THESIS

COMPARISON OF DOSE-DEPENDENT OUTCOMES IN INDUCTION OF CYTOGENOTOXIC RESPONSES BY NOVEL GLUCOSYL FLAVONOIDS

Submitted by

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ABSTRACT

COMPARISON OF DOSE-DEPENDENT OUTCOMES IN INDUCTION OF CYTOGENOTOXIC RESPONSES BY NOVEL GLUCOSYL FLAVONOIDS

The flavonoids quercetin, and its glucosides isoquercetin and rutin, are phytochemicals commonly consumed in plant-derived foods. They are associated with potential health-promoting effects such as anti-inflammation, anti-viral, anti-carcinogenesis, neuro- and cardio-protection, etc. Semi-synthetic water soluble quercetin glucosides, maltooligosyl isoquercetin (MI), monoglucosyl rutin (MO) and maltooligosyl rutin (MA) were developed to overcome solubility challenges for improved incorporation in food and medicinal applications. Quercetin and its glucosides are known to induce genetic instability and decrease cell proliferation, which are possible mechanisms of anti-carcinogenesis in *in vitro* and animal studies. Using an *in vitro* system of Chinese hamster ovary (CHO) cells, this thesis project examined the differences in cytogenotoxic responses induced by natural and novel flavonoids. Treatments with flavonoids at a concentration range of 0.1 and 1,000 ppm induced sister chromatid exchanges (SCE) and micronuclei (MN) in CHO cells. Compared to spontaneous occurrences, significant increases in SCE and MN were observed in both natural and synthetic flavonoid-treated cells in a dose-dependent manner. The natural flavonoids exhibited greater potency than the synthetic compounds, where quercetin was most potent. An analysis of the effects of flavonoids on DNA repair via the poly(ADP ribose) polymerase (PARP) pathway using ELISA showed that all three natural flavonoids along with MI and MO were capable of inhibiting PARP activity by 50%. Quercetin was observed to be the strongest natural inhibitor of PARP. In growth studies using the same treatment dosages as the SCE-MN experiments, colony formation data corroborate those of the growth inhibition studies, in which all flavonoids exerted varying inhibitory effects on cell proliferation. These cytogenetic studies demonstrated that quercetin, isoquercetin and rutin generally exerted more potency than the synthetic compounds, requiring lower doses to achieve

efficacy. The ability of both the natural and the synthetic flavonoids to cause genomic instability and impair cell growth may have human health implications for chemoprevention.

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Introduction

1.1 Flavonoids

Flavonoids are naturally occurring polyphenolic compounds ubiquitous in plants, where they serve a multitude of physiological functions [1-2]. In plants, flavonoids neutralize oxidative stress, regulate growth, deter feeding by herbivores, and provide protection against pathogens [2-3]. These compounds contribute to the bright hues of yellow, orange, red and blue, in foliage, flowers and fruits [4]. Flavonoids are common constituents in the human diet as they are consumed in plant-derived foods and beverages, such as fruits, vegetables, tea, cocoa, and wine [5-6]. In food applications, these compounds are used as coloring agents, flavor enhancers and antioxidants [7]. In the United States, typical consumption of dietary flavonoids is approximately 1 g/day, while intake fluctuates with geography, seasons, and individual preference [8-9].

All flavonoids share a common benzo-γ-pyrone molecular core which comprises 15 carbon atoms arranged in three rings (Fig. 1) [1]. Classes of flavonoids are organized by the oxidation and substitution patterns of the heterocyclic C ring, whereas compounds within a class are distinguished by the substitution patterns on the A and B rings (Fig. 1) [10]. The variations in

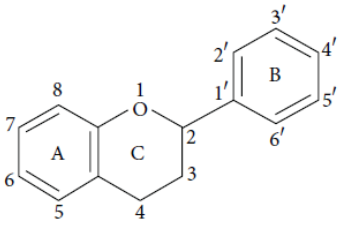


Figure 1 Basic structure of flavonoid

oxidation levels and substitution patterns have led to the identification of over 4,000 flavonoid compounds and characterization of five major classes [10]. Flavone, flavanone, isoflavanone, flavonol, and flavan-3-ols make up the major classes of flavonoids (Table 1). A contributing factor to the great variety of flavonoids is that these polyphenolic compounds occur as aglycones, glucosides[1], and methylated derivatives [7]. Glucosides tend to form on the glucosidic linkage at positions 3 or 7 (Fig. 1), where the carbohydrate is often glucose but can also be rhamnose, glucorhamnose, galactose, or arabinose [11]. Some common food sources associated with various classes of flavonoids are shown in Table 2. Quercetin, catechin, and genistein are some of most common dietary flavonoids [11].

[1] Glucosides and glycosides both signify carbohydrate residues attached to aglycone flavonoids and are used interchangeably.

Table 1 Structures of Common Flavonoids. Reference: [7]

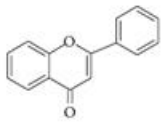
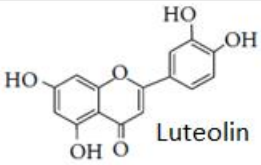
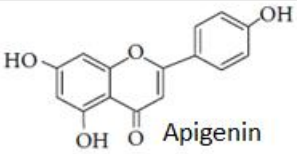
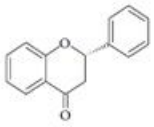
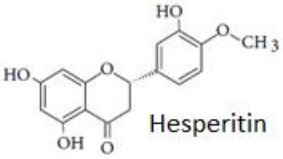
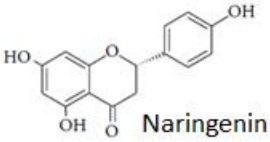
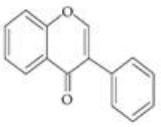
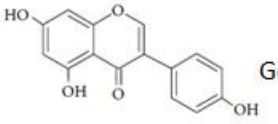
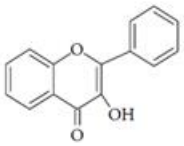
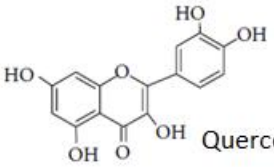
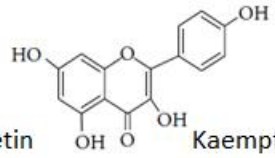
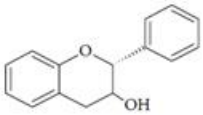
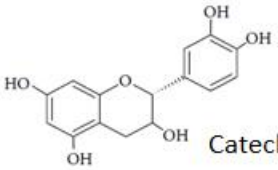
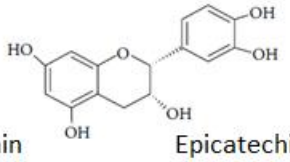
Class	Class Structure	Representative Flavonoids	
Flavone		 Luteolin	 Apigenin
Flavanone		 Hesperitin	 Naringenin
Isoflavone		 Genistein	
Flavonol		 Quercetin	 Kaempferol
Flavan-3-ols		 Catechin	 Epicatechin

Table 2 Flavonoids and common dietary sources

Class	Flavonoid	Food Source	References
Flavone	Luteolin Apigenin Chrysin	Fruit skins	[12-13]
Flavanone	Hesperedin Naringenin	Citrus fruits	[14]
Isoflavone	Genistein Daidzin	Soybean	[15]
Flavonol	Quercetin Kaempferol Myricetin Rutin	Onion, berries, olive oil, grapefruit, red wine	[16]
Flavan-3-ols	Catechin Epicatechin Epigallocatechin	Tea	[17]

1.2 Biological Properties

In plants, the biological properties of flavonoids have been well established. As photoreceptors, they regulate auxins, a class of hormones responsible for growth and differentiation, through stimulation, or inhibition [18,19]. The pigments of flavonoids are often reflected in plant foliage and flowers where they influence the feeding behaviors of pollinators [20]. Flavonoid compounds have an unpleasant taste which contributes to plant defense against herbivores [3,20]. They also provide protection against pathogens by inhibition or crosslink of microbial enzymes essential to proliferation [21]. Furthermore, flavonoids serve as UV screens by absorbing UV radiation and shielding plants from damage to internal tissues [22]. The best known physiological property of flavonoids in plants is antioxidation. Flavonoid compounds can be induced in response to oxidative stress, brought on by metabolic processes, or environmental stress, as well as, depletion of antioxidant enzymes [23]. Oxidation occurs when

the electron transport chain becomes uncoupled, resulting in the transfer of unpaired single electrons from one atom to another [2]. The outcome of this process is the generation of free radicals, which are also known as, reactive oxygen species (ROS). Common examples of ROS include superoxide ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and nitric oxide ($NO^{\cdot}$). Under normal conditions, eukaryotic organisms manage ROS with a system of endogenous antioxidant enzymes [24]. However, when an imbalance of ROS production and radical scavenging activities arises through depletion of antioxidant enzymes, oxidative stress occurs [24]. Accumulation of ROS damages lipids, proteins, nucleic acids, and tissues [24].

Flavonoids combat oxidation with diverse mechanisms. They scavenge free radicals and arrest initiation of lipid peroxidation, and chelate transition metals [25]. Chelated metals are removed as electron donors from the process of reactive oxygen species (ROS) formation via the Fenton reaction. Flavonoids inhibit oxidases, catalysts of superoxide radical production, through their molecular configuration and hydroxylation patterns [26]. Reduction of alpha-tocopherol radicals via hydrogen donation by flavonoids is another major antioxidative mechanism [27]. The protective antioxidant effects in plants yield potentially significant health benefits in humans and are the focus of research in the medical, pharmaceutical, and food industries.

1.3 Human Health and Therapeutic Implications

The concept of the French paradox, in which epidemiological studies highlighted historical consumption of diets high in saturated fat and a low occurrence of cardiovascular disease in the French population, generated immense interest in flavonoids [28]. The paradox was attributed to large consumption of fruits and vegetables on top of high saturated fat intake

[29]. Following the French paradox, a surge of extensive investigations into possible beneficial health effects consequent to flavonoid-rich diets and supplements resulted in *in vitro*, *in vivo*, and epidemiological evidence, which suggests a myriad of benefits (Fig. 2). Flavonoids have

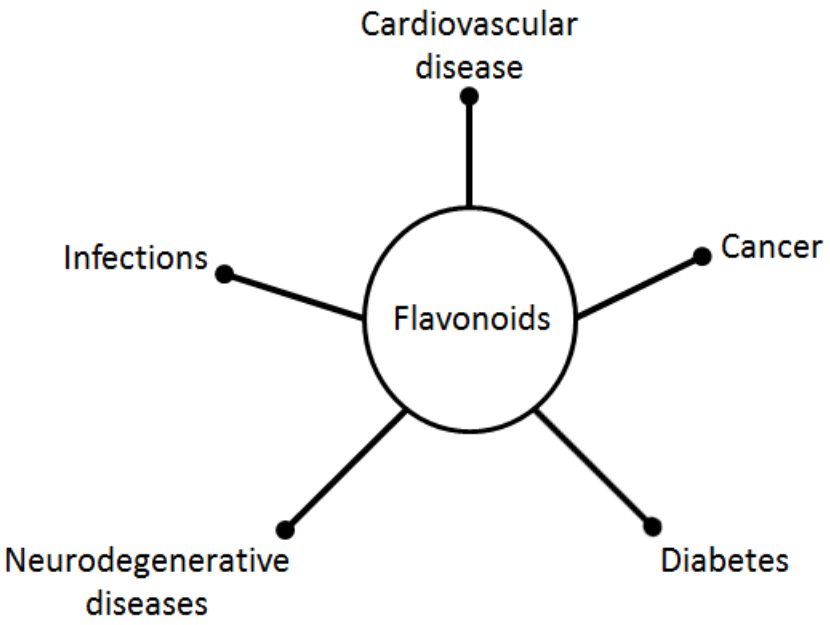


Figure 2 Protective health benefits associated with flavonoids.

been shown to impede development of atherosclerosis by inhibiting oxidation of low-density lipoprotein (LDL) [31]. Catechins which are found in tea, may delay the development of atheromatous lesions in arterial walls by inhibiting invasion of smooth muscle cells [32]. Resveratrol, a prominent flavonoid in wine, inhibits activities of cyclooxygenase 1, which subsequently, prevents platelet aggregation [33]. Quercetin, the most abundant flavonoid, disrupts atherosclerotic plaques via suppression of metalloproteinase 1 [34].

Flavonoids, such as quercetin, resveratrol, flavanones, isoflavones, and catechins have demonstrated chemopreventive effects in numerous studies. Carcinogenesis is a multi-phase process, characterized by three distinct phases: initiation, promotion, and progression. Initiation involves genetic alteration, whereas, promotion requires non-genetic stimului to trigger cell proliferation. The sequence of initiation and promotion events can lead to progression to malignancy. Flavonoids exert chemopreventive effects that interfere with various stages of carcinogenesis through a variety of mechanisms. Cell cycle arrest and apoptosis are anti-proliferative and anti-angiogenic; induction of detoxification enzymes prevent oxidative stress and inflammation; alteration of cellular signaling and immune system regulation contribute to anti-tumorigenic activities [30]. The catechin flavonoid, theaflavin interferes with metastasis in human breast cancer cells via p53 downregulation of the metastatic proteins, metalloproteinase 2 and 9 [35]. The soy-based isoflavone, genistein induces cell cycle arrest of human breast cancer cells at physiologically relevant concentrations [36]. In mice, quercetin is shown to protect against lung cancer induced by benzo(a)pyrene through its radical scavenging mechanism [37]. Resveratrol was found to induce apoptosis through activation of the caspase cascade in human pancreatic cancer stem cells [38].

Anti-viral activities have been reported in studies which explored flavonoid treatments against infections. Theaflavins, common flavonoid constituents in black tea, were found to protect against infections from the human immunodeficiency virus (HIV) by preventing formation of the viral helix bundle necessary for fusion with the host cell membrane [39]. Analogs of theaflavins have exhibited anti-viral activities against the corona virus responsible for Severe Acute Respiratory Syndrome (SARS) [39]. The protective effect is attributed to disruption of the viral replication process via inhibition of proteolytic processing [39]. Protection from neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases may be associated with consumption of flavonoids. In rat studies, rutin, a flavonoid abundant in buckwheat and

wine, was shown to improve memory retrieval through protective activity from β-amyloid toxicity, a key factor in Alzheimer's disease [40]. Nobiletin, a citrus peel flavonoid, exhibited enhancement of dopamine release resulting in improved motor functions in rodent models of Parkinson's disease [41].

Diabetes mellitus, a disease indicated by hyperglycemia due to defective glucose metabolism, is associated with long term progressive health complications when the disease is not maintained. Impaired immunity, retinopathy, neuropathy, nephropathy, and cardiovascular conditions are serious complications of diabetes, which can be debilitating and life threatening [30]. Epigallocatechin gallate (EGCG), a flavan-3-ol flavonoid, has expressed anti-diabetic activities in *in vitro* and animal studies [42]. Under glucotoxic conditions, EGCG promoted insulin release through upregulation of insulin receptors, and survival of pancreatic β-cells by modulating oxidative stress and cytokine-induced apoptosis through inhibition of pro-apoptotic proteins [42]. Kaempferol, a flavonol, was also shown to promote cell survival under cytotoxic conditions by rescuing β-cells and pancreatic human islet cells from lipid peroxidation-induced apoptosis [43].

Extensive evidence provides a rationale in support of using dietary flavonoids in the prevention of various diseases, in conjunction with either conventional drug therapy, or dietary intervention. Although solubility may pose hindrance to therapeutic efficacy as most flavonoids are poorly solubilized in water [7,44]. This limitation can be attributed to the crystalline structure of the flavonoid compounds, which exerts strong intermolecular forces that cannot be broken by the release of energy during solvation [44]. The solubility issue has been circumvented to an extent by the development of partially synthetic flavonoids with increased water solubility [44]. However, the low solubility property of flavonoids restricts bioavailability, and consequently, toxicity from consumption [7].

1.4 Bioavailability of Flavonoids in Humans

The implications of flavonoid therapeutic value appear to be diverse, but in order to optimize the health benefits, the bioavailability of flavonoid compounds must be considered. The physicochemical properties of flavonoids dictate absorption from either the small intestine or the colon [3]. Aglycone flavonoids are freely absorbed by the small intestine, while hydrolysis of glucosidic flavonoids is required for transformation to aglycones prior to absorption [45-47]. Preferential flavonoid glucoside hydrolysis takes place in the lumen of the small intestine via β-glucosidases [45-47]. Glucosides without substrates specific to β-glucosidases are hydrolyzed by microflora in the colon, where absorption capacity is much more limited, and free aglycones are degraded [45-47]. Once absorbed, flavonoids are metabolized in the liver to phenolic acids or conjugated to glucuronides, sulfates, or methylated compounds [3]. The metabolites are primarily excreted in feces, with a small fraction in urine [3]. Figure 3 summarizes flavonoid metabolism in humans. Hydrolysis is essential to the absorption of flavonoid glucosides, but it is one of several factors that determine bioavailability. The sugar moiety of glucosides, as well as, functional groups on the molecular core influences the structural and physiological location of hydrolysis [48-49]. Dose, delivery vessel, diet, gender differences, and colonic microflora population, may also affect absorption, and subsequent bioavailability of flavonoids [49].

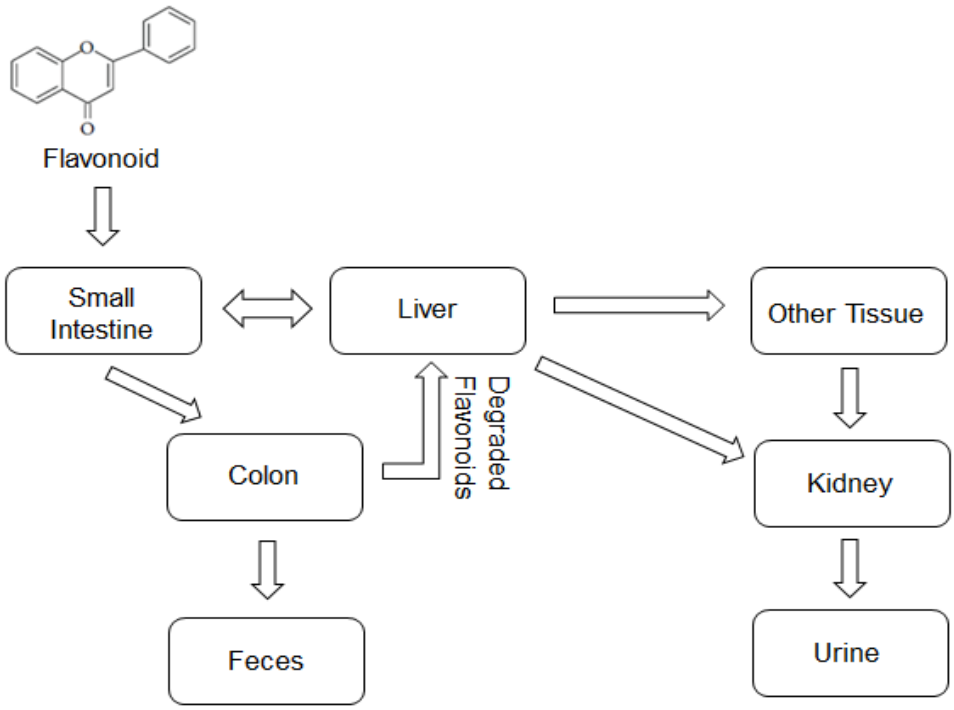


Figure 3 Simplified scheme of flavonoid metabolism. Reference: [3].

1.5 Quercetin and its Glycosides

1.5.1 Quercetin, Isoquercetin and Rutin

Quercetin is the most abundant flavonol, a subclass of flavonoids, which occurs in nearly all parts of fruits and vegetables [18]. Quercetin-rich dietary sources are cranberries, apples, blueberries, onions, black tea, red wine, and fruit juices [50]. The average daily intake of quercetin is estimated to be in the range of 25 mg to 500 mg per day, which makes up approximately 2.5% to 50% of the estimated total consumption of flavonoids per day [9]. In plants, quercetin commonly occurs as glycosides, such as isoquercetin and rutin. Isoquercetin and rutin each has a monosaccharide and a disaccharide residue on the glycosidic linkage, respectively (Fig. 4). Isoquercetin is widely distributed in mangoes, apples, and a variety of fruits

and vegetables [51]. Buckwheat, citrus fruits, fruit skins, and red wine are typical sources of rutin [52].

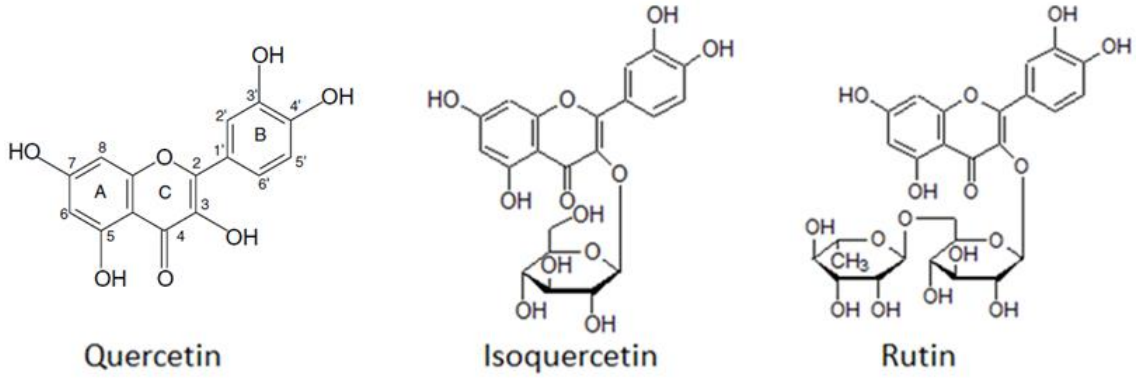


Figure 4 Molecular structures of quercetin and its glycosidic derivatives.

Similar to reported benefits of many flavonoids, studies in cell systems and animals show that these flavonols are potentially beneficial to human health [53-59]. Quercetin has been shown to be cardioprotective through reduction of oxidative stress associated with ischemia and reperfusion [53]. It is reported to modulate inflammation by decreasing production of inflammatory cytokines [54]. The anti-proliferative effects of quercetin have been demonstrated in various types of cancer cells including breast, cervical, gastric, and skin [5,55-57]. Isoquercetin has exhibited neuroprotective effects against lipid peroxidation in neurotoxic induction of Parkinson's disease, and oxidative stress produced by ischemic brain injury [51,58]. Anti-allergenic and anti-arthritic activities have been expressed by rutin. Rutin was shown to be effective in inhibiting histamine release during immunoglobulin E-mast cell activation in mucosal cell studies, and in septic arthritis, suppressing production of ROS by macrophage [52,59].

1.5.2 Pharmacokinetics

Following ingestion, quercetin is primarily absorbed and metabolized in the small intestine [18,50]. However, the only form of quercetin that can be absorbed is as an aglycone [18,50]. The glycosides, isoquercetin and rutin, must undergo hydrolysis and transformation to the quercetin aglycone in either the small intestine or the colon [7,50]. The aglycone is transported into enterocytes via sodium-dependent glucose transporters, where it is methylated, glucuronidated, or sulfated at one of the hydroxyl sites on the aglycone [50]. Conjugated and free quercetin then enter systemic circulation via the hepatic portal vein [50]. Once in the liver and kidney, the metabolites and untransformed quercetin can be further metabolized by methylation, sulfation and glucuronidation [18,50]. Unabsorbed quercetin glycosides and free quercetin are subject to metabolism and degradation by colonic microflora to phenolic acids and carbon dioxide [18,50]. Rodent studies indicate that a mere 3% of quercetin aglycone is absorbed from the colon [50]. Animal and human studies suggest that up to 60% of the ingested dose is absorbed and bioavailable [50]. Systemic distribution of quercetin affects all organs and tissue types [50]. The majority of quercetin metabolites is excreted in feces and respired, with urine as a minor elimination pathway [18,50]. Human studies have reported the elimination half-life of quercetin to be in the range of 31 – 50 hours [50].

1.5.3 Pharmacodynamics

The health benefits linked to quercetin, isoquercetin and rutin are generally associated with their antioxidant properties [18,51,58]. The antioxidant property of these flavonols is largely attributed to their chemical structure which has a hydroxylation pattern of 3, 5, 7, 3', and 4', and a catechol B ring (Fig. 4) [60]. Bors and colleagues characterized an antioxidant as having a catechol group in the B ring, a 2,3-double bond, and hydroxyl substitutions at positions 3 and 5 [61]. These features take part in the radical scavenging and metal chelating activities of

flavonoids [62]. Quercetin preferentially chelates transition metals, such as iron and copper, at C3-OH, C4=O, C5-OH, and the catechol moiety [62]. Metal chelation prevents oxidative stress through removal of transition metals from the Fenton reaction, where their presence in a low oxidation state with hydrogen peroxide produces hydroxyl radicals [62].

Quercetin also has pro-oxidant properties that are rooted in the same structural attributes which give rise to its antioxidant properties. It can be metabolically bioactivated to the reactive products, semi-quinone intermediate, and subsequently, *ortho*-quinone. *Ortho*-quinone can bind DNA causing oxidative damage, which is proposed to be a mechanism of mutagenicity of quercetin. The mutagenic property of quercetin has been well established in positive reverse mutation tests in various strains of *Salmonella typhimurium*, though it has not exhibited carcinogenic effects in humans [3,16]. Such mutagenic property may confer protection against carcinogenesis [64]. Glycosylation of aglycones, on the other hand, tends to diminish pro-oxidant activities, resulting in less mutagenicity [64].

Genetic stability is crucial to cell proliferation and survival, and as a mutagen, quercetin has been shown to induce genomic instability in the form of sister chromatid exchange (SCE) and micronuclei [65]. It was also demonstrated to impair DNA repair capability by inhibiting poly(ADP ribose) polymerase (PARP), an enzyme critical to DNA single strand break (SSB) repairs, in various cancer cell lines [5]. Aside from causing genomic instability, quercetin has exhibited cytotoxicity through growth inhibition and cell cycle arrest in various types of cancer cells [7]. Collectively, these anti-proliferative mechanisms make quercetin and its glycosides ideal candidates in anti-cancer therapy and chemoprevention.

1.6 Statement of Project

Quercetin and its glucoside derivatives have demonstrated potential health benefits and therapeutic value in *in vitro* and animal studies. Increased consumption of these dietary flavonols may provide protection against various diseases, particularly cancer. However, the poor water solubility property of quercetin poses an issue in manufactured food and beverage applications, which has led to the development of semi-synthetic versions of this compound. This thesis is an examination of the cytogenotoxic responses induced by three novel glucosyl flavonoids, in comparison to those of quercetin, isoquercetin, and rutin. Using known cytogenotoxic endpoints of quercetin, an analysis of growth inhibition, viability, sister chromatid exchange, micronuclei, and inhibition of PARP activity was completed in an *in vitro* system of Chinese hamster ovary (CHO) cells.

Chapter 2

Comparative Studies of Cytogenotoxic Effects of Natural and Synthetic Flavonoids: Quercetin, Isoquercetin and Rutin versus Maltooligosyl Isoquercetin, Monoglucosyl Rutin and Maltooligosyl Rutin.

2.1 Background and Rationale

As human health stands to benefit from the biological properties of quercetin and its glycosides isoquercetin and rutin, there is an increase in interest to incorporate these flavonols into various food and beverage products, as well as, therapeutic applications. Before these flavonol compounds can be employed in food and therapeutic applications, the challenge of water solubility must be overcome. Quercetin, isoquercetin and rutin are not readily miscible with water which lends difficulty to their incorporation into dietary products, and utilization in medicinal applications [7]. The miscibility issue has led to the development of semi-synthetic flavonoid glycosides with improved water solubility: maltooligosyl isoquercetin (MI), monoglucosyl rutin (MO), and maltooligosyl rutin (MA) (Fig. 5). Enzymatic modifications to the natural aglycone and glycosides increase the number of carbohydrates that are attached to the glycosidic linkage of the novel glycosides. MI and MA contain one to seven glucose modifications, and only a single glucose modification on MO.

Currently, no cytogenotoxic information is available for MI, MO and MA. The aim of this thesis is to examine the cytogenetic responses through comparison of known cytogenotoxic endpoints induced by quercetin with those of the novel glucosyl flavonoids. Endpoints under evaluation are cell viability and growth inhibition, SCE, micronuclei, and PARP inhibition. It is hypothesized that the novel glucosyl flavonoids will mimic the cytogenotoxic effects exhibited by quercetin in a dose-dependent manner, based on similarities in structural composition of these

compounds. Using a system of wild-type CHO cells, treatments with reconstituted flavonols at a concentration range of 0.1 – 10,000 ppm were applied to CHO cell cultures in viability, growth, SCE and micronuclei assays. PARP activity was determined using ELISA and the same treatment dosages.

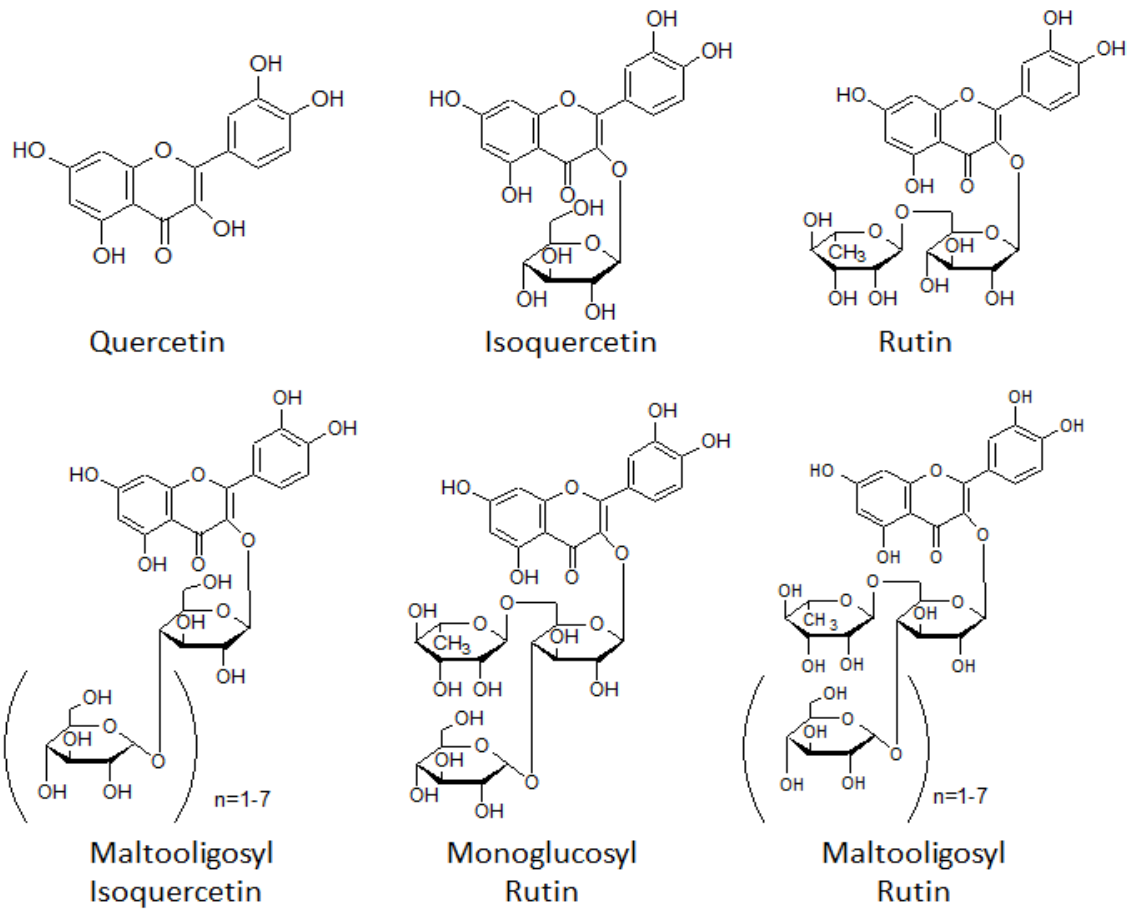


Figure 5 Chemical structures of natural (top row) and synthetic (bottom row) flavonoids.

2.2 Materials and Methods

2.2.1 Cell culture

CHO cells were cultured and maintained in minimum essential medium (MEM-α, Gibco, Grand Island, NY) and supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) and 1% antibiotics and antimycotics (Gibco, Grand Island, NY) in a humidified incubator at 37^{o}C and 5% CO_2.

2.2.2 Flavonoids

Quercetin, isoquercetin, maltooligosyl isoquercetin, rutin, monoglucosyl rutin and maltooligosyl rutin were provided by the Toyo Sugar Refining Co., Ltd. (Tokyo, Japan). 10% (w/v) solutions of all flavonoids were prepared in dimethyl sulfoxide (DMSO, Fisher Scientific, Pittsburgh, PA). The treatment dosage range utilized in this study was 0.1 ppm to 10,000 ppm.

2.2.3 Colony formation

Trypsinized CHO cells were plated on P-60 dishes to obtain approximately 100 colonies per dish. Cells were treated with various dosages of natural and synthetic flavonoids. After a 7-day incubation period, cells were washed in 0.9% sodium chloride and fixed in 100% ethanol, then stained with 0.1% crystal violet dye (Sigma). Colonies with greater than 50 cells were recorded as viable.

2.2.4 Growth inhibition

In 24-well plates, 4,000 CHO cells were seeded per well, then treated with various dosages of flavonoids, and incubated for 3 days. Trypsinized cells were counted using the Coulter Z1 (High Wycombe, United Kingdom), and the numbers were normalized.

2.2.5 PARP inhibition

PARP colorimetric assay kits (Trevigen, Gaithersburg, MD) were used to measure PARP activity. 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/l 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 is directly proportional to absorbance at 450 nm and measured by the NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA).

2.2.6 Sister chromatid exchange

CHO cells were synchronized into G1 phase using a mitotic shake-off procedure [66]. Synchronized mitotic cells were sub-cultured in T25 flasks and incubated for two hours at 37°C. Cells were treated with various concentrations of flavonoids and incubated with 10 uM of 5-bromo-2'-deoxyuridine (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 4 ml of a 75 mM KCl solution warmed to 37°C and placed in a 37°C water bath for 20 minutes. A fixative solution of 3:1 methanol to acetic acid was added to the samples according to the standard protocol [67]. 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 [68]. Differentially stained metaphase chromosome images were taken using a Zeiss Axioplan microscope equipped with Q-imaging Aqua CCD camera and Q-capture Pro 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

G1 synchronized CHO cells were incubated with various concentrations of flavonoids and 8 ug/ml of Cytochalasin B (Sigma) for 22 hours. Harvested cells were suspended in 5 ml of 75 mM KCl solution, centrifuged, and fixed in 3:1 methanol:acetic acid solution and formaldehyde (Fisher Scientific). Cells were dropped onto slides and allowed to air dry at room temperature. Slides were stained in 10% Giemsa (Gibco) in GURR (Gibco) solution for 5 minutes. 300 binucleated cells (BNC) were scored per treatment dosage, and images were analyzed using a Zeiss Axioplan microscope.

2.2.8 Statistics

All experiments were repeated at minimum three times, and error bars indicate standard errors of the means. Statistical significance was assessed by the t test using Prism 5 software (GraphPad Software, Inc., San Diego, CA). For all data, p values of <0.05 indicate statistical significance.

2.3 Results

2.3.1 Cell viability and growth inhibition

Assessment of cell viability was based on qualitative colony formation and quantitative growth curve studies. Figure 6a shows the effect of 1 and 10 ppm quercetin treatments compared to the control sample, where a reduction in the number and size of colony formations was observed. The effects of rutin, MO and MA treatments are shown in Figure 6c. The colony size and formations decreased with increasing concentrations when compared to the control. Isoquercetin and MI also exerted effects with similar trends as quercetin and rutin (Fig. 6b). No colony formations were observed at the 1,000 ppm treatment dose for all flavonoids, except for

MA, where very small colonies formed. Growth curve studies show that quercetin is the most potent in growth inhibition with an extrapolated IC50 (concentration at which 50% activity is inhibited) of 5 ppm, where isoquercetin and MI had an IC50 of 200 ppm (Fig. 7a). The IC50 of rutin is 500 ppm and MO 2,000 ppm, while MA does not reach IC50 at the highest dose of 1,000 ppm (Fig. 7b).

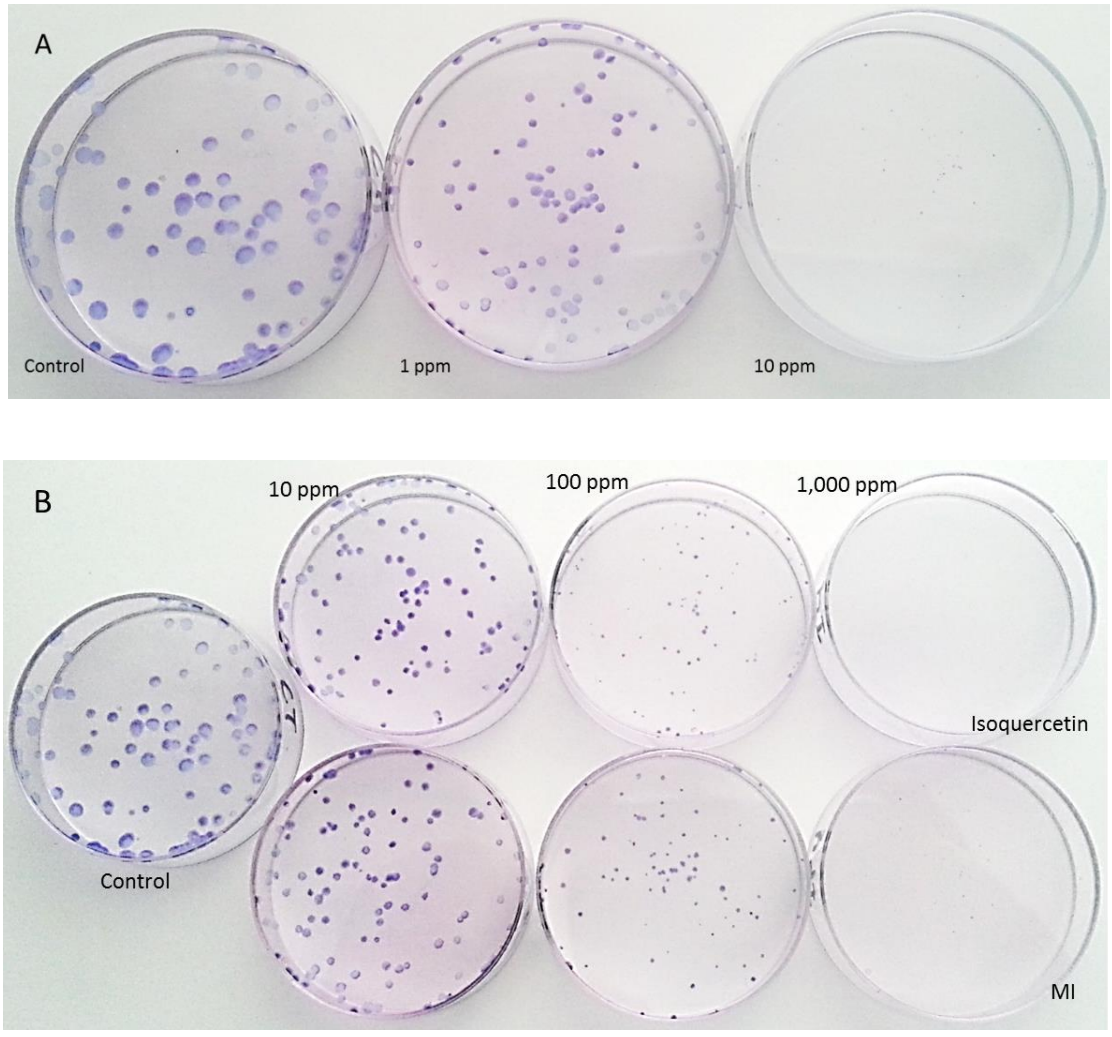


Figure 6 Effects of flavonols on CHO cell colony formations 10 days post treatment: (a) quercetin at 1 and 10 ppm; (b) isoquercetin and MI at 10, 100, and 1,000 ppm.

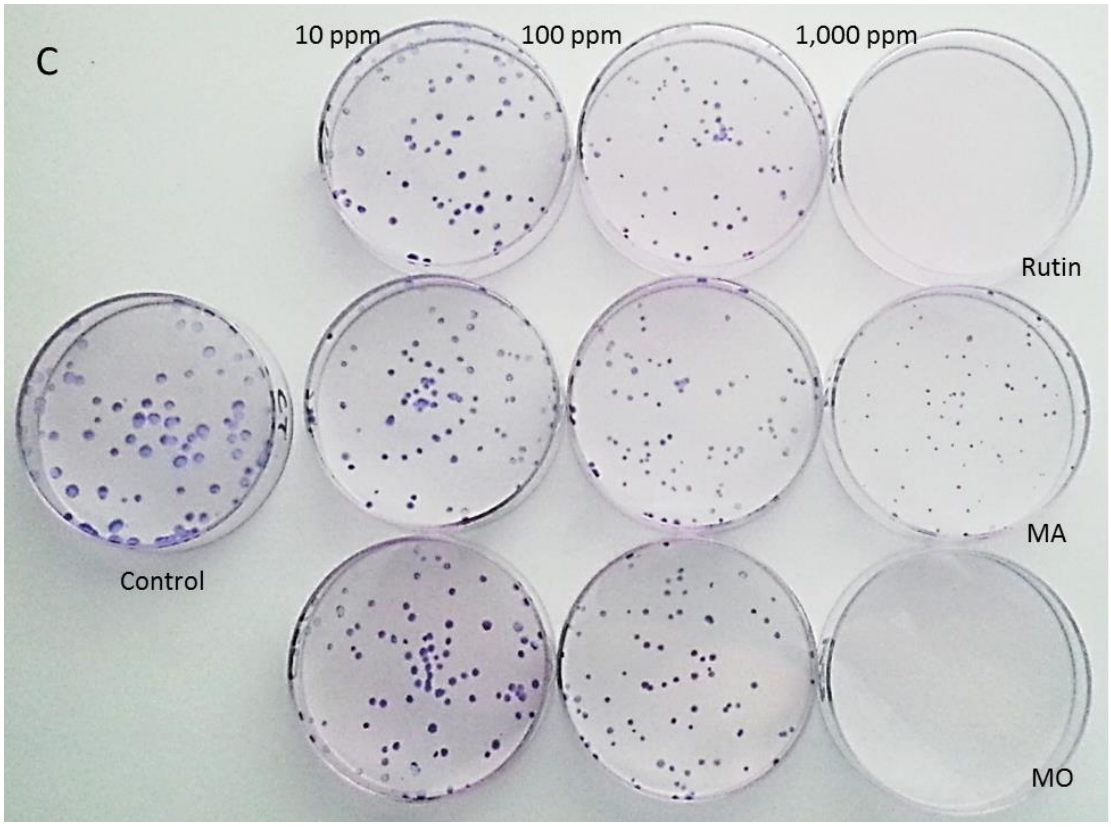


Figure 6 *continued* Effects of flavonols on CHO cell colony formations 10 days post treatment: (c) rutin, MO and MA at 10, 100 1,000 ppm.

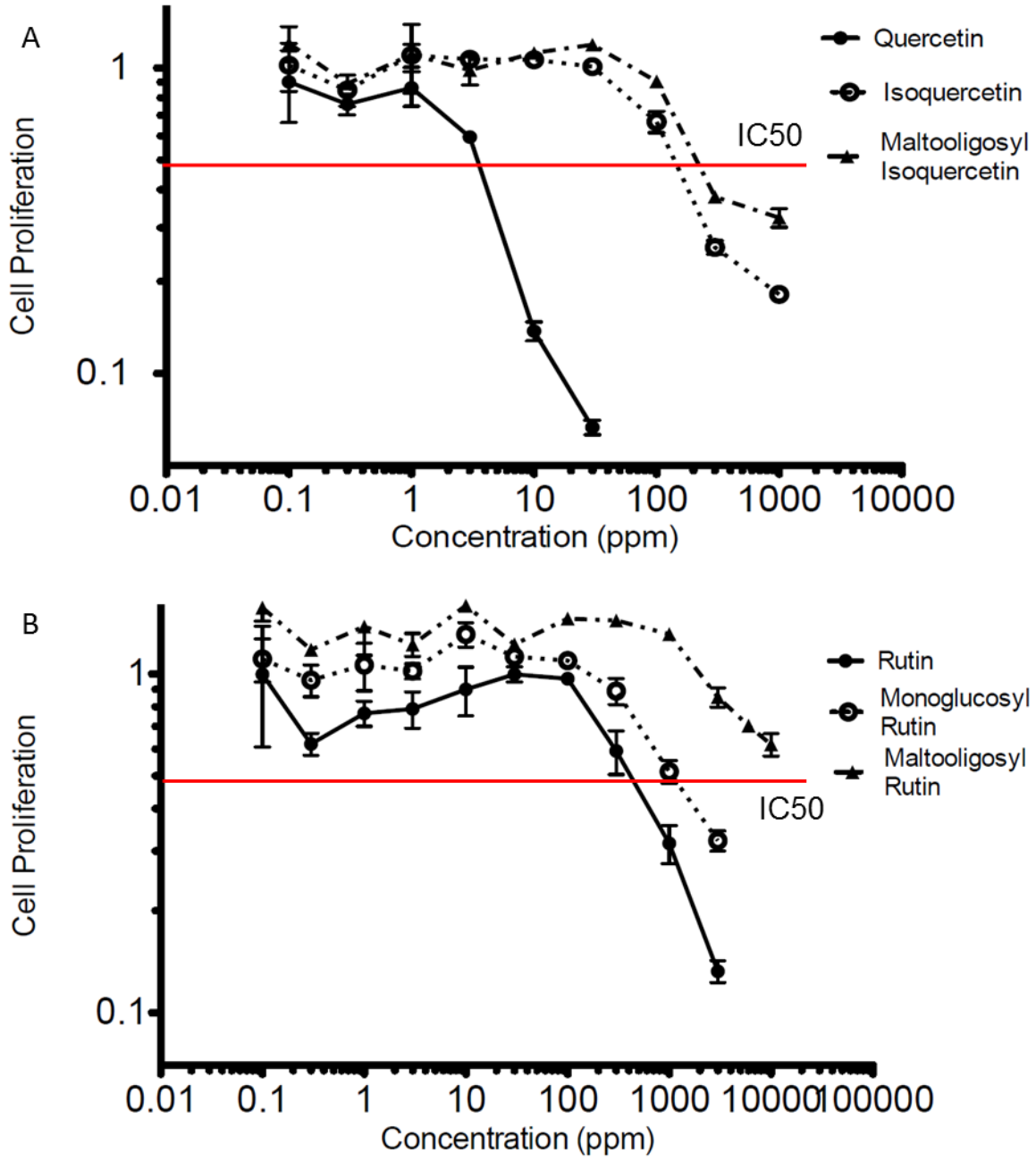


Figure 7 Growth curves of CHO cells in response to treatments with flavonoids after 3 days: (a) quercetin, isoquercetin and MI over a treatment range of 0.1 to 1,000 ppm; (b) rutin, MO and MA dosing range of 0.1 to 20,000 ppm.

2.3.2 Sister chromatid exchange

The frequency of SCE was determined through incorporation of BrdU into DNA during cell replication, followed by the arrest of cells in metaphase, chromosome extraction, and differential staining of chromosomes. Figure 8 illustrates images of SCE in metaphase chromosomes. Figure 9 shows SCE induction frequency per chromosome. Untreated CHO cells exhibited a mean background level of 0.21 SCE per chromosome. Figure 9a shows the SCE frequency in cells treated with 0.1 – 10 ppm quercetin, isoquercetin and MI. All three compounds induced SCE at a range of 0.22 – 0.27 SCE per chromosome. Isoquercetin induced the largest and significant SCE frequency of 0.27 per chromosome at 1 ppm (p<0.05). While quercetin induced slightly less SCE frequency of 0.26 per chromosome at 0.1 ppm, and the increase is significant (p<0.05), which makes it more potent than isoquercetin and MI. The SCE induction frequency by MI is the same as that of quercetin, though this increase required 10 ppm to achieve the same response. No SCE was observed at doses above 10 ppm for these three flavonoids.

Rutin, MO and MA also induced SCE responses across all three doses of 1, 10, and 100 ppm (Fig. 9b). Treatments with 0.1 ppm were not used in this study based on previous screenings, which did not result in responses above spontaneous background levels of SCE. Rutin, MO and MA induced SCE frequencies at a range of 0.19 – 0.26 per chromosome. Of these three, rutin exerted the most potency with a significant SCE frequency of 0.26 per chromosome at 1 ppm (p<0.05). Rutin maintained its potency at 10 ppm with a slightly less but significant SCE frequency of 0.25 per chromosome (p<0.05). MO achieved the same response of 0.26 SCE frequency per chromosome (p<0.05) as rutin, but at 100 ppm. MA treatments did not result in statistically significant responses. No SCE was observed at treatments with 1,000 ppm for rutin, MO and MA. Based on order of potency demonstrated in this study, the flavonoids can be ranked in the following order: quercetin>isoquercetin>rutin>MI>MO>MA.

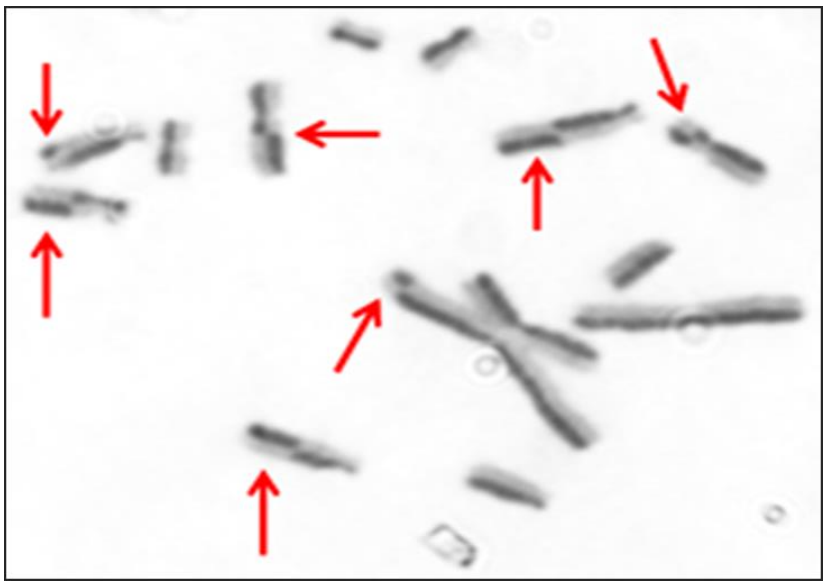
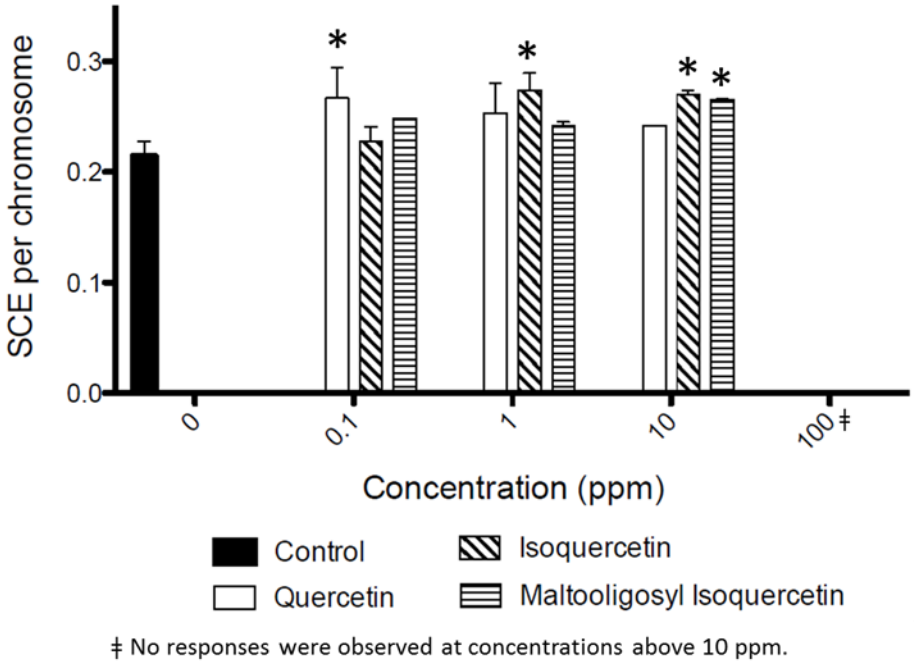


Figure 8 Differentially stained metaphase chromosomes with arrows indicating SCE.



‡ No responses were observed at concentrations above 10 ppm.

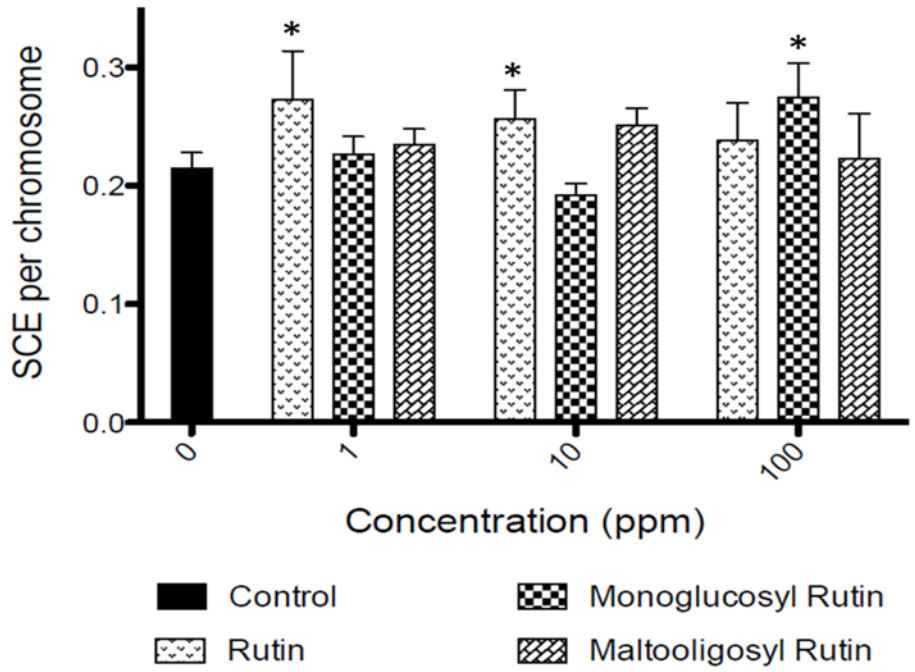


Figure 9 SCE frequency per metaphase chromosome after treatment with flavonoids for 25 hours, (* indicates $p<0.05$): (a) responses to quercetin, isoquercetin and MI at 0.1, 1, and 10 ppm; (b) responses to rutin, MO and MA at 1, 10, and 100 ppm.

2.3.3 Micronuclei

Micronucleus frequency was quantified by arresting untreated and treated mitotic CHO cells in telophase with cytochalasin B, which resulted in binucleated cells. Untreated cells had a background level of 0.01 MN per binucleated cell. An image of a micronucleus in a binucleated cell is shown in Figure 10. All flavonoid treatments resulted in varying degrees of MN induction. Induced responses by quercetin, isoquercetin and MI range from 0.005 – 0.075 MN per binucleated cell (Fig. 11a). Of these three, 10 ppm quercetin resulted in statistically significant (p<0.05) and the highest induction of MN frequency at 0.075 MN per binucleated cell (Fig. 11a). All four treatment doses of isoquercetin induced significant frequencies of MN (p<0.05), where the 1,000 ppm treatment induced 0.06 MN per binucleated cell (Fig. 11a). MN frequencies for MI occurred at or below control levels for doses of 1 – 100 ppm, however, at 1,000 ppm, 0.065 MN per binucleated cell (p<0.05) were observed (Fig. 11a). The frequencies of MN induced by rutin, MO and MA range from 0.008 – 0.04 MN per binucleated cell (Fig. 11b). Rutin significantly increased MN frequencies at 1 and 1,000 ppm with 0.016 and 0.04 MN per binucleated cell (p<0.05), respectively (Fig. 11b). Both MO and MA induced significant frequencies of MN at 100 ppm, where MO treatment resulted in 0.02 and MA 0.016 MN per binucleated cell (p<0.05) (Fig. 11b). Similar to the SCE studies, quercetin demonstrated to be the most potent MN inducer. The relative potency of each flavonoid is summarized as follows: quercetin>isoquercetin>rutin>MO>MA>MI.

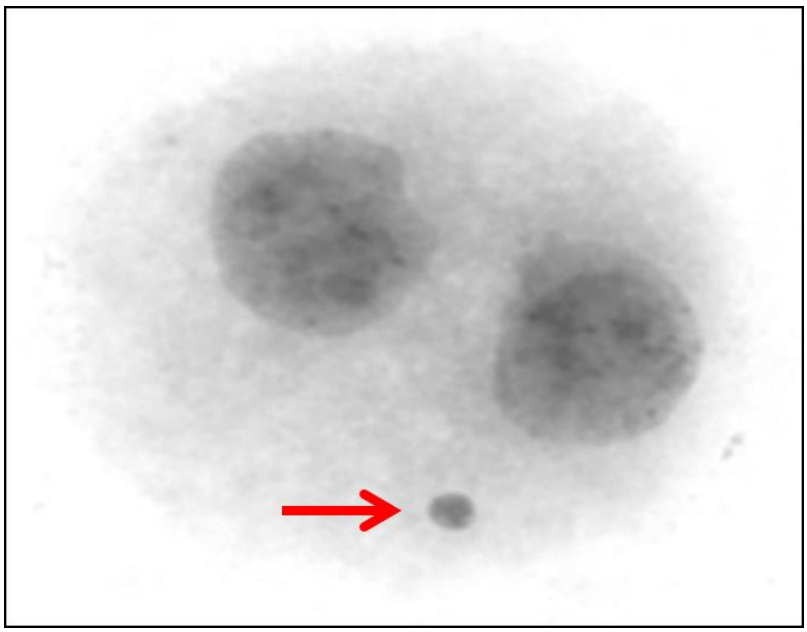


Figure 10 A binucleated CHO cell with a micronucleus (indicated by the red arrow).

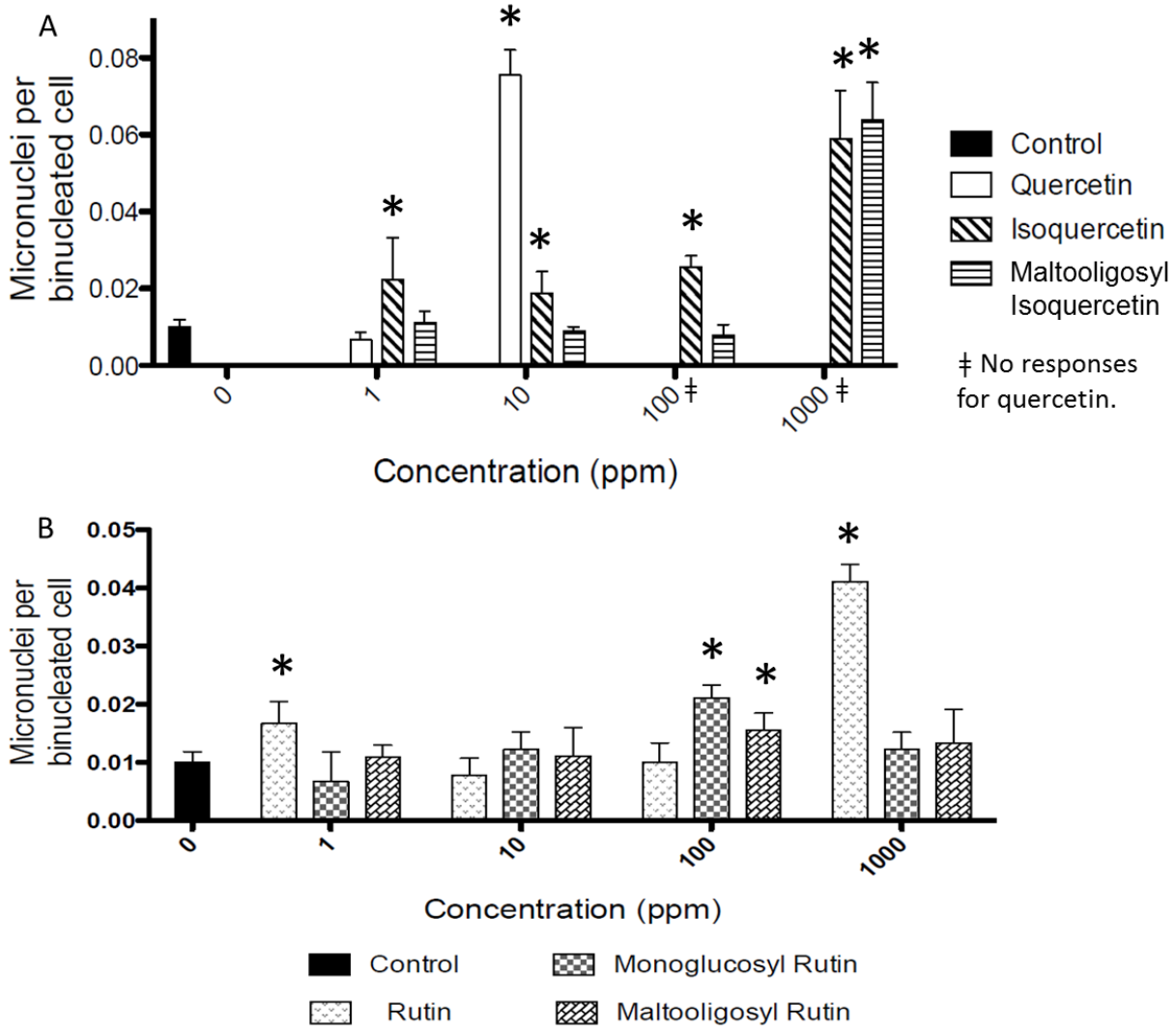


Figure 11 Frequency of micronuclei per binucleated cell after a 20-hour treatment with flavonoids: (a) treatment with quercetin, isoquercetin and MI at concentration range of 1 to 1,000 ppm; (b) treatment with rutin, MO and MA at concentrations of 1 to 1,000 ppm. Asterisk indicates $p<0.05$.

2.3.4 PARP activity

Flavonoid influence on PARP activity was quantified using a PARP ELISA assay which measured poly ADP-ribosylation of histone proteins. 3-aminobenzamide (3-AB), a known PARP inhibitor was used as a positive control. Quercetin exerted the greatest inhibitory effect on PARP activity (Fig. 12a) with an extrapolated IC50 of 30 ppm, followed by 3-AB at IC50 of 100

ppm. Rutin and isoquercetin share the same IC50 at 1,000 ppm, whereas treatments of 10 and 100 ppm with both flavonoids show rutin as the stronger inhibitor of PARP (Fig. 12a-b). All three synthetic flavonoid glycosides exhibited less inhibitory effects on PARP activity than the natural flavonoids (Fig 12a-b). The IC50 for MO is 5,000 ppm, while MI and MA do not reach IC50 even at 20,000 ppm (Fig. 12a-b). The apparent potency of each flavonoid in PARP inhibition is summarized as shown: quercetin>rutin>isoquercetin>MO>MA>MI.

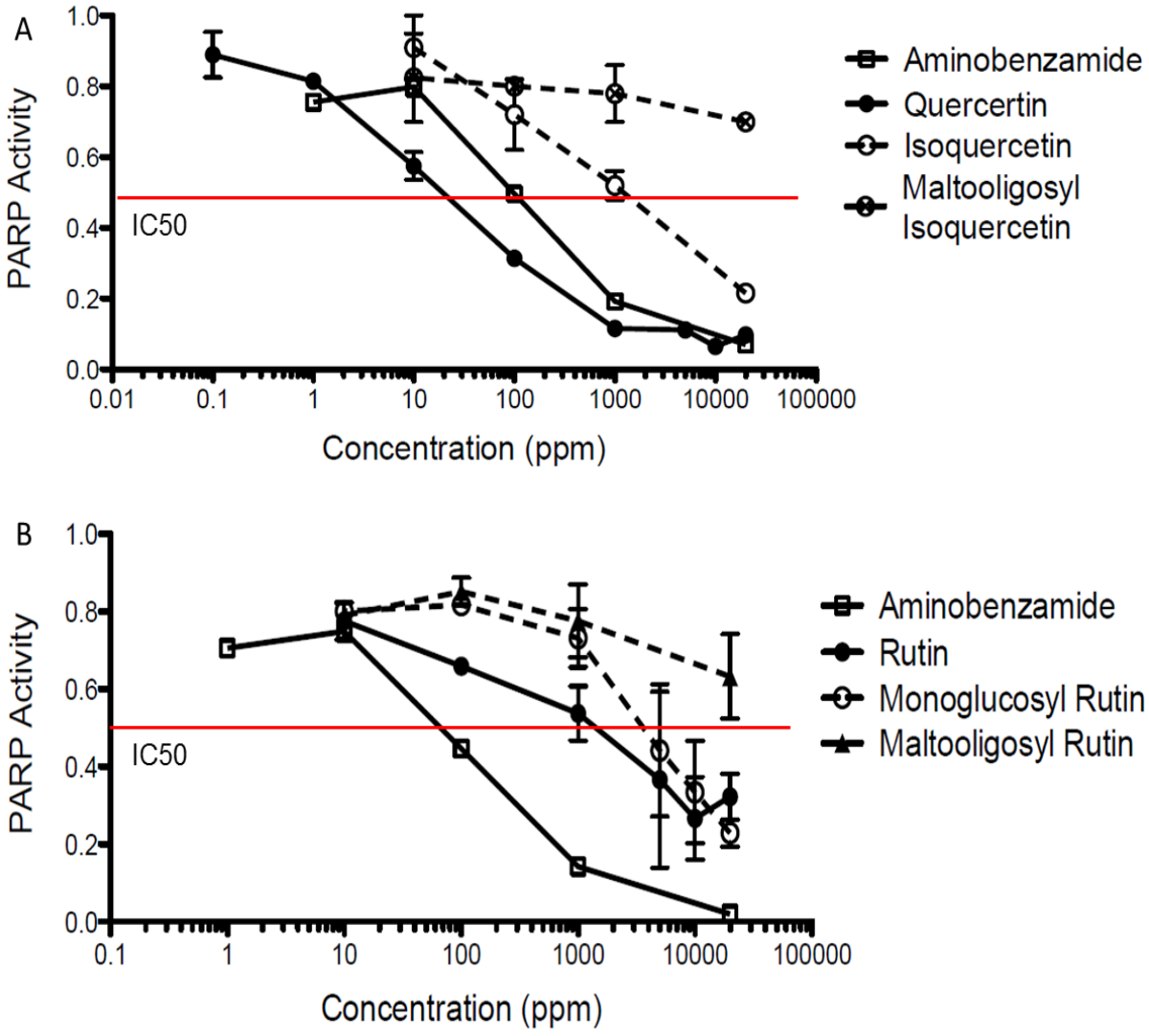


Figure 12 Inhibitory effects of flavonols on PARP activity: (a) treatments with quercetin, isoquercetin and MI at concentrations of 0.1 to 20,000 ppm; (b) treatments with rutin, MO and MA at concentrations of 1 to 20,000 ppm; aminobenzamide is a positive control.

2.4 Discussion

The results of the present study support the hypothesis that synthetic flavonoids mimic the cytogenotoxic responses induced by natural flavonoids. The degree of responses, however, appears to be modulated by the structure activity of each flavonol, as determined by the number of glucosides contained in each compound. Both the natural and synthetic flavonoids exhibited

weakened cytogenotoxic effects in accordance with the level of glycosylation. Based on findings in this study, they are ranked as follow in order of overall potency, which also corresponds with the number of glucosides: quercetin>isoquercetin>rutin>MI>MO>MA. Quercetin, the aglycone flavonol, exhibited the most potency across all cytogenetic assays in this study, requiring the lowest treatment dose to induce significantly high frequencies of SCE and MN (p<0.05) and effectively inhibit growth and PARP activity. Its poor water solubility property is most likely responsible for high bioavailability, since aglycones do not contain glucosides that prevent, or require enzymatic hydrolysis for absorption into cells. MA, a manufactured quercetin rutinoside derivative with 1 – 7 glucosyl modifications, exerted the least cytogenotoxicity in this study. MA did not significantly induce SCE or produce IC50's in growth and PARP experiments at the highest doses. In colony formation assays, MA was the only flavonol which did not completely prevent colony formations at the highest treatment dose of 1,000 ppm after 7 days. While a significant induction of MN frequency was observed at 100 ppm (p<0.05), MA produced the lowest frequency of all the significant observations. These results suggest that MA is substantially less bioavailable than other flavonols. As a consequence of having more glucosyls and increased water solubility over natural flavonoids, these properties most likely contribute to poor absorption into cells, thus reducing bioavailability.

Consistent with other studies, the high frequencies of SCE and MN indicate that quercetin is able to bind DNA, possibly causing oxidative damage through its pro-oxidant property that resulted in DNA damage and disruption to DNA repair processes [65,69]. SCE and MN occur under different mechanisms, and together, the effects may drastically lower chromosomal stability resulting in growth arrest or apoptosis. SCE is a product of DNA homologous recombination in response to collapse of the DNA replication fork due to strand breaks [70]; whereas MN are fragments of chromosomes, or whole chromosomes that failed to be incorporated in the daughter nuclei during nuclear division, as a result of defective DNA

lesion repairs or mitotic malfunction [71]. The reduction in cell proliferation evident in the growth curves (Fig. 7) and colony formation studies (Fig. 6) may also be attributed to failure to maintain genetic stability. PARP is an enzyme crucial to DNA single strand break (SSB) repair, in which PARP detects and binds to a site of SSB, then synthesizes poly (ADP) ribose (PAR) proteins; PAR recruits other repair proteins to the site of damage to complete DNA repair [72]. Quercetin and rutin have previously been reported to preferentially inhibit PARP in BRCA-mutant cells associated with ovarian and breast cancers, which subsequently impairs cell viability [5]. In agreement with literature on PARP inhibitors, the current study demonstrated that quercetin and rutin were both effective in inhibiting PARP activity [5]. Isoquercetin and MO also inhibited PARP, although requiring much higher doses than quercetin. While MI and MA decreased PARP activity, they did not achieve IC50, even at the highest doses. Studies of PARP-deficient cell systems show that exposure to genotoxic chemicals resulted in a substantial increase in SCE and MN frequencies, which suggest an increase in recombination repairs [73]. The ability of natural and synthetic flavonoids to inhibit PARP indicates that the absence of this enzyme may be an underlying factor in the decline in genomic stability of exposed CHO cells. Furthermore, PARP has been shown to play a role in proper chromatin conformation of newly synthesized DNA [73]. Thus, elevated SCE and MN frequencies induced by flavonoids may involve a combination of mechanisms related to PARP inhibition: failure of DNA repair and alterations of chromatin conformation. Additionally, PARP is reported to be essential in cell proliferation, and its inhibition can negatively affect cell growth through several mechanisms: decreased cell recovery after oxidative damage; increased genomic instability from loss or gain of chromatin delays proliferation; and failure of the activation cascade of DNA repair proteins [73].

Interestingly, all flavonoids in this study share a common trend, where an inverse relationship between dose and responses in induction of SCE is observed. Instead of a dose-

dependent increase in SCE inductions, SCE frequencies declined at higher treatment doses. Examinations of stained chromosomes show a decreased proportion of cells in mitosis to those which are not. The lack of SCE responses appears to correspond with impairment of cell proliferation and viability. As evident in the growth studies, cell proliferation is impeded by increased flavonoid concentrations. Failure at the DNA replication fork must take place in order for SCE to form, and when cells cease to divide, SCE are not generated. These observations, on the other hand, are generally not expressed in MN inductions with exception of quercetin. Quercetin induced a peak MN frequency of at 10 ppm and no responses at 100 and 1,000 ppm. These observations may be explained by PARP inhibition, defective DNA repairs, and cessation of cell growth, which are evident in the present study. PARP is reported to be important in maintaining chromatin conformation, and its inhibition can result in an increase in MN frequency [73]. MO and MA induced a peak MN frequency at 100 ppm, although low but significant responses; and at 1,000 ppm, the MN inductions reduced to insignificant levels. A low MN response at 1,000 ppm following a peak response at 100 ppm, may be attributed to cytotoxicity associated with genomic instability.

The relevance of these findings for potential utilization in food and therapeutic applications as a chemopreventive agent is efficacy. This study shows that increased water solubility by glucosidic modifications corresponds with reduction in efficacy, which is most likely the result of poor absorption. The novel flavonoid glucosides have a large number of carbohydrate modifications which require enzymatic hydrolysis for absorption into cells. While hydrolysis can transform glucosides to aglycones, the site of cleavage may differ from one compound to another [3,7]. Although a flavonoid glucoside is cleaved, it may still retain some carbohydrates, preventing its absorption. Utilization of the novel glucosyl flavonoids for the purposes of chemoprotection would likely require a higher dose with repeated consumption to achieve similar efficacies as quercetin. The completion of this study is only the preliminary step

to more in depth cytogenetic studies, as animal studies still need to be conducted and evaluated for physiological relevance.

Chapter 3

Conclusions

A comparison of the cytogenotoxic responses induced by the natural flavonoids, quercetin, isoquercetin and rutin, and the manufactured flavonoids, MI, MO and MA, show that the natural flavonoids exhibited greater cytogenotoxicity than the synthetic flavonoids. It was apparent that the cytogenetic effects were modulated by the structure activity associated with the level of glycosylation of each flavonoid. An increase in the number of glucosides corresponds with a decrease in cytogenotoxic effects. Quercetin, the aglycone demonstrated to be most potent, inducing significant responses in all cytogenetic endpoints. It was readily absorbed by cells and made bioavailable. The water soluble MI, MO and MA which contain 3 – 9 glucosyls, induced less cytogenetic effects than the natural flavonoids. The increased water solubility property hinders absorption into cells and decrease bioavailability, as these flavonoids require enzymatic hydrolysis for conversion to the aglycone form. The relative potency of flavonoids are summarized as follow: quercetin>isoquercetin>rutin>MI>MO>MA.

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