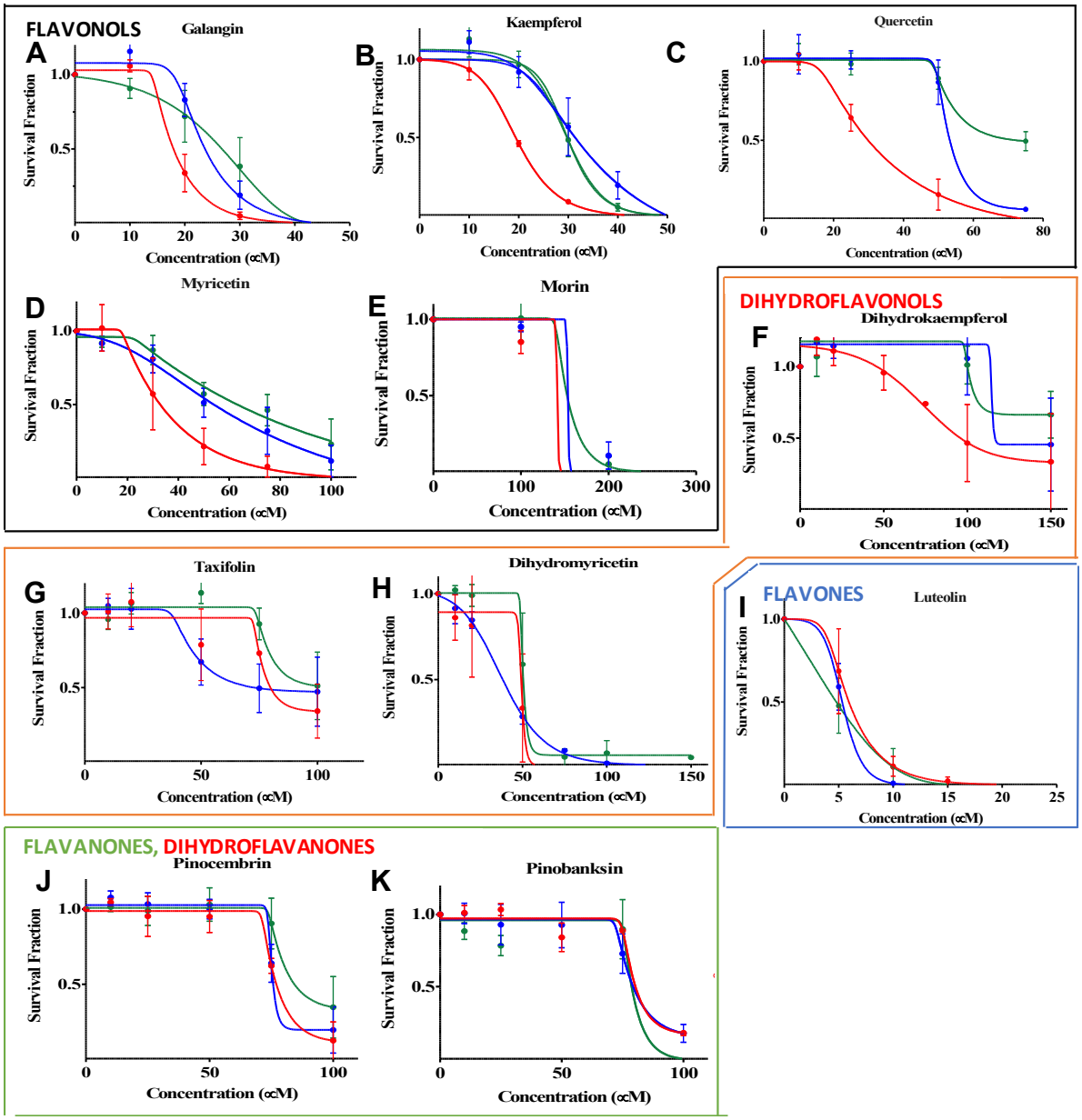


Figure 3.3: Clonogenic cell survival curves for eleven different subclasses of flavonoids in V79 cells, V-C8 cells and V-C8 gene complimented (♦ V79, ♦ Gene corrected V-C8, ♦ V-C8.)

For CHO and *rad51D* mutant 51D1, Kaempferol suppressed clonogenic activity for 51D1 cells compared to wild type CHO cells (Figure 3.4B). The IC_{50} values were 55.2 μM for CHO but were 21.6 μM for 51D1 cells (2.56 times selective). Treatment of cells by Quercetin also suppressed the clonogenic activity for 51D1 cells compared to wild type CHO cells in a similar manner. The IC_{50} values were 55.2 μM and 26.4 μM for CHO and 51D1 cells, respectively (2.1 times selective). Noticeable selective cytotoxicity toward 51D1 was not observed another flavonoid. Based on these hamster cells' studies, Kaempferol and Quercetin can result selective cytotoxicity toward homologous recombination repair deficient cells.

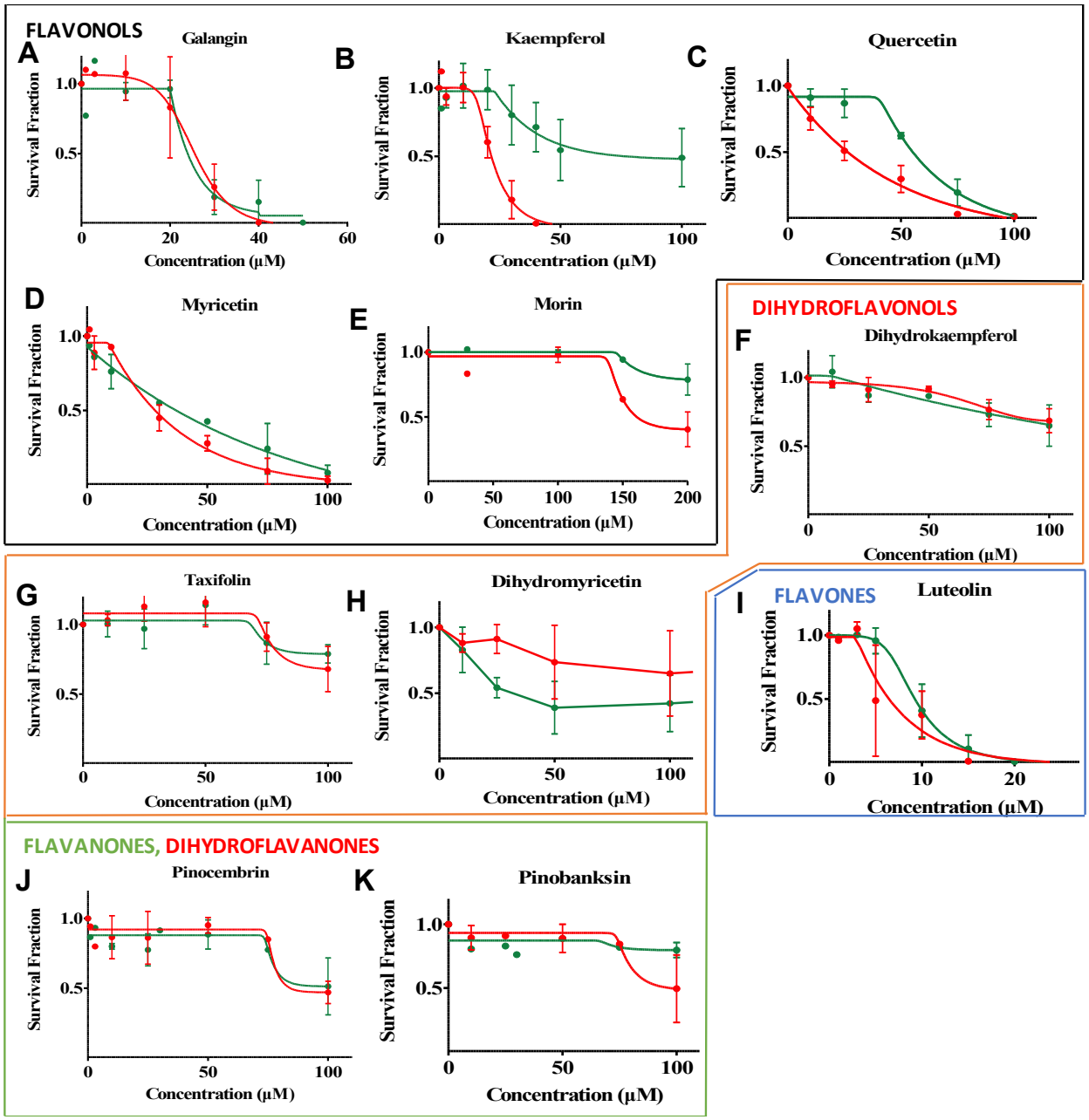


Figure 3.4: Clonogenic cell survival curves for eleven different subclasses of flavonoids in Chinese hamster cells (CHO) [♦] and *rad51D* mutated 51D1 cells [♦]

3.3.3 Doubling Time

Cellular growth delay was tested by adding 10 µM of flavonoid to cell cultures. The number of cells was recorded daily and population doubling hours were recorded (Figure 3.5). The basal doubling time was 14.5 hours for CHO and 16.6 hours for 51D1. Luteolin was toxic in both cell lines, similar to previous results, and showed a severe increase in doubling time (Figure3. 5). Doubling time for Luteolin was 36.9 hours in CHO and 51.4 hours in 51D1. It was a significant difference from the basal time for both wild type CHO cells (p-value is 0.0001), and the mutant 51D1 cells (p-value is 0.0048). The other subclasses of flavonoids such as flavanols (Galangin, Kaempferol and Myricetin) also caused an increase in the doubling time in 51D1 mutant cell lines compared the control, while maintaining a near basal doubling time in wild type CHO cells. Of the tested compounds, Kaempferol had the most drastic effect, increasing the doubling time in 51D1 cells from 18.05 hours to 41 hours (Figure 3.5).

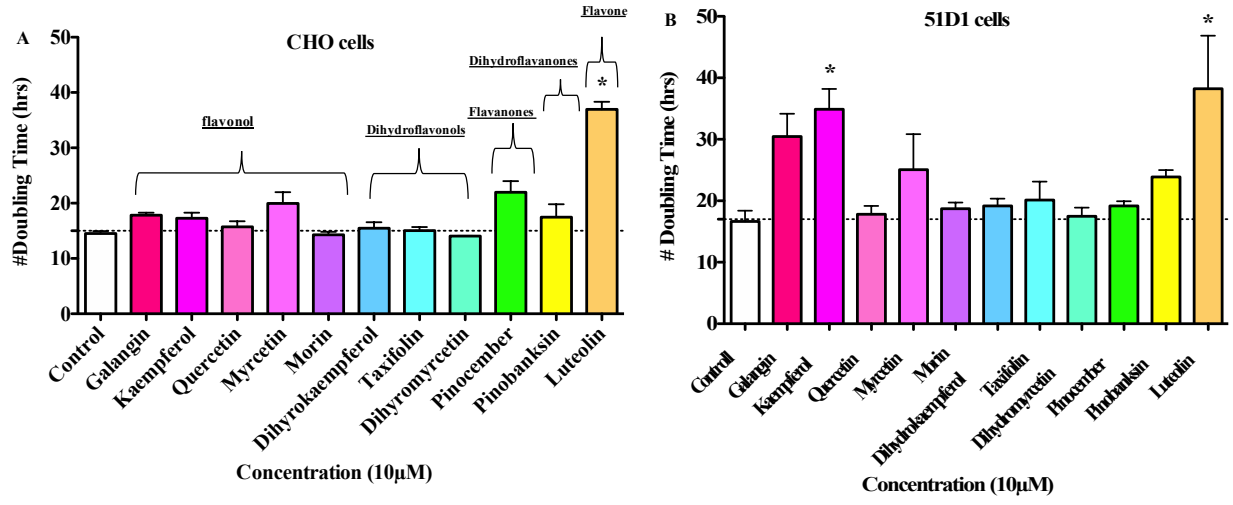


Figure 3.5: Cell population doubling time after flavonoid treatment: (**A**) CHO cells, (**B**) 51D1 cells. Values are mean ± standard error of the means. standard. (*) indicates statistically significant differences from control ($p < 0.05$).

3.3.4 Cell cycle

There was an overall decrease in population between wildtype and mutated cell lines, with 51D1 decreasing below the 60% line (Figure 3.6A and B). However, only Kaempferol-treated cells showed an abnormally large difference when it came to population differentiation between the various phases of the cell cycle. While the 51D1 mutant cell line showed a general increase in cell population in G2/M phase compared to CHO wild type, only Kaempferol-treated cells showed a statistically significant increase with a p-value of 0.0001 (Figure 3.6D).

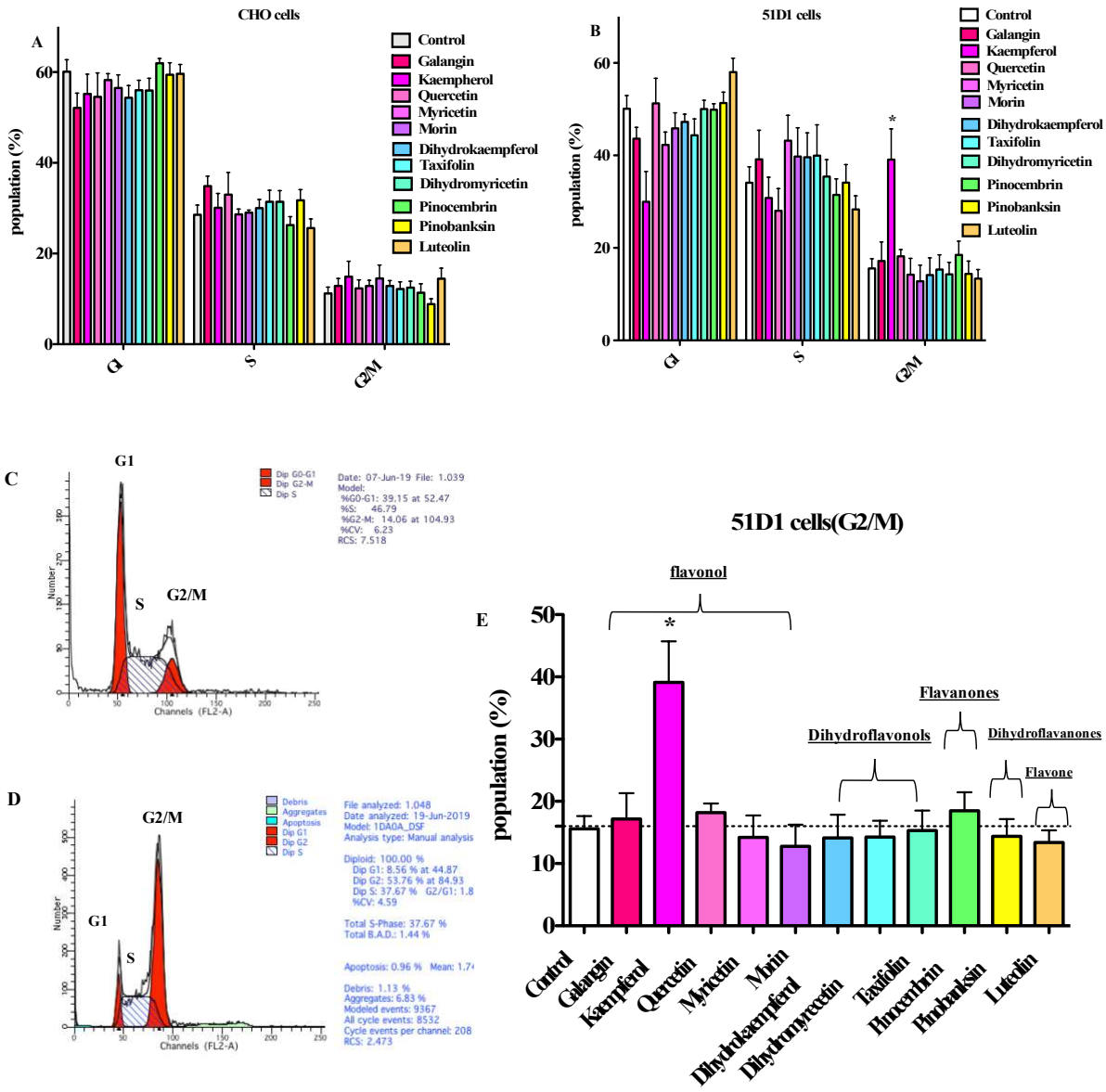


Figure 3.6: Effect of selected flavonoids on the cell cycle of (**A**) CHO (wild type) and (**B**) 51D1 (HR mutant) cells. Cells were incubated with or without 10 µM of eleven various flavonoids for 24 hours. The percentage of cells in each phase of the cell cycle was assessed by flow cytometry. Three independent experiments were conducted. Bars indicate standard deviation of the mean. **P*<0.05 (ANOVA test). (**C**) Distribution of cells between cell cycle phases for control sample. (**D**) Distribution of cells between cell cycle phases for 51D1 (HR mutant) cells after treatment with 10 µM Kaempferol. Accumulation of 51D1 cells in the G2/ M phase as compared to control is observed. (E) effect of flavonoids on G2/M phase of 51D1 cells.

3.3.5 Sister Chromatid Exchange:

A sister chromatid exchange (SCE) assay was performed to determine if these flavonoids increased the genetic chromatid instability exchange in the cells. This assay was performed because a higher rate of SCE implies that PARP inhibition might play a role in the chemical's mechanism of action [32]. The measured background SCE frequency of the CHO cells was 6.04 SCE per chromosome. The flavanols Kaempferol and Quercetin were the most potent, inducing a high SCE frequency at treatment dose (10µM), but these differences were not statistically significant. The measured Kaempferol SCE frequency of the CHO cells was 10.24 SCE per chromosome. Cells treated by Luteolin did not reach the second mitosis for SCE formation due to high toxicity of Luteolin at 10 µM.

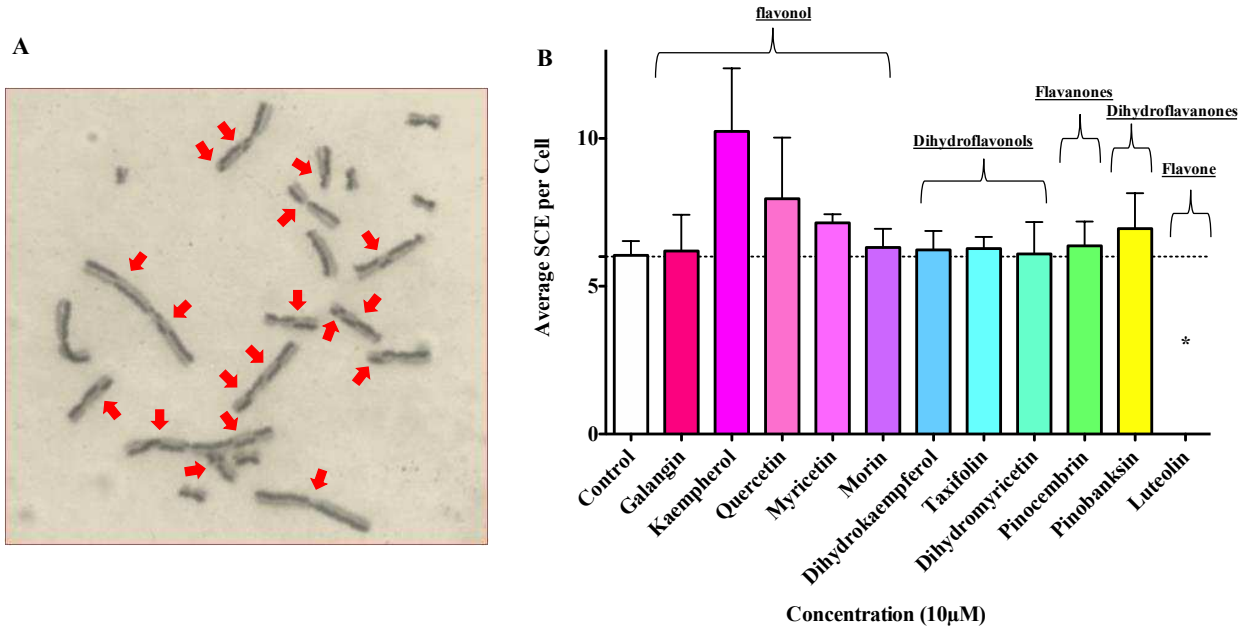


Figure 3.7: Sister chromatid exchange (SCE) induction by flavonoid treatment, arrows indicate SCE formation (**A**). Average SCE after 10 µM of Kaempferol (**B**).

3.3.6 Micronuclei Formation

DNA damage can occur during the replication period of the cell cycle, which can result in cellular lethality in certain mutant cell types. This DNA damage can be observed at the chromosomal level as fragmented DNA or micronuclei (MN) (Figure 3.9A). However, there were no statistically significant changes in cellular DNA damage within cells treated with 10 µM flavonoid (Figure 3.9B). While, Kaempferol did increase MN formation in 51D1 mutant cell two times compare to control, it was not to a statistically significant amount (Figure 3.9C).

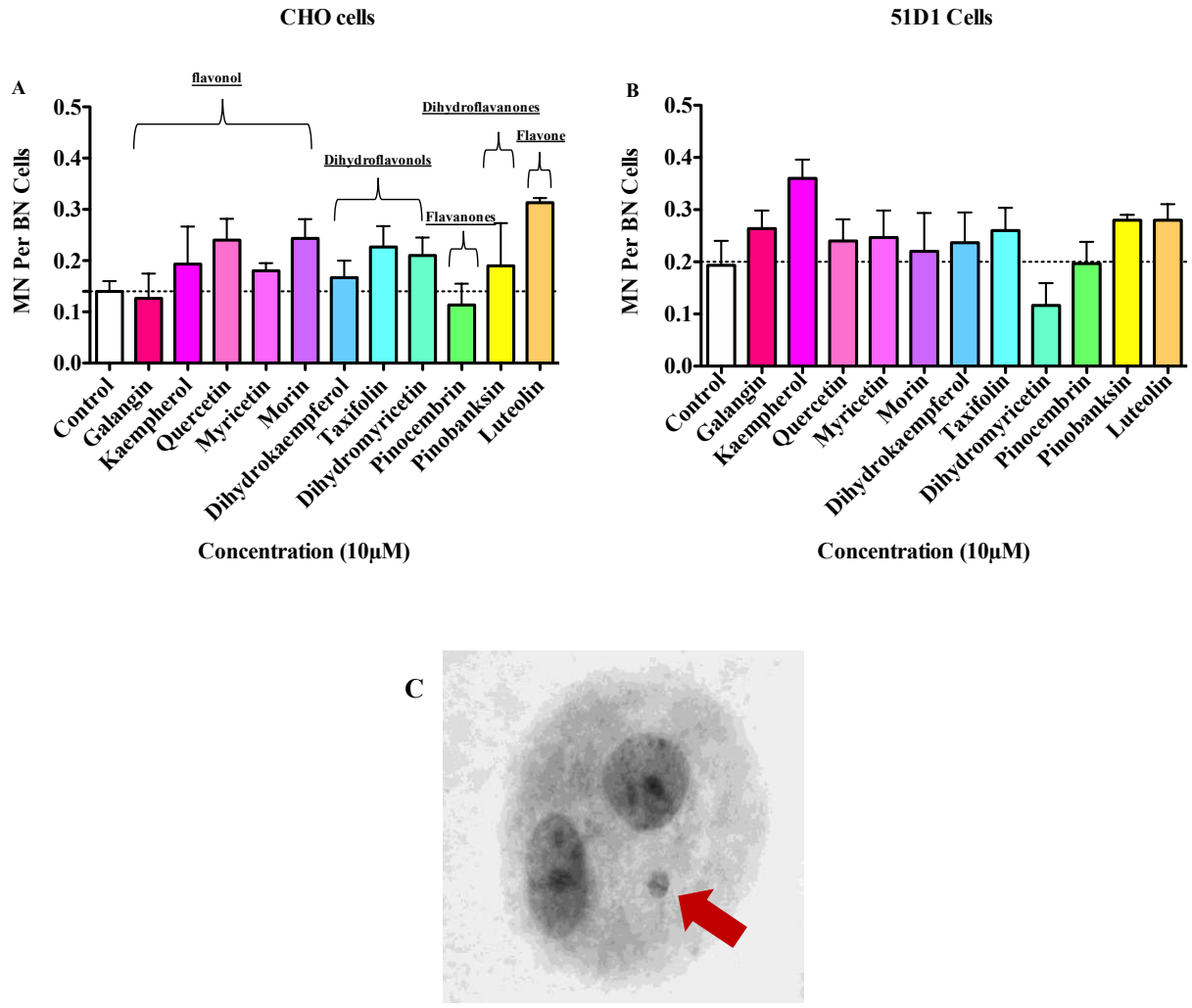


Figure 3.9: Micronuclei formation in CHO cells after treatment with 10 µM flavonoid (**A**).Micronuclei formation in51D1 cells after treatment with 10 µM Kaempferol (**B**). Micronuclei formation after flavonoid treatment (**C**).

3.3.7 In Vitro PARP Activity Inhibition

The effect of flavonoids on PARP activity were analyzed with *in vitro* PARP activity kits (Figure 3.10). Flavonols inhibition Quercetin, Kaempferol, Myricetin showed statistically significant PARP inhibitory effects at concentrations of 10 µM ($p < 0.05$). Statistically, Myricetin showed the strongest reduction of PARP activity among the test flavonoids in this assay (0.3, $p < 0.0073$). Dihydromyricetin also showed statistically significant PARP inhibitory effects at 10 µM, but its cytotoxic effects were not consistent throughout the other assays like the flavonols

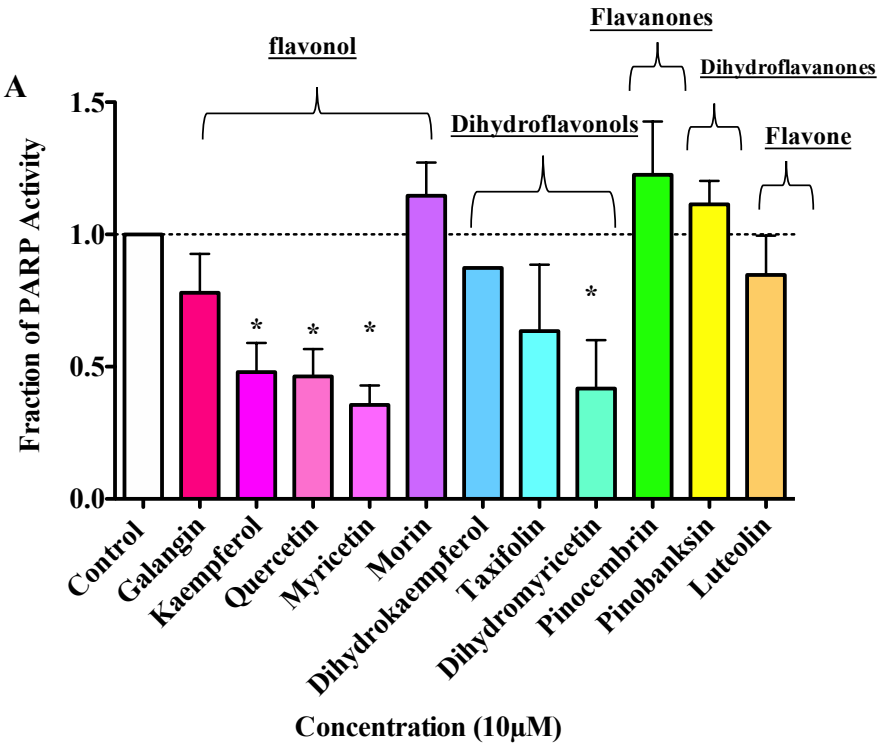


Figure 3.8: The measured PARP inhibitory effect of flavonoids after treatment of 10 µM. Reduction of activity below 1.0 indicates PARP inhibition.

3.3.8 Clonogenic Cell Survival for human cell lines:

After analyzing the results from the *in vitro* PARP activity, we test the most effective subclass: the flavonols. Kaemperol, Quercetin and Myricetin all strongly suppressed clonogenic activity for DLD1 *BRCA2*$^{-/-}$ compared to their wild type colorectal adenocarcinoma cells (DLD-1) (Figure 3.9.A, B, and C). The IC_{50} values for Kaempferol-treated DLD-1 wild type cells was 47.1µM and DLD1 *BRCA2*$^{-/-}$ was 12.4 µM. The IC_{50} values for Quercetin-treated DLD-1wild type cells was 42.020µM and DLD1 *BRCA2*$^{-/-}$ was 35.635 µM. The IC_{50} values for Myricetin-treated DLD-1wild type cells was 103.4 µM and DLD1 *BRCA2*$^{-/-}$ was 11.7µM. The differences between survival fractions after exposure to 20 µM of the flavonols Kaempferol and Myricetin were statistically significant between DLD-1 wild type cells and DLD1 *BRCA2*$^{-/-}$ cells ($p < 0.05$). However, Quercetin only showed a statistically significant difference between survival fractions of DLD-1 wild type and DLD1 BRCA2$^{-/-}$ after exposure to 40 µM of the flavonol ($p < 0.05$). On the other hand, the flavonol Galangin did not have any selective cytotoxic effect on DLD1 *BRCA2*$^{-/-}$ cell survival, indicating that their mechanism of action may be independent of the *BRCA2* pathway. Galangin had IC_{50} values of 35.5µM for DLD-1 wild type cells and 41.2 µM for DLD1 *BRCA2*$^{-/-}$.

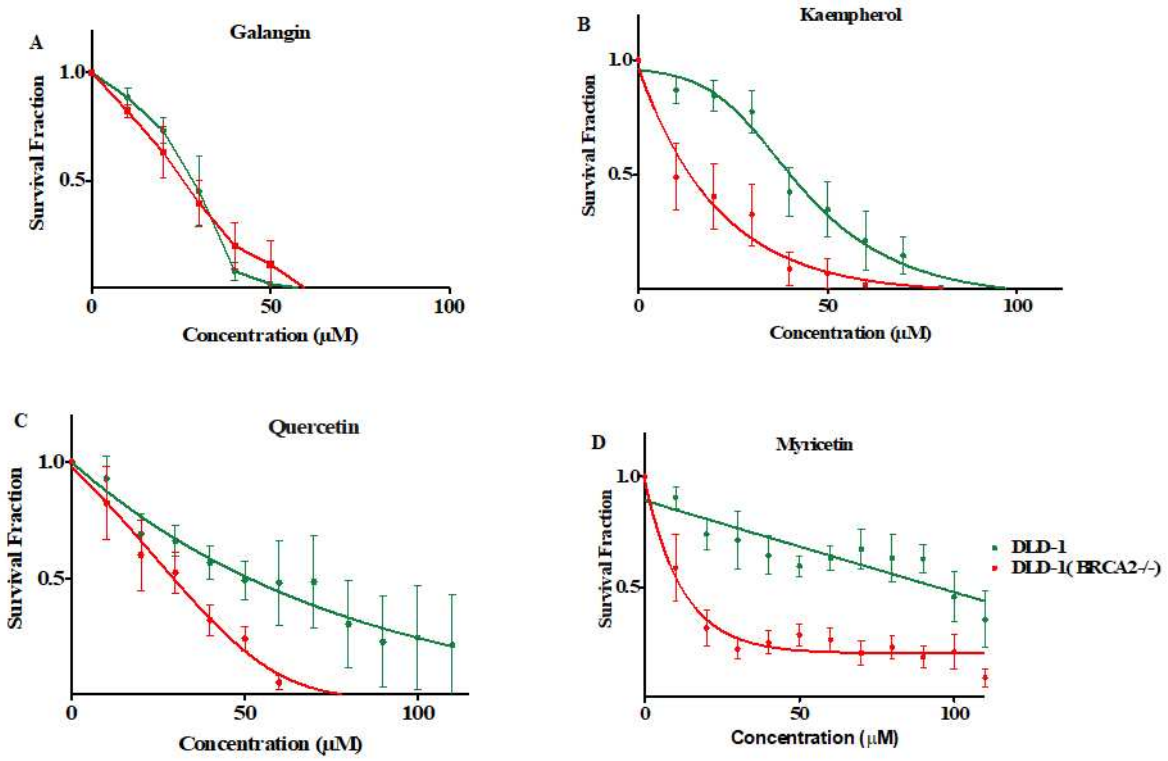


Figure3.9: Clonogenic cell survival curves for eleven different subclasses of flavonoids using Human colorectal adenocarcinoma cells (DLD-1) [♦], and their *BRCA2* -/- cells (DLD1 *BRCA2*-/-) [♦].

3.4 Discussion:

In this study, we examined eleven flavonoids, in particular its subclass of flavonols (Quercetin, Kaempferol, Myricetin) that have shown the most potential to cause synthetic lethality in HR deficient cell lines through PARP inhibition. The clonogenic assay showed that Kaempferol had a strong cytotoxicity that was induced in HR deficient cell lines (Figure 3.3,4 B). Data from human cell line experiments confirmed this *BRCA2* cytotoxicity dependent deficiency. The cytotoxic effects of other flavonols, like Quercetin, occurred at lower concentrations in our study and were consistent with published reports in CHO and human cells with HR repair deficiency(Figure 3.9) [116]. In treated cells, cell doubling time was extended because the flavonoids likely interfered with cell cycle progression and postponed cellular growth. This

observation is consistent with previous studies, which have shown that flavonoids are capable of inducing perturbation of cell cycle progression in human prostate cancer cells(Figure 3.5) [119]. In our tested condition, normal cells did not show any noticeable alteration of cell cycle distribution to CHO wild type cells. However, homologous recombination deficient cells treated Kaempferol showed clear G2M accumulation most likely due to DNA damage occurred by synthetic lethality. Additionally others report Kaempferol can down regulate Cyclin Dependent Kinase signaling pathway (Figure 3.6) [120, 121].

Evidence from the present studies indicate that genotoxicity is associated with increased SCE and MN induction by both Kaempferol and Quercetin. The genotoxic effects that resulted from exposure to these two flavanols suggest that they are capable of binding DNA(Figure3.7,8) [116]. In addition to PARP inhibition, flavonoids have other mechanisms they can utilize to prevent cancer growth such as radical scavenging, detoxification of mutagenic xenobiotics, and inhibition of topoisomerases, cyclin-dependent kinases, and protein kinases -- including phosphatidyl inositol 3-kinase [122]. Most flavonoids have demonstrated an ability to inhibit proliferation in many kinds of cultured human cancer cell lines, with less or almost no toxic side effects observed in normal human cells [121]. In a previous study, Luteolin was shown to arrest cells in G2/M and cause apoptosis in colon cancer cell lines [123]. In this study, Luteolin presented severe cytotoxicity but was also not selective to HR deficiency, making it undesirable as a therapeutic treatment of *BRCA2* mutated cancers.

With regard to the structure-activity relationship of the flavonoids that are shown in Figure 3.2, it can be noted that the flavonoids that significantly inhibit PARP (Quercetin, Kaempferol, Myricetin) all contain hydroxyl groups on the 5 and 7 position of the A-ring. Furthermore, there are hydroxyls present on the B ring in the 3, 4, and 5 positions. Additionally, a C-2,3 double bond

(a 4-ketone function) is present. All these structural components together can potentially inhibit PARP. Tea polyphenols (Theaflavin and Epigallocatechin Gallate), which show a degree of PARP inhibition, contain an ortho-OH substitution pattern in the B ring where the OH groups are not glycated [41]. Another study examined variations in cytotoxic responses arising from short and continuing exposure to natural and synthesis flavonoids [124]. The most cytotoxic flavonoids have been confirmed through other, similar studies to be Quercetin: the aglycone form of several other flavonoid glycosides that contains a hydroxyl group on position 3 in ring C. The least cytotoxic was determined to be the highly glycosylated maltooligosyl Rutin while another flavonoid, the highly glycosylated Isoquercetin, demonstrated an intermediate cytotoxicity, comparable to that of un-glycosylated Isoquercetin [124]. Neither of these structures had a hydroxyl group on position 3 in ring C, which highlights how important this structure is to flavonoids being selectively toxic.

Luteolin has a similar chemical structure to the previously mentioned flavanols, but is missing the hydroxyl group on position 3 in ring C. This could explain why it was showing toxicity in both mutant and wild type cell lines. Similar things happened with other subclasses; such as dihydroflavonols, dihydroflavanones and flavanones; where flavanones are also missing the hydroxyl group on position 3 in ring C and there is no hydroxyl group on the B ring in the 3, 4, and 5 positions. Dihydroflavonols and dihydroflavanones have similar chemical structure to flavonols and flavanones, the only difference being the trans positioning for hydroxyl on ring C, which could be responsible for its ability to inhibit PARP.

In this study, Kaempferol was the most potent PARP inhibitor, consistently effecting HR repair mechanism deficient cell lines while having no significantly noticeable effect on wild type cells. This implies that Kaempferol could serve as the best potential therapy for mutant or cancerous cells that already have a HR deficiency, targeting them through synthetic lethality.

Kaempferol contains hydroxyl groups on both the 5 and 7 position of the A-ring on the B ring in the 4 position. Additionally, it exhibits a C-2,3 double bond, a 4-ketone functional group, all of which are structural markers of effective PARP inhibitors. Kaempferol also typically exhibits semipro-oxidant activity after administration, which can produce ROS and increase the activity and toxicity of anticancer agents. Generally, a flavonoid can reduce a free radical by donating a hydrogen atom and transforming it into a phenoxyl radical. This radical phenoxyl can either react with a second, stable radical (antioxidant effect) or interact with oxygen, leading to ROS (prooxidant effect) production [35]. Further research could yield interesting results in the testing and analysis of other flavonoids with similar structural components, to induce PARP inhibition.

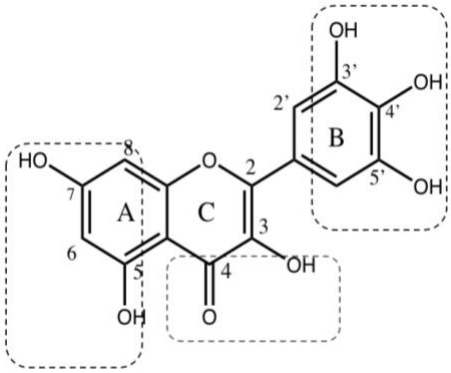


Figure 3.11: Depiction of the most effective chemical structure for flavonoid subclasses as determined by our research.

3.5 Conclusions:

In conclusion, this chapter identified flavonols (Kaempferol, Quercetin and Myricetin) as having PARP inhibitory activity and selective cell toxicity towards *BRCA2* deficient cells through synthetic lethality. This study supports the hypothesis that synthetic lethality function is related to the functional groups present in the Flavonoid. Specifically, the presence of hydroxyl groups on the 5 and 7 position of the A-ring, and on the B ring in the 3, 4, and 5 positions. Additionally, the

presence of a C-2,3 double bond, a 4-ketone function, is hypothesized to be important in PARP inhibition.

CHAPTER FOUR:

Conclusion

Flavonoids are a group of polyphenols that are widely distributed throughout the plant kingdom and are common constituent of fruits, vegetables, and herbs. Flavonoids have been known to play a role in reducing the risk of chronic diseases, especially in those who partake in a plant-rich diet. Studies have shown possible mechanisms in both *in vitro* and *in vivo* environments by which flavonoids can confer cancer and cardiovascular protection. All flavonoids have the same basic chemical structure: a 15-carbon skeleton consisting of two benzene rings (A and B) bound through a heterocyclic pyrene ring (C). In our studies, we examined different subclasses of flavonoids with different chemical structures to determine which chemical structure would be more selectively cytotoxic towards *BRCA2* deficient cell lines via PARP inhibition.

A comparison of the cytogenotoxic responses including by naturally occurring flavonoids (Galangin, Quercetin, Kaempferol, Myricetin, Dihydrokaempferol, Taxifolin, Dihydromyricetin, Pinocembrin, Pinobanksin, Luteolin, Epigallocatechin Gallate, and Theaflavin) showed that the flavonol Kaempferol had the greatest PARP inhibitory activity and selective cell toxicity in *BRCA2* deficient cells through the mechanism of synthetic lethality. We could determine that based on ability of Kaempferol to increase SCE at 10 μM compare to Epigallocatechin gallate and other tested compounds. Kaempferol is a common flavonoid in the plant kingdom, with most edible plants containing some amount of it. Human dietary intake of this polyphenol has been reported to be up to 10 mg/day. Both *in vitro* and animal studies support the hypothesis that Kaempferol functions in preventing and treating diseases such as a reducing the risk of developing cardiovascular diseases and certain types of cancer, inflammation, neurodegenerative

diseases, diabetes, infectious diseases, and several other ailments [35]. It was readily apparent throughout this study that flavonoids that exhibited both PARP inhibitory activity and selective cell toxicity in *BRCA2* deficient cells had similar structural moieties. Kaempferol contained hydroxyl groups on the 5 and 7 position of the A-ring and another hydroxyl on the B ring in position 4 (Figure 3.11). Kaempferol also has a C-2,3 double bond and a 4-ketone present in its structure.

Further research could yield interesting results in the testing and analysis of other flavonoids with similar structural elements, such as Cyanidin or Pelargonidin, and their ability to induce PARP inhibition. Moreover, the PARP inhibition that induced synthetic lethality was only tested through individual chemicals in this study. It is reasonable to speculate that polyphenols may show a synergistic effect for PARP inhibition and improvement of bioavailability due to forming complexes[34]. Further research should be done to investigate *in vivo* synthetic lethality cancer preventative activities and the PARP inhibitory mechanisms of these polyphenols in a systemic, rather than an isolated *in vitro* environment.

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LIST OF ABBREVIATIONS

CHO -- Chinese Hamster Ovary

DLD-1 -- Colorectal Adenocarcinoma Cells

DLD1 *BRCA2*$^{-/-}$ -- *BRCA2* Human knockout cells

HR -- Homologous Recombination

BRCA2 -- Breast Cancer Associate Gene 2

PARP -- Poly (ADP-ribose) polymerase

SCE -- Sister Chromatid Exchange

MN -- Micronuclei

DPPH -- 2,2-diphenyl-1-picrylhydrazyl

ANOVA -- Analysis of valiance