DISSERTATION

FLAVONOIDS HYDROXYL GROUP POSITION EFFECT ON INDUCING ssDNA DAMAGE MEDIATED BY CUPRIC IONS

Submitted by

Suad Ahmad Elmegerhi

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Doctoral Committee:

Advisor: Takamitsu Kato

Susan Bailey
Douglas H Thamm
Mary-Keara Boss

ABSTRACT

FLAVONOIDS HYDROXYL GROUP POSITION EFFECT ON INDUCING ssDNA DAMAGE MEDIATED BY CUPRIC IONS

Flavonoids are polyphenol compounds characterized by 2-phenyl-1-benzopyran-4-one structure. Some flavonoids possess strong antioxidant activity. In nature, flavonoids are found as glycosides in most plant families, as the water solubility of the hydrophobic flavonoids is enhanced by the sugar. Also, flavonoids can be synthesized to enhance the bioavailability and water solubility of flavonoids. As of today, over 8000 different flavonoids have been isolated from plants. Flavonoids are arranged in seven main classes and different subclasses depending on the degree of the γ-pyranone ring oxidation. The variation of the flavonoids' structures is correlated to many biological and pharmaceutical roles, including anti-microbial, anti-proliferation, cardiovascular, and protection against diabetes.

Many flavonoids have wide benefits to both human and animal health this is because of the structure different among flavonoids, consequently it will lead to variation of its biochemical properties, this makes them play different roles such as such as anti-proliferation, anti-inflammatory, antimicrobial, antioxidant, reduction in cardiovascular diseases, anti-viral, anti-osteoporotic effects. These effects increase the enquiring if the flavonoids are promising tools to treat different diseases including cancers.

Although flavonoids can protect cells against oxidative damage due to their well-known antioxidant properties, under certain conditions flavonoids acts as prooxidant that in turn induce cell damage. The prooxidant property is affected by several factors: the number of hydroxyl

groups, presence of hydroxyl groups at specific rings such as the B ring, the presence of 2-keto and 4-oxo groups in the flavonoid structure, and the presence of free metal ions such as copper or iron.

The prooxidant activity induced by flavonoids in the presence of metal ions is well documented with variable results in the literature due to the use of different methods. To follow up with these studies, and as a part of this dissertation. The main hypothesis is that the presence of specific hydroxyl groups at specific rings in flavonoids will induce free radicals that causes DNA damage in presence of cupric ions. We investigated the ssDNA damage induced by different structures of natural and synthetic flavonoids in the presence of cupric ions at different temperatures. To get better knowing of the prooxidant activity of flavonoids in presence of cupric ions, we try to understand the mechanism underlying ssDNA damage induced by flavonoids in presence of cupric ions. We studied the cupric ion chelating capability of Quercetin and compared to other 11 flavonoids that differ in its hydroxyl group's positions, and we analyzed the oxidative damage induced by flavonoid-cupric ion interaction to all tested flavonoids. This was done as a part of an approach to investigate the mechanism of ssDNA induced by the flavonoid Quercetin in presence of cupric ions.

To understand the role of hydroxyl groups at specific residue positions at B and C rings of the flavonoids, we compared three residues positions in 12 flavonoids at different temperatures: residue 3 (glycosylated quercetin) at C ring and residues 1 and 2 at B ring represented in naringenin and hesperetin groups respectively. We found that the 3-position hydroxyl group of the C ring is required to induce DNA damage at low temperatures while the 1 and 2 position of the B ring is required only at high temperatures.

Additionally, to identify the most potent flavonoids to cause ssDNA damage and to understand the mechanisms by which quercetin can induce a lot of ssDNA damage compared with other flavonoids in the presence of cupric ions. We compared six flavonoids that are like the quercetin because they all possess the 3-position hydroxyl group of the C ring beside different hydroxyl position at B rings. We studied the effect of the hydroxyl number at B ring in flavonoids that have hydroxyl group at position 3 of the C ring, and we investigate if the hydroxyl group number at B ring is correlated with inducing more ssDNA damage in presence of cupric ions. Our results showed that DNA damage was more related to OH distribution in B ring. Flavonoids with a pyrogallol structure in the B ring such as myricetin induced more DNA damage than quercetin with a catechol group in ring B or kaempferol with a phenolic ring.

To determine the role of potent flavonoids that showed ssDNA damage toward cancer cells, we studied if the DNA damage induced by flavonoids is P53 dependent or not. Results showed that flavonoid myricetin which possess a pyrogallol structure of OH groups at B ring have a slightly larger anticancer activity against cancers carry loss of function mutations in P53 in presence of cupric ions than other tested flavonoids. This could be attributed to the DNA damage enhanced by the extra free radicals' formation of flavonoids that have pyrogallol structure in presence of cupric ions, and consequently could act as a potential against cancers carry loss of function mutations in p53.

Overall, we identified the specific positions and number of hydroxyl groups on flavonoids to cause ssDNA damage with metal ions. results presented here indicated that ssDNA damage induced by flavonoids in the presence of cupric ions is correlated with presence of specific hydroxyl positions at B and C rings; positions 1 and 2 at B ring and position 3 at C ring. Also, the number of hydroxyl groups at B ring has an impact on inducing more ssDNA damage in presence

of cupric ions. Myricetin showed the highest ssDNA damage capacity and very slightly larger cytotoxic ability against p53 cancer cells in presence of cupric ions. This is increasing the curiosity for more studies of the anticancer effect of myricetin in presence of metal ions in future.

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DEDICATION

I dedicate this dissertation to my first teacher, intriguer, and affectionate, my father Ahmed Elmegerhi.

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CHAPTER 1/ INTRODUCTION

Flavonoids

Flavonoids are important polyphenol plant metabolites. They are widely distributed in nature and found in different parts of plants like leaves and seeds. Flavonoids are known to produce the pretty colors of flowers. At the time of their discovery in 1930 flavonoids were thought to be a new class of vitamin [1, 2]. This subset of polyphenol plant metabolites was given the name "flavonoids" because of their natural yellow pigmentation --- this nomenclature was derived from the Latin word Flavus which means yellow [3].

Flavonoids possess nutritional properties; they are considered an integral part of our daily diet and are found in many vegetables, cereals, and fruits [4,5]. These secondary metabolites are made by plants to fight some microbial infections and protect the plant against ultraviolet radiation [1, 6]. As of today, over 8000 different flavonoids have been isolated from plants as well as synthesized in laboratories. Scientists have partitioned these 8000 distinct metabolites into seven classes based on their chemical properties. [6, 7].

In addition to the nutritional value of flavonoids in our daily food, flavonoids also have medicinal properties. In the last decades there has been more consideration in studying the benefits of the antioxidant properties of flavonoids to scavenge free radicals or chelate metals. Thousands of publications underline this interest and report the importance of many flavonoids for health benefits for humans. There is hardly any flavonoid structure without a biological use of interest in both ancient and modern herbal medicines. Flavonoids possess many biochemical properties that allow them to serve in different clinical roles such as anti-proliferation, anti-inflammatory, anti-microbial, antioxidant, reduction in cardiovascular diseases, anti-viral, anti-osteoporotic, and anti-

thrombotic effects [8]. These effects give rise to ask if these polyphenolic compounds are health promoting tools to prevent and treat different diseases including cancers [1, 9].

The biological significance of flavonoids

Multiple studies have demonstrated that eating foods rich in flavonoids supports the immune system, and may help reduce the severity of some common chronic diseases such as cardiovascular disease [10], type II diabetes [11], neurodegenerative diseases [12], and some kinds of cancers [13]. The ability of flavonoids to prevent and cure disease is attributed to possessing various physiochemical properties related to biological activities; these activities let flavonoids play different clinical roles.

Anti-microbial effects

Previous papers suggested different mechanisms of anti-bacterial and anti-fungal effects of flavonoids [14-17]. Flavonoids can stop the growth of bacteria through blocking transcription and translation [18] or through impairing the function of the cytoplasmic membrane of the cells [19]. Moreover, some previous data demonstrated that the presence of metal ions with flavonoids enhance the antimicrobial activity more than the presence of the parent flavonoids [20].

Studying the anti-viral effect of flavonoids against common viral infections and HIV is the point of interest. Previous studies suggest the anti-viral role through the interference of flavonoids with different stages of the viral replication cycle [21]. Although the mechanism is not clear, flavonoids can inhibit viruses directly or indirectly via inhibiting the immune system

Anti-inflammatory effects

Numerous papers highlight the impact of the anti-inflammatory effect of flavonoids [22]. Flavonoids are likely to inhibit the inflammatory mediators. Cyclooxygenase is an inflammatory meditator that involved in releasing arachidonic acid from membranes of neutrophil cells which constantly prevent immune cell degranulation and considered the start point for inflammatory response [22-24]. Additionally, flavonoids prevent the biosynthesis of some molecules involved in inflammation like eicosanoids, such as prostaglandin E2 which are involved in various immunological responses [25]. Another anti-inflammatory mechanism of flavonoids is represented by the inhibition of inflammation in the beginning by constraining inflammatory mediators that play a role in the early stage of inflammation such as cyclooxygenase and lipoxygenase [26-28].

Anti-diabetic effects

Many flavonoids possess antidiabetic activity because of its ability to inhibit enzymes involved in glycogenolysis. Also, flavonoids could change the gene expression of genes that work in metabolic pathways in the glycogenesis process [29-31].

Anti- proliferative effects

Oxidative stress is responsible for inducing pathophysiology of several types of cancers. Flavonoids found in fruits and vegetables play a role in preventing cancers [13, 32, 33]. Although the exact anti-proliferative mechanism of flavonoids is still unclear, many opinions refer to the antioxidant activity of flavonoids and its ability to scavenge reactive oxygen radicals which otherwise leads to the creation of DNA damage. A paper suggest an inverse association between flavonoids intake and lung cancer incidence [34], the anti-tumor effect of some flavonoid functions by different inhibiting mechanisms, by inhibiting the cell growth of cancer such as ovarian cancer via inhibiting the vascular endothelial growth factor (VEGF) expression at mRNA level [35]. The

flavonoid demonstrated antiinvasive ability by inhibiting the metastasis of cancer like melanoma, the antiinvasive mechanism of flavonoid is not clear [36]. Other papers suggest the inhibition of angiogenesis of cancer [37].

Hepatic protective effect

Many flavonoids such as silibinin, anthocyanins, quercetin, rutin, and naringenin [38-40] protect the liver against damage and protect hepatic cells against disorders that may progress to hepatic disease. Some flavonoids can regenerate damaged liver tissue; the flavonoid silymarin can regenerate the damaged tissue by stimulating protein synthesis in hepatocytes by enhancing the activity of the RNA polymerase 1 enzyme [41].

Chemical structure of flavonoids

The general phenolic structure of all flavonoids is 2-phenyl-1,4benzopyrone. This structure consists of fifteen-carbon atoms arranged in three rings (C_6-C_3-C_6); A and B rings are benzene rings and in between those rings is a heterocyclic pyrene C ring (Fig. 1). Those three rings build the basic structure of flavonoid and are formed from two pathways. The A ring is synthesized from the acetate metabolism pathway, whereas that the B ring is produced from a shikimic acid pathway and C ring is formed from both pathways [6].

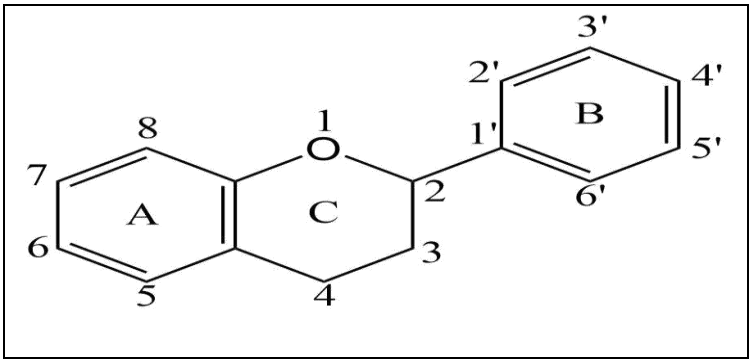

Figure 1. 1. The basic general structure of flavonoids illustrated with ChemDraw Version pro 16.0.

Bioavailability of Dietary Flavonoids

Metabolism

Bioavailability is the proportion of a substance that reaches systemic circulation unchanged (active ingredient), after a particular route of administration at the site of action [10]. In general, flavonoids have low bioavailability and differ between flavonoids in every class in flavonoid classification and even among flavonoids in a specific class [42].

The real doses of flavonoids that target specific organs could be done via *in vivo* experiments. Most researchers use *in vitro* methods with large doses (μM) then real concentrations of flavonoids in plasma to investigate the properties and activities of flavonoids. These doses are larger than the real concentration of flavonoids in plasma (nM to μM); a previous *in vivo* study demonstrates that the intake of 50 mg of aglycone will yield a plasma concentration of 4 μM of flavonoid metabolites [43]. Another study showed that the intake of anthocyanins with various doses ranging from 150 mg to 2 g yielded very low plasma concentration of flavonoid metabolites, ranging between 10 to 50 nM. Therefore, studying the effects of different flavonoids on target organs requires knowledge of concentrations reflective of achievable concentrations in the body [43].

Oral administration is the main route of dietary flavonoids; some flavonoids such as naringenin and hesperetin have the weak ability to penetrate layers in the skin and enter the body. This percutaneous route is only used in cosmetics after adding compounds to increase the solubility of flavonoids [44, 45].

In general, the metabolism of absorbed flavonoids occurs in the small intestine and liver. In the small intestine, ingested flavonoids conjugate to O-glucuronides, sulfate esters and O-methyl esters [46]. The produced metabolites such as glucuronides and sulfate derivatives are transferred via the hepatic portal vein and undergo further metabolism in the liver. The metabolites produced

from the liver will target specific tissues, and finally, the flavonoids metabolite elimination will occur via urine and through recirculation via the bile duct. Unabsorbed metabolites undergo microbial degradation in the colon and eliminate via feces [47].

Factors Affecting Bioavailability

Different parameters affect the bioavailability of flavonoids inside the body such as metabolic conversion rate as well as the breakdown rate of flavonoids by microflora in the colon [10]. However, bioavailability is affected by other factors related to the flavonoid itself. The most significant factors that affect flavonoids are their chemical structure, molecular weight, and glycosylation [10].

1. Chemical Structure of Flavonoids

Stability of flavonoids is one of the main reasons that limit the bioavailability. The stability of flavonoids varies, and it is affected by the difference in the chemical structure of classes and substitution [48]. In general, flavonoids with complex chemical structures usually have low bioavailability [49]. The simple structure flavonoids possess higher bioavailability than complex flavonoids. Presence of hydroxyl group at position C3 in flavone give them more stability than Flavonols [41]. The stability is related to the poor bioavailability of glucosides flavonoids

2. Molecular Weight

Some flavonoids possess large molecular weight such as proanthocyanidins; these have less bioavailability than flavonoids that have small molecular weight such as apigenin and quercetin [10, 43]. Therefore, most large flavonoids degraded into the small molecular weight of active compounds to make the absorption of flavonoids easier. But some large flavonoids are not able to degrade to small sizes [50] and break down in the colon.

3. Glycosylation

The presence of attaching sugar to flavonoids, or in other words called glycosylation, influences the bioavailability of flavonoids because the sugar moiety increases the absorption rate of flavonoids inside the small intestine. Thus, glycosylated flavonoids absorb more rapidly than many other flavonoids [10].

Biosynthesis of flavonoids

The biosynthesis pathway of flavonoids is well known, and preceding papers demonstrated that the phenylpropanoid metabolic pathway (shikimate) is the common responsible pathway for biosynthesis of all flavonoids [51]. In this pathway the flavonoid in chalcone form (2', 4', 6', 4-tetrahydroxychalcone) is formed by the condensation of one molecule of 4-hydroxycinnamate-coenzyme A (4-coumaroyl-CoA) (B ring) with three molecules of malonyl-CoA (A ring) [52] .

The reaction of flavonoid synthesis started by producing of 4-coumaroyl-CoA via Co-S CoA which is formed by phenylalanine. 4-coumaroyl-CoA can make the true backbone of flavonoids when combined with the co-enzyme called malonyl-CoA (Fig. 2). Then, a two-ring compound called chalcone catalyzes the condensation and conjugation of three rings. A ring of flavonoid formed from the three molecules of malonyl-CoA, together with one 4-coumaroyl-CoA molecule forming B and C rings, altogether creating a common intermediate called 2', 4, 4', 6'-tétrahydroxychalcone. This represents the familiar form of flavonoids with the three-ringed structure of a flavone. The chalcone molecule stimulates the final common flavonoid C6-C3-C6 skeleton form through catalyzing the cyclization via intermediate molecule to produce an (S)-4', 5, 7-trihydroxyflavanone molecule [52-55].

Furthermore, the intermediate compounds (chalcones and flavanones) will synthesize other final compounds of flavonoids via different reductase, hydroxylase, or methylase enzymes [52, 56]. These enzymes contribute to creating a series of modifications to biosynthesize different classes of flavonoids such as flavanols and dihydroflavonols, flavones etc.

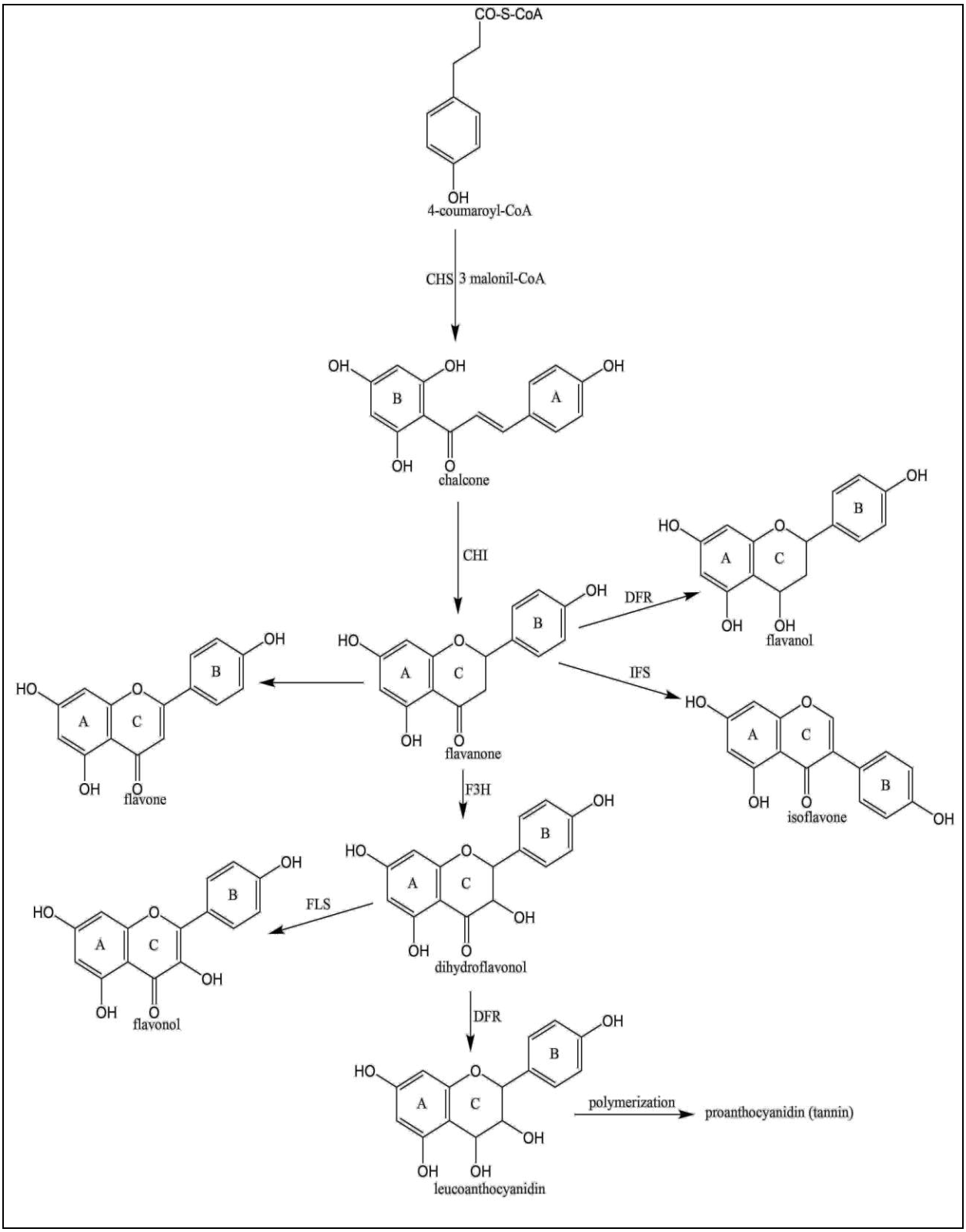

Figure 1. 2. Biosynthesis of flavonoids illustrated with ChemDraw Version pro 16.0.

Classification of flavonoids

A. *Main classes of flavonoids*

There are over 8000 types of flavonoids that have been identified and classified into eight groups and different subgroups. The classification depends on different factors: [1] The carbon of the C ring, [2] the number of hydroxyls in different rings, [3] and the degree of oxidation and unsaturation of the C ring. The flavonoids differ in the pattern of substitution of the C ring. It could be hydroxyl, glycosyl, methyl or acyl groups. Consequently, flavonoids differ in the level of antioxidant activity. Flavonoids are divided into a variety of significant classes (Fig. 3). The flavonoid classes are:

Flavones are one of the important subgroups of flavonoids. Flavones are widely found as glycosides in leaves, flowers, and fruits. They are characterized by the presence of a ketone group at C4 of the C ring and a double bond in between a C2 and 3 positions. Also, the B ring of flavones could have hydroxyl group substitution patterns at positions C3', C4' or C5'. Most flavones in vegetables and fruits have a hydroxyl group in position 5 of the A ring.

Flavonols are widely distributed in a variety of fruits and vegetables. Many flavonoids that belong to this class are associated with health benefits such as quercetin and kaempferol. This subgroup is characterized by having the flavones structure with the hydroxyl group at the position C3 of the C ring.

Flavanones are a subgroup mainly found in citrus fruits such as oranges. Flavanones differ from flavones in having a saturated C ring. This subgroup is characterized by a ketone group at C4 without the C2, 3- double bond that is found in flavones. They possess a chiral center at C2. Flavanones are also called dihydroflavone when substituted with a hydroxyl group at C3. They

provide with two couples of mirror image or enantiomers because of having C2 and C3 asymmetric carbons.

Isoflavones represent a large group of flavonoids; besides their presence in plants such as soybeans and other leguminous plants, they are also found in microbes [57]. Isoflavonoids have the potential to prevent diseases, and previous papers showed that one of the Isoflavonoids called genistein can cause metabolic changes that interfere with disease paths [58, 59]. Some flavonoids that belong to this group such as daidzein have estrogenic hormonal activity [60]. Structurally, in isoflavonoids both B and C rings are linked at C3 instead of C2 to which other flavonoids are linked usually.

Flavan-3-ols or Flavanols are widely distributed in bananas and apples. This group is characterized by the permanent binding of a hydroxyl group at position 3 of the C ring. They are characterized by missing the double bond between C2 and C3, and absence of the 4-ketone function. There are two classes called flavan-3, 4-diol and flavan-3-ol, the difference between them is attributed to occurrence of hydroxylation status at C4 or not.

Anthocyanidins are flavonoids that represent pigments in the outer cell layers of plants, red grapes, different kinds of berries, and the color of flowers. The color of these flavonoids occurs due to methylation or acylation at the hydroxyl groups on the A and B rings [61]. Anthocyanidines are not found as a free Aglycon, and stereotypically are present in a cationic charge.

Aurones and Chalcones. Tomatoes and berries are the most abundant sources of these flavonoids, and chalcones are well known as one chain subclass of flavonoids because of the absence of the C ring on the basic structure of flavonoids. On the other hand, aurone, known as a

chalcone-like group is unlike the basic structure of all other flavonoids because it is characterized by a 5-atom ring instead of the 6-atom ring of the central C ring.

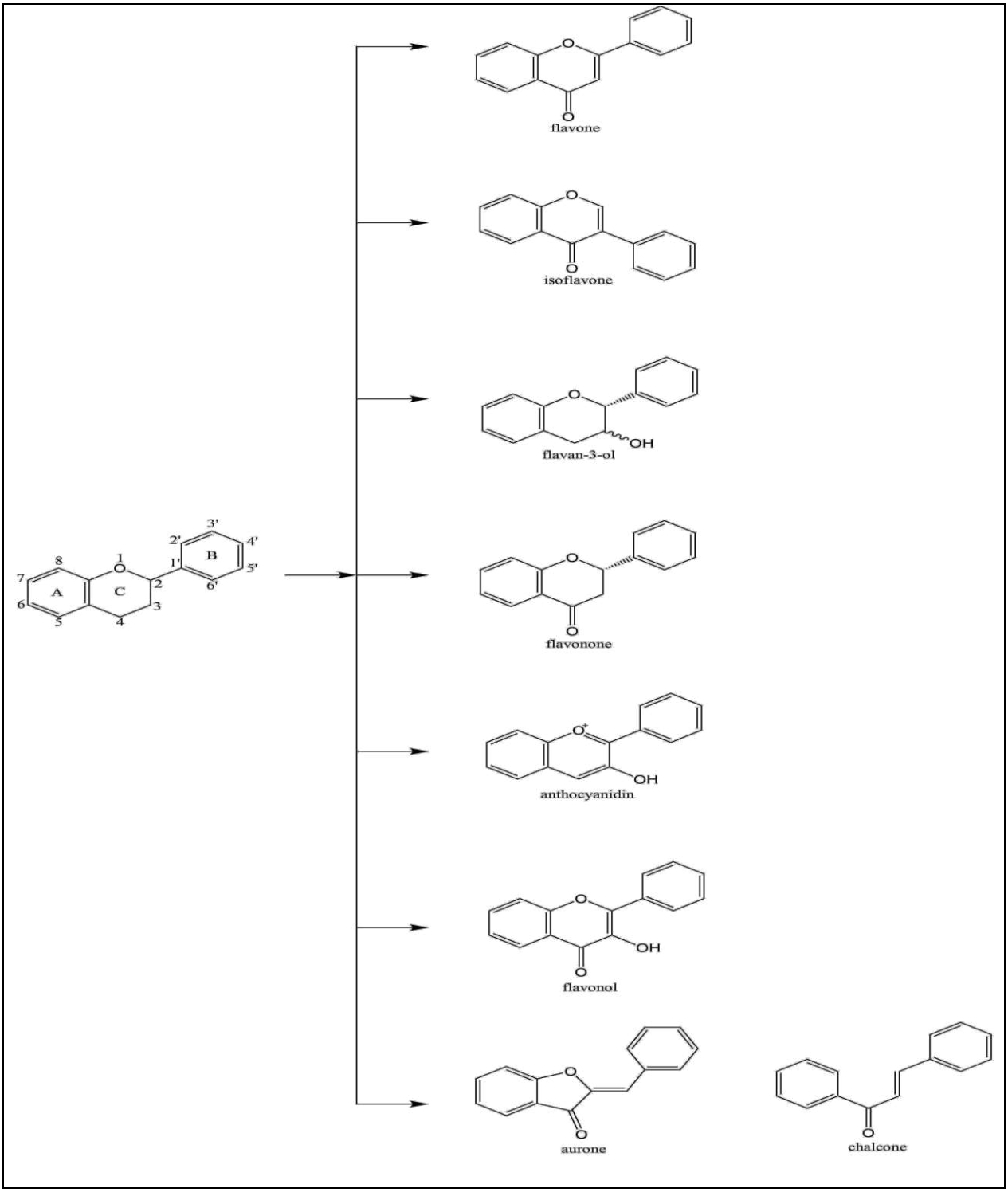

Figure 1. 3. Main classes of flavonoids illustrated with ChemDraw Version pro 16.0.

B. Aglycone and glycosidic flavonoids

The presence or adding sugar chains to flavonoids leads to a variation of the flavonoid properties. Glycosylation is a biocatalytic reaction that affects the water solubility of flavonoids. In other words, glycosylation enhances the hydrophilic character of flavonoids. Moreover, glycosylation affects the stability of flavonoids [48], and a previous study showed that an aglycone such as kaempferol or luteolin are less stable during extraction from honey samples than glycosylated flavonoids such as rutin, naringin, and hesperidin [62]. The difference of the oxidative activity between them is attributed to the number of the hydroxyl groups; while aglycones possess more hydroxyl groups and have higher antioxidant activity than glycosides, flavonoids which have low hydroxyl groups [63]. Depending on the sugar, flavonoids can be classified into two forms; aglycone or glycosides form (Fig. 4):

The aglycone form represents the basic natural structure of flavonoids. When no sugar is attached to their backbone, they are referred to as aglycone. Aglycone flavonoids could be found in the natural or syntactic form with none or few glucosyl residues [1].

The glycoside form is obtained by glycosylating the original glucosyl flavonoids. Glycosylation alters the properties of flavonoids and precisely raise the water solubility and minimize the cell toxicity and genotoxicity in tissue culture system [64, 65]. According to the number of sugars added to aglycone form, the glycosylated form could be monoglucosyl (MG) or maltooligosyl (MO), where MG form represents glycosylated flavonoids with 1 to 3 sugars added, while MO form represents glycosylated flavonoids with 4 to 7 sugar residues.

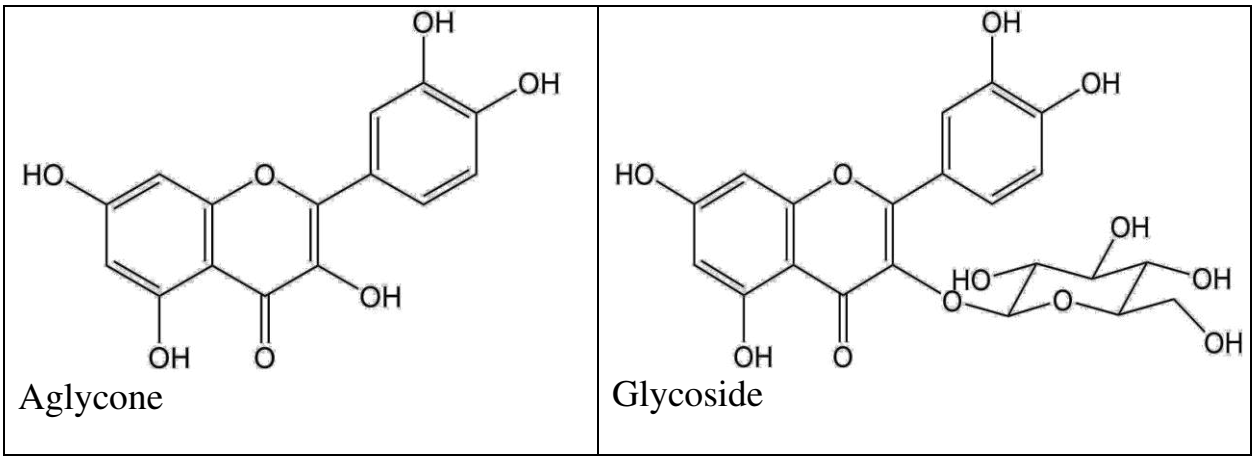

Figure1. 4. The difference in the structure between glycoside and aglycone flavonoids. illustrated with ChemDraw Version pro 16.0.

Targeted flavonoids in this dissertation

Quercetin; QN

Quercetin is one of the most important flavonols that has dual activities, acting as an antioxidant or prooxidant under different conditions. It is abundant in more than twenty plant families [3, 66]. Because of its high free radical scavenging, this bioactive flavonoid has high interest to be used in food. It has broad beneficial health properties and plays many biological roles. A paper reported that long-lasting consumption of quercetin has an anti-obesity effect in C57/BL6J mice by limiting hepatic fat accumulation [67]. Also, quercetin has anti-inflammatory and immunosuppressive activity on dendritic cell function, and anti-atherosclerotic effects [67-69]. On the other hand, quercetin has been reported as a mutagenic not carcinogenic compound. previous papers showed that the urine of Fischer rats that fed diets containing quercetin at 0.2% w/w is mutagens to Salmonella typhimurium TA100. On the other hand, these diets did not cause any carcinogenicity when fed to rats [70-72]. QN has prooxidant activity in presence of metal ions. Its prooxidant activity seems to be flavonoid concentration-dependent [73]. A study showed that the flavonol quercetin maintains the highest prooxidant activity in human lymphocyte among tested flavonoids: naringenin, hesperetin, and morin [73].

Myricetin; MN

Myricetin is a phenolic compound belonging to the flavonol group. It is used as a food additive because of its antioxidant activity. It displays many beneficial health roles, such as anti-inflammatory, anticancer, antidiabetic [74-78], and high doses of myricetin can lower hypertension caused by deoxycorticosterone acetate (DOCA) in rats [79]. It also has immunomodulatory effects by stimulating antibody formation [77, 80]. On the other hand, other studies referred to the ability of myricetin to act as a prooxidant and to cause cell damage at 450 µM to isolated enterocyte cells of guinea pigs [80]. Generally, researchers referred to the superoxide produced by autoxidation as responsible for the toxicity of myricetin at high concentrations in the presence of metal ions [81-83].

Kaempferol; KL

At a low concentration of 10 µM, kaempferol did not show any antioxidant activity, whereas at 60-100 µM kaempferol showed potent antioxidant activity [51]. This flavonol is well known for its broad health benefits, especially in fighting cancer. Several kinds of literature described that kaempferol can control the apoptosis, angiogenesis, and metastasis of cancer cells [84]. A previous paper demonstrated that kaempferol can impair the metastasis of extremely aggressive MDA-MB-231 breast cancer cells, via inhibiting MMP-3 protein activity that is responsible primarily for destroying extracellular matrix proteins that support cells and tissue in the body [85]. Additionally, kaempferol can suppress *in vitro* angiogenesis in ovarian cancer cells via inhibition of a primary proangiogenic mediator called (VEGF) [86]. Also, kaempferol can induce apoptosis by several ways; the most significant ones are; (A) blocking proteins involved in apoptosis, such as RSK2 protein through direct binding to functional site in the protein [87], (B) activating the ERK subfamily of mitogen-activated protein kinase (MAPK) cell growth regulation pathway, which

enhances apoptosis in breast cancer cells [88], (C) arresting cell cycle; a study showed that kaempferol can arrest cells at G2/M phase and may induce apoptosis via p53 phosphorylation, and decreased CDK1 levels in human breast cancer cells [89].

Luteolin

Luteolin is a member of the flavone subclass. Like many flavonoids, luteolin has both antioxidant and prooxidant activities. Elucidating how luteolin can regulate both activities will help to understand its anticancer, anti-inflammatory, and cardiac protective effects [90] [91]. A previous study referred to the impact of ferrous (Fe) metal ions on the redox regulation process in hepatic cells; at over 100 μM concentration of Fe, luteolin acts as an antioxidant, whereas at a lower concentration, less than 50 μM luteolin acts as prooxidant [92]. Additionally, luteolin might regulate the redox system through inhibiting the c-Jun N-terminal kinase (JNK), which is involved in a pro-inflammatory signaling pathway, and induce apoptosis. Luteolin suppresses the activity of this pathway in normal macrophages and activates the JNK kinase in lung cancer cells via prooxidant activity [93, 94].

Morin

Morin belongs to flavone subclass (3,5,7,2',4'-pentahydroxyflavone) of flavonoids; it has significant anti-carcinogenic activity, and oral feeding of rats showed that morin clearly suppresses tongue carcinoma development by 44% [95]. The halt of carcinogenesis could be attributed to the apoptotic activity of morin, and a previous paper showed that morin can induce apoptosis via upregulating the pro-apoptotic BCL2 associated agonist of cell death (BAD) protein in human leukemic cells [96]. Otherwise, the prooxidant activity of morin was reported to be dependent on

the presence of Cu^{2+} and Fe^{3+}, and morin induced DNA damage, and lipid peroxidation in a concentration-dependent way [97].

Taxifolin or Dihydroquercetin; TAX

Structurally, taxifolin belongs to the flavanol subclass (3, 5, 7, 3′, 4′-pentahydroxy-flavanone). Taxifolin can activate ERK1/2, Akt, and JNK signaling pathways and lead to Nrf2 nuclear translocation. This translocation will protect cells against DNA oxidative damage by enhancing the phase II antioxidant and detoxifying enzymes [98]. Because of its effective antioxidant activity, taxifolin has been reported to have many biological, and promising therapeutic roles in cardiovascular, live, neurodegenerative diseases [99-102], and particularly in fighting cancer, as shown by several papers [103-105] There is evidence that taxifolin could be used in conventional therapy as effective chemotherapy drug through disturbing many signal transduction pathways, in different types of cancers; a recent study showed that the *in vivo* and *in vitro* administration of taxifolin can down-regulate Wnt/ β-catenin signaling, and induce apoptosis in colorectal cancer cells [106]. Also, taxifolin can suppress UV induced cancer cells via direct interaction with epidermal growth factor receptor (EGFR) and phosphoinositide 3-kinase (PI3K) at the ATP-binding pocket [107].

Galangin; GN

Galangin (3, 5, 7-trihydroxyflavone) is rightly placed in the flavonol subclass. Besides its antioxidant activity against carcinogens through activation phase II detoxifying enzymes, GN can induce apoptosis mediated by a mitochondrial pathway in hepatocellular carcinoma [108], and GN can protect against sulfation-induced carcinogenesis by suppressing sulfotransferase 1A1 [109]. Although some researches point to the mutagenicity of GN and its ability to cause chromosome

aberration in CHO cells, other studies refer that GN does not exhibit any mutagenicity through modulating phase I enzyme like CYP1A and phase II detoxifying enzymes such as glutathione S-transferase and quinone reductase [110]. GN showed mutagenicity with metabolic activation, but without metabolic mechanism did not showed mutagenicity. On the other hand, a previous study showed that the GN can produce the lowest oxidative DNA breaks compared with myricetin, and quercetin, in the presence of Cu^{2+} [111].

Naringenin; NAR

Naringenin belongs to the citrus flavanones (4,5,7-trihydroxyflavanone). It is a glycol form of naringin and it derives from naringin hydrolysis [112,113].This flavonoid has lower antioxidant activity compared with other flavonoids such as apigenin because it does not have the hydroxyl group in the position 3 at C ring, and there is no catechol structure in the B ring [51]. Naringenin is insoluble in water, and soluble in organic solvents [114]. This feature could affect its properties and mode of action. A previous paper indicated that naringenin and its glycosylated form can act as an antioxidant in hydrophilic solvents, and this action can convert to prooxidant activity in a lipophilic environment [115]. Also, the prooxidant activity of naringenin seems to be affected by concentrations. There is evidence that naringenin can induce DNA damage, and lipid peroxidation in a concentration-dependent manner, and also at above 100 μM naringenin will be cytotoxic to human lymphocyte cells [73, 97]. Overall, naringenin plays several biological roles, including decreasing cholesterol via inhibition of 3-hydroxy-3-methylglutaryl–o enzyme A (CoA) reductase in rats fed a high cholesterol diet, enhancing carbohydrate metabolism, anti-atherogenic, anti-inflammatory effects, and promoting antioxidant activity that modulates the immune system [114, 116, 117].

Hesperetin; (HT orHET)

Hesperetin is a citrus flavanone (3, 5, 7-trihydroxy-4′-methoxyflavanone). The potency of antioxidant action is affected by the solvent. Although it acts as an antioxidant in both hydrophilic and lipophilic environments, it showed lower antioxidant activity in a lipophilic environment [115]. It has many health benefits; topical treatment with hesperetin can protect against UVB induced damage in mice [118]. Another interesting feature is the anti-inflammatory effect, since nitric oxide is one of the inflammation mediators, a previous study reported that adding hesperetin in the media (250 μg/mL) can reduce nitrate levels by ≈75% in cells induced over 9-fold with nitrite [119]. On the other hand, hesperetin above 100μM acts as a prooxidant, and DNA damage in human lymphocyte cells induced by Fe^{3+} and hydrogen peroxide can be elevated by increasing the concentration of the flavonoid [73].

Flavonoids with the glycosylated form used in the dissertation

Rutin; R

This flavonoid (quercetin-3-rhamnosyl glucoside) is one of the glycosylated derivatives of quercetin. It is a powerful free radical scavenger; it scavenges 100–300 times more efficiently than mannitol, which is typical OH scavenger [120, 121]. The ability of rutin to scavenge radicals is attributed to its inhibitory activity on the enzyme xanthine oxidase (XO) [120]. Different studies refer to its biological benefits via an anti-inflammatory effect; for instance, rutin reduces radicals in antigen–IgE activated mast cells and mast cells mediate cytokines responsible for inflammation and defense [122]. Besides its powerful antioxidant effect, rutin is reported to have anti-carcinogenic activity; a study of rutin demonstrated that rutin caused cytotoxicity to hepatoma tissue culture (HTC) cells at a high concentration of 810 μM after 72 h, and DNA damage was produced without inducing apoptosis following 24 h [123].

Monoglucosyl rutin; MG-R

Monoglucosyl rutin a high solubility semi-synthetic flavonoid glycoside of quercetin. It is synthesized by using glycosyltransferase to add glucose to rutin because of the limited solubility of rutin itself in water (130 mg/L) [124]. Whereas the aglycone form represented in QN and R are considered harmful to cells, MG-R is a very safe antioxidant compound since it is not reported as having a mutagenic effect in cells [125]. Otherwise, MG-R showed weaker anticancer effect compared to other flavonoids, and a previous paper reported that this flavonoid causes less inhibition efficiency to PARP activity and induces less synthetic lethality in BRACA2 deficient cells compared with its natural aglycone forms QN and R [126].

Maltooligosyl rutin; MO-R

Maltooligosyl rutin is semi-synthetic quercetin and developed by synthetic glycosylation to improve the water solubility. It has less efficacy than quercetin in inhibiting PARP [126].

Isoquercetin; IQ

Isoquercetin is a glycosylated derivate of quercetin, with a beta-D-glucosyl residue attached at position three. It is naturally occurring with much better bioavailability than quercetin with the same biological *in vivo* action because of its fast hydrolysis in the body, and it enters the circulation in its aglycone form (quercetin) [127]. Besides its antioxidant activity, IQ has shown a protecting role in a dose-dependent way against endothelial injury from inflammation mediators in platelets and granulocytes *in vitro* [128, 129]. Additionally, IQ is considered a better anti-inflammatory than QN [127].

Maltooligosyl Isoquercetin; MO-IQ

Maltooligosyl isoquercetin_is synthesized form of isoquercetin by glycosylation of 4 to 7 glucosides to increase their water solubility [130].

Hesperidin; HES

Hesperidin is a citrus fruit flavonoid (7-O-rutinoside of hesperetin). It represents a more bioavailable glycosylated form of hesperetin. Like its aglycone form, hesperidin exhibits many pharmaceutical roles, reducing lipids, antidiabetic, and cardio protective effects. The Consumer Affairs Agency of Japan allow to use of hesperidin powder as a drink for health benefits because of its safety; its LD_{50} is more than 2000 mg/kg [131].

Monoglucosyl- Hesperidin; MG-HES

MG-Hesperidin or Alpha glucosyl hesperidin (αGH) is known for decreasing body fat through the induction of brown-like adipocyte formation which can suppress fats. It is a synthetic from hesperetin that is synthesized via transglucosylation of 1 to 3 glucosides by cyclodextrin glucanotransferase enzyme [132].

Naringin; N

Naringin is a flavanone glycoside form of naringenin (flavanone-7-O-glycoside). It is abundant in citrus fruits giving a bitter taste to grapefruit. It has an extra added disaccharide, neohesperidose, linked through a glycosidic connection at the 7-carbon position. Although naringin is considered a strong antioxidant, its efficacy to scavenge radicals is lower than its aglycone form because the sugar moiety causes steric interruption of the scavenging group [133].

Monoglucosyl-Naringin; MG-N

MG-Naringin (MG-N) is synthetized by adding 1 to 3 glucose to naringin by glycosyltransferase enzyme in order to improve the water solubility of naringin [134].

Mechanism of action of the flavonoids

A. Antioxidant action

This mechanism of antioxidant action is the main focus of the flavonoids field. The human body is exposed to different kinds of reactive oxygen species like superoxide anions, hydroxyl radicals, and hydrogen peroxide. Hydroxyl radicals (•OH) are the main oxidative agent produced in the body and cause damage to the DNA, lipid, and protein in the cells at the site of formation [135]. The ability of flavonoids to delay or remove oxidative damage on a target molecule characterizes flavonoids as typical antioxidants. The oxidant protective effects of flavonoids work by stabilizing free radicals via donating hydrogen from their hydroxyl groups to •OH, or via the transfer of electrons from free radical species to flavonoids. Ultimately, this stabilization leads to the prevention of cellular damage.

Antioxidant properties depend on the number and distribution of hydroxyl groups throughout the flavonoid rings. Hydroxyl groups of flavonoids can scavenge reactive oxygen species, with the most reported being ones arranged in the B ring [1, 136].

There are different mechanisms by which flavonoids act as antioxidant molecules. They either scavenge radicals directly, or through interaction with different enzymes. Furthermore, they may scavenge radicals through a combination of both mechanisms, and by protecting the antioxidant defense system [8, 137-142].

1. Direct scavenging of the free radicals

Because of the high reactivity between hydroxyl groups on the most effective positions in flavonoids and reactive oxygen species (Fig. 5), flavonoids can directly scavenge radicals by

exchanging electrons to free radicals [137] or donating hydrogen atoms. The common less reactive and stable radicals' reaction is defined by the following equation [8]:

Flavonoid (OH) +R▪ > Flavonoid (O▪) +RH

Some flavonoids can scavenge superoxide's anions directly, where (R▪) represents the free radicals, and appreciation (O▪) refer to oxygen-free radicals attached to flavonoid.

The ability of flavonoids to scavenge radicals depends on the arrangement of hydroxyl groups at specific positions on the three rings of the basic structure of the flavonoid. Previous studies demonstrated that the hydroxyl groups in the B ring are the most important in scavenging radicals over those in the A and C rings [143, 144]. Specifically, the presence of the two hydroxyl groups at neighboring positions C3' and C4' on the B ring (a catechol moiety structure) have more impact in scavenging free radicals. Catechol moiety structures such as quercetin and myricetin are the most effective radical scavengers when compared with other flavonoids in the same class that are lacking the catechol structure [145].

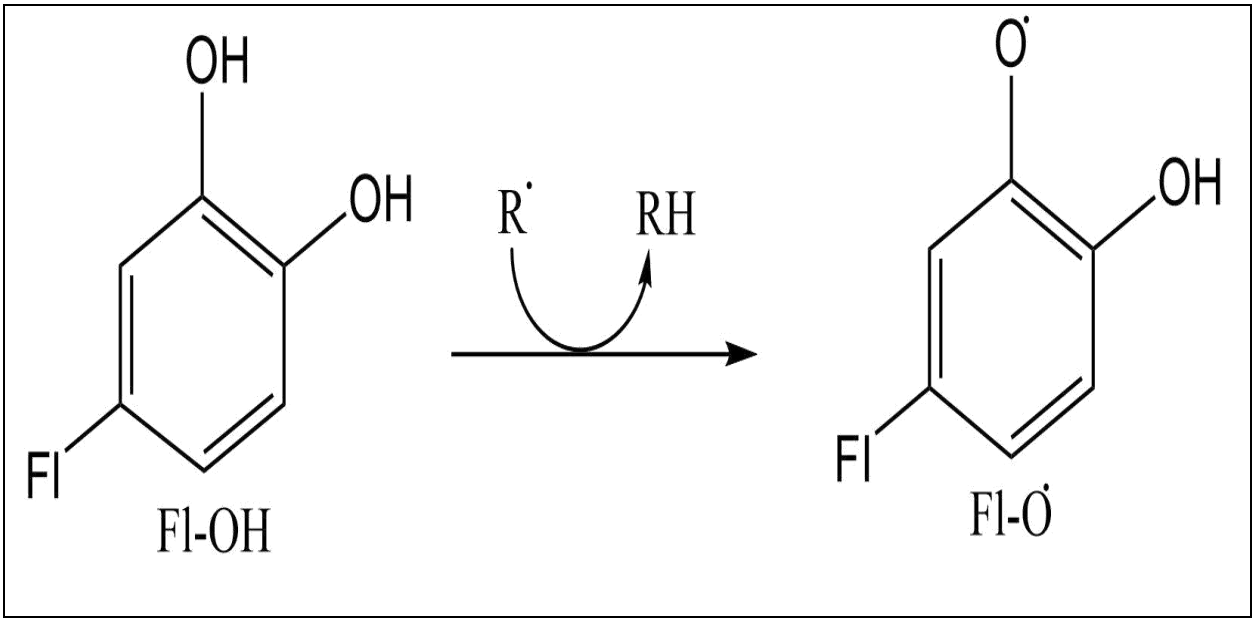

Figure1. 5. Direct scavenging of the free radicals illustrated with ChemDraw Version pro 16.0.

2. *Interaction with enzymes involved in free radical formation*

Flavonoids like quercetin can inhibit enzymes involved in the production of the reactive oxygen species (ROS). This includes microsomal monooxygenase, cytochrome P450 and xanthine oxidase [138-142]. Quercetin helps to reduce oxidative damage produced by free radicals, while silibin interferes with nitric oxide synthetase (NO), which is involved in inducing oxidative damage in different cells. Moreover, by decreasing peroxidase release, flavonoids inhibit the production of free radicals produced by neutrophil cells [8].

3. *Reinforcement of the antioxidant protection system*

All organisms have protective mechanisms to defend their cells against damage caused by exposure to oxidative stresses. Flavonoids can donate hydrogen atoms to lipids, which in turn deters the free radical reaction from transpiring, enhancing the defense system against free radicals [146]. Furthermore, free metal ions such as Cu^{2+} or Fe^{3+} could increase the production of free radicals. Flavonoids possess properties that allow them to chelate transit metal ions, which protects cells against cellular membrane damage and lipid peroxidation [147].

Methods used to measure the scavenging activity of flavonoids

There are many different methods to measure the scavenging activity of flavonoids. The level of accuracy of scavenging activity in one flavonoid differs from method to method. Not all of these methods yield the same results [6]. Antioxidant assays can be simple, such as DPPH assay, or complex, like the more expensive pulse-radiolysis [6, 148].

It is impossible to measure the concentration the •OH directly because of their high reactivity. However, the first reaction of hydroxyl radical formation can be measured by different methods. In this dissertation, we used two simple methods to estimate the scavenging activity of flavonoids. Both methods work on the same basic principles; reacting the •OH with a specific

probe such as;(1) The indoxyl-β-glucuronide probe which is used with chemiluminescence method, and (2) Fluorescent dye probe termed 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radicals which used with DPPH method. The probes followed by using physical parameters to detect the changes that happened in the reaction.

1. *Chemiluminescence method*

This enzyme-free assay is sensitive and widely used to estimate the •OH scavenging activity of antioxidant compounds and their reactive metabolites [149-151]. This method is a time-dependent assay; chemiluminescence solution could last from seconds to minutes depending on the amount of produced free radicals. Chemiluminescent light (430 nm) emitted during reactive oxygen species production, and light emission can be monitored and amplified via luminometers [152]. Different probes with different emission levels have been developed and used in this chemiluminescence assay; the indoxyl-β-glucuronide probe has a low emission compared to the luminol probe. In chapter two of this dissertation, we used a luminol solution as a probe to detect and monitor •OH formation and direct scavenging, allowable by the high reactivity of •OH. We also measured the scavenging activity of flavonoids in the presence of Cu^{2+} within seconds to minutes after interaction with chemiluminescence's solutions [153].

2. *DPPH Antioxidant Assay*

One of the methods widely used to compare the antioxidant activity among flavonoids is the DPPH assay. This assay is suitable to measure the free radical scavenging activity of newly discovered natural compounds. The antioxidant activity can be measured by estimating the ability of flavonoids to scavenge a stable and purple-colored fluorescent dye probe termed 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radicals. Flavonoids can donate hydrogen, which will produce a stable

non-radical reduced form called diphenyl picrylhydrazine, and subsequently will scavenge DPPH radical [154].

The basic idea of this as assay is simple compared to other methods and can measure the antioxidant activity in a short time by measuring the color of the sample at 517 nm wavelength; At an absorption light of 517 nm and in ethanolic solution, DPPH gives a maximum absorption with an odd number of electrons and remains stable with a dark purple color. On the other hand, when antioxidants such as flavonoids are added to the DPPH compound [155]. DPPH decolorizes with the presence of a pale-yellow color due to the picryl group [156] (Fig. 6).

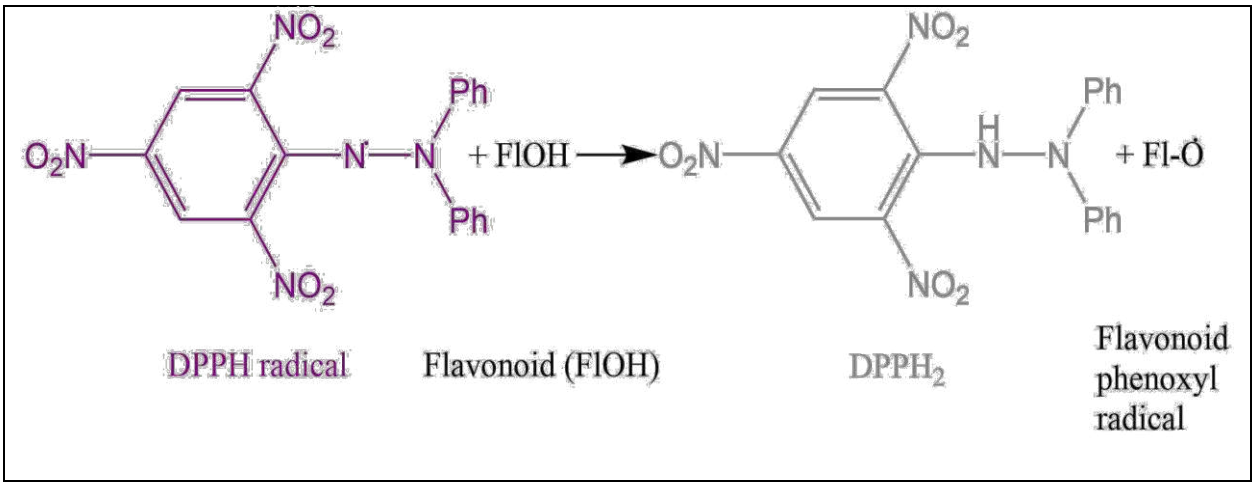

Figure1. 6. Reaction of the DPPH free radical with flavonoids illustrated with ChemDraw Version pro 16.0.

B. Prooxidant action

Because of the specific chemical structure of flavonoids, its ability to chelate metals is connected to the prooxidant activity, which is exerted in the presence of free transition metal ions such as Cu^{2+} or Fe^{3+}. Flavonoids can form redox complexes with metal ions leading to production of free radical molecules. Some flavonoids such as quercetin and myricetin can reduce Cu and Fe and produce radicals through a Fenton- like reaction. Free radicals enhance DNA damage and can produce hydroxyl radicals which have prooxidant activity [157]. Some papers demonstrate

evidence of the prooxidant properties of the flavonoid metal complex, and its ability to cause oxidative damage to DNA and induce apoptosis in cells [158-160].

Flavonoid metal ions interaction

In order to clarify the structure, chemical properties, prooxidant and antioxidant action of flavonoid-metal ion interactions, different peer reviewed studies have utilized various *in vitro* methods. These studies demonstrate that the binding of flavonoid to metal ions make a change in flavonoid's properties and affect the kinetics of the reaction.

Optical properties of flavonoid metal ions interaction

All flavonoids can be analyzed by spectrophotometric methods because flavonoids are considered a colored compound, and every flavonoid has a defined region of absorption bands in the ultraviolet/visual spectra (UV/VIS). The creation of bands could happen due to highest occupied molecular orbital (HOMO) - lowest unoccupied molecular orbital (LUMO) energy gaps [161]. In general, benzoyl and cinnamoyl bands are the two main absorption bands of most flavonoids, especially flavonoids that have 3-OH moiety (flavonol) [1]. The absorption band of the benzoyl band (band II) is found in the range between 240-280 nm, while in the cinnamoyl band (band I) the absorption is found in the range 320-385 nm (Fig. 7) [1].

When metal ions bind to flavonoids, a change in absorption bands will occur. The HOMO-LUMO gap in the flavonoid will decline and lead to a shift in the absorption bands of the flavonoid metal complex [162]. Likewise, the change in the energetic state could also result in changes in both absorption regions; a strong charge will transfer from flavonoid to metal ions and lead to a change of both absorption bands to different absorption regions when metal ions coordinate with flavonoids and show a bathochromic shift [163, 164].

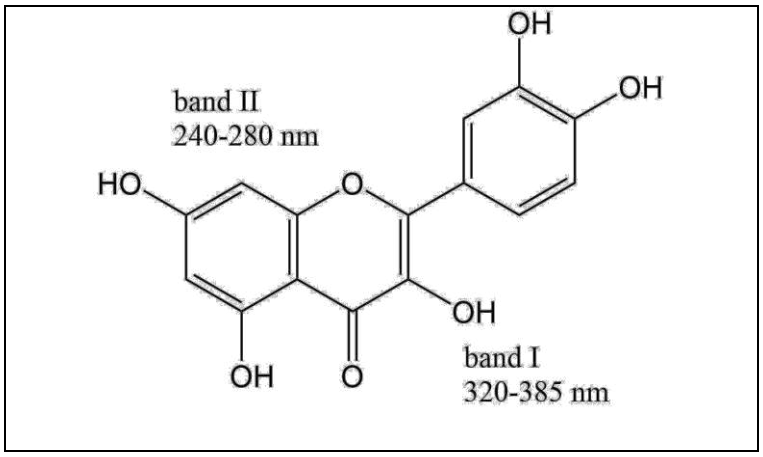

Figure 1. 7. Two main absorption bands of flavonoids illustrated with ChemDraw Version pro 16.0.

Factors affecting the properties of flavonoid-metal ion interaction

Flavonoids are natural compounds with dual actions; some flavonoids may act as antioxidants or prooxidants under different conditions. Different factors cause rerouting action of the same flavonoid from antioxidant to prooxidant in the same kinds of cells; for example, a previous paper reported that myricetin can act as an antioxidant, and repair oxidative damage induced by Fe in rat liver cells [165]. Conversely, myricetin can act as prooxidant in the presence of Fe^{3+} or Cu^{2+} and enhance DNA damage in liver cells [158]. The switch from one activity to another may depend on flavonoid concentration. The primary factor that promotes the transition to the prooxidant activity of flavonoids, and induces radical formation is the nature of flavonoid structure; for example, the number and distribution of hydroxyl groups at the B ring, and the existence of 2-keto and 4-oxo groups on the flavonoid skeleton [166, 167]. Furthermore, the existence of the keto group at the C ring of the flavonoid induces prooxidant activity [168]. Flavonoid-metal ion interaction is affected by the concentration of flavonoids, solvent, pH, and presence of other antioxidant and metal ions in the surrounding zone [169]. Some flavonoids such as quercetin and its glycosylated forms, morin, hesperetin, and kaempferol showed dose-dependent prooxidant activity [170].

Metal-binding sites of flavonoids

Transit metals interact with flavonoids at specific sites. There is more than one chosen site on flavonoid rings to chelate metals ions. Figure 8 demonstrates the typical metal chelating positions on flavonoids. The positions of hydroxyl groups at 3′-4′ site of B ring, and site 3 hydroxyl group and 4 carbonyl group at the 3-4 position of the C ring and the position A ring at the 4-carbonyl-5-hydroxyl site [5].

Preferential specific sites of interaction between flavonoids and transit metals is mostly affected by the properties of both flavonoids and metal ions. The choice of these sites depends on several factors: the amount of metal ions, the oxidative state of metal ions, and the reaction conditions such as pH of the body and temperature. Factors related to flavonoids include the type of solvent used to prepare specific concentrations of flavonoids and the number and position of hydroxyl groups distributed at different rings [171-173]. Also, the sites of interaction between flavonoids and metal ions is mostly affected by the ratio of both. Flavonols such as quercetin, Kaempferol, rutin, and morin prefer to interact with metal ions at a 1:2 (Melal: Favonol) specific ratio compared with other ratios [18, 174, 175]. Furthermore, some flavonoids can interact with metal ions at different sites, and within a different ratio. Previous studies showed that quercetin can interact with metal ions at different sites in two ratios, 1:1 and 1:2 [172, 176]. This can be explained by the preferential binding of metals to specific structures. At a ratio of 1:1 the metal ions bind with the keto group of the flavonoid skeleton structure at position 4, and with hydroxyl groups at position 3 or 5. However, metal ions at the 1:2 ratio prefer different positions, especially at hydroxyl groups placed at the 3 and 4 positions [174, 175].

In general, flavonoids possess a wide variety of structures and have a different number of metal-binding sites, and there are many studies concerning the binding sites on flavonoids to

chelate metals. The study of the effects of these binding on DNA damage needs more investigation with different flavonoids.

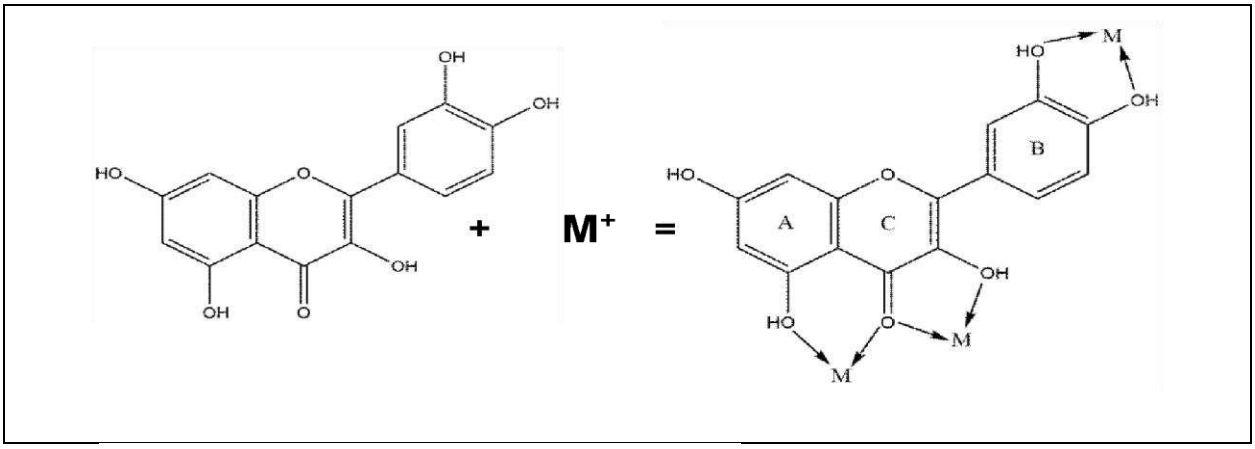

Figure1. 8. Preferred chelating sites in flavonoids illustrated with ChemDraw Version pro 16.0. M^+ indicate to metal ions that interacts with flavonoids.

The stability of flavonoid-metal ions interaction

The stability of flavonoid -metal interaction is different, ranging from medium to high. According to the prediction of the electron's placements in the atom in molecular orbital theory in which two atoms combine to form molecular orbitals, the stability attributed to the linking between flavonoids and metal ions happens due to the transfer of the electron to the flavonoids' orbital from the metal ion's orbital [176]. Flavonoids donate hydrogen to free radicals during the interaction of the metal ions with flavonoids, and rings in the flavonoid ligand become accustomed to some non-polar twisted conformation. Also, the stability is influenced by other factors; there is evidence that pH and ratio between flavonoid and metal ions could affect the stability *i.e.,* previous study mentioned that most flavonoids tend to create a stable complex with metal ions at ratio of 1:2 compared to other ratios [18]. Some flavonoids prefer acidic medium to interact with specific metals [18].

Interaction of flavonoid metal complex with nucleic acids

Introduction to oxidative DNA damage

Several endogenous or exogenous factors can cause damage to different molecules in the body including DNA; some endogenous factors continuously occur in the body during DNA synthesis, and due to exposing cells to reactive oxygen and nitrogen-based reactive species. On the other hand, exposure to exogenous factors such as ionizing radiation or ultraviolet lead to the formation of ssDNA and dsDNA breaks [177, 178].

Reactive oxygen species (ROS) are an endogenous factor that induce DNA damage. They are highly reactive molecules with unpaired electrons known as free radicals. Also, ROS can exist as non-radicals that can form radicals such as hydrogen peroxide. Both forms lead to oxidative stress which is considered one of the most important causes of the DNA breaks.

Oxidative DNA breaks occur normally inside the body when ROS are produced during normal physiological processes such as metabolism (cellular respiration in mitochondria), or during the production of macrophages during bacterial killing. Additionally, ROS could be associated with a pathological condition such as arthritis, heart disease, or certain types of cancers. Despite oxidative damage occurring in both normal and cancer cells, it causes alteration in redox signaling pathways in various cancer cells rather than normal cells [179].

There is evidence that ROS in and of themselves are not toxic, and the body can repair the DNA breaks induced by ROS, and antioxidant compounds such as dietary flavonoids help to repair DNA damage caused by oxygen species, and protect against DNA break formation and base alteration in DNA [180]. Nevertheless, in the presence of metal ions such as Cu^{2+}, the ROS can be

more toxic due to the production of •OH that nucleophilically attacks DNA, and lipids and proteins in the cell membrane and consequently leads to aging and cancer [181, 182].

Detection of oxidative damage by electrophoresis

Extensive production of free radicals might produce oxidative stress and could induce DNA damage developing to serious diseases. Tracing oxidative DNA breaks can be done by different methods in the laboratory. One of these methods is the agarose gel electrophoresis assay. Besides its reliability and sensitivity, this assay is not time-consuming and characterized by simplicity, and it does not need multiple steps. Also, it is a cheap assay; it does not need expensive instruments [183, 184].

In this dissertation, the protocol of gel electrophoresis assay was adapted from former literature to estimate single strand instead of dsDNA breaks [185]. The protocol is based on measuring the size of the intact DNA fragments, while broken DNA fragments migrate through the gel pores faster and may disappear. In this literature, the protocol of electrophoresis was used to estimate dsDNA breaks in order to investigate if the glycosylation of flavonoids affected the radical scavenging and radio protective properties.

Flavonoid metal complex with DNA

DNA is one of the cellular targets for the free radicals produced by flavonoid metal ion interactions. Flavonoids interact covalently with DNA or non-covalently via an intercalation mechanism, in which a flavonoid as a small molecule will bind to the DNA internally in between base pairs of minor and major grooves, or externally, on the external sides of the helix [186].

Flavonoids interact with DNA with various affinities [187]. Flavonoids can intercalate with DNA via hydrophobic interaction with DNA's nitrogenous bases [188], or through electrostatic

interaction between the negatively charged phosphate backbones of the DNA with charged metal ions. Both hydrophobic and electrostatic interactions showed better intercalation interaction to the flavonoid metal complexes than parent flavonoids [187]. Different kinds of flavonoids such as hesperetin, apigenin, and quercetin interact with metal ions like Cu^{2+}, and showed better and different affinities of intercalation with DNA compared with unaccompanied flavonoid [189, 190].

The affinity of the reaction with DNA depends upon the category and structure of the flavonoid [191]. For instance, quercetin has a higher affinity to intercalate with the DNA than rutin, which possess the lowest affinity. Overall, the interaction of flavanone with metal ions exhibits the highest intercalation with DNA, according to previous studies that demonstrated that naringin and naringenin showed a higher affinity of intercalation with DNA [192, 193]. Furthermore, indistinct information about the role of DNA sequences in intercalation between flavonoid metal interaction and DNA. A study showed that the grooves of DNA with adenine/thymine-rich regions (AT) with isoflavones genistein intercalate more than guanine/cytosine-rich GC [194, 195]. Conversely, other studies have suggested that DNA sequences rich with GC base pairs have a higher affinity to bind to flavonoid metal complexes [196].

Moreover, other conflicting data about the role of increasing flavonoid concentrations in enhancing DNA breaks showed that increasing the flavonoid concentration in the presence of cupric ions did not show an increase in DNA breaks [197]. Furthermore, other data showed the flavonoids enhance more DNA breaks at high concentrations [170]. Inducing ssDNA or dsDNA breaks could be dependent on the concentration of metal ions that interact with the flavonoid. Previous work demonstrated that quercetin with a low concentration of cupric ion induces ssDNA breaks, while a high concentration of cupric ions promote DSB formation; at concentration of 100 μM of cupric

ions induce the formation of DSB of the plasmid DNA, when compared with DNA breaks formed at 25 μM of cupric ions, in which quercetin showed protective role by decreasing the formation of ssDNA breaks [197].

Interaction of flavonoid metal interaction with RNA

There is an insignificant number of flavonoid metal complex interactions with RNA studies in the literature. In a previous study, quercetin and lanthanum oxide metal showed a higher affinity for RNA than flavonoid on its own in the reaction [198]. Most likely, the adduction with a higher affinity of flavonoid metal complex to bind DNA and RNA is attributed to the electrostatic reaction. The negative charge of phosphate groups (anionic) in DNA will bind strongly to the positively charged metal ions (cation). This will significantly stabilize the flavonoid metal complex compared to free flavonoid and help DNA base pairs to remove the flavonoid in the metal complex [199, 200].

Anticancer activity of flavonoid metal ions interaction

The ability of flavonoid metal ion complexes to degrade DNA has encouraged scientists to study its anticancer activity. There are many studies evaluating the effect of flavonoid metal interaction at different signaling cascade steps in cancer cells to determine which classes of flavonoids give the most potent toxic effect in different cancer cells [168, 189, 192, 201].

The anticancer effectiveness of the flavonoid metal complex is larger than the parent flavonoid [18]. The preceding paper showed that hesperetin, naringenin, and apigenin can be two times toxic to hepatoma and gastric cancer in the presence of Cu^{2+} [189], compared to the parental flavonoid. The greater toxicity effect of flavonoid metal complex in cells could be attributed

mainly to the prooxidant activity of the flavonoid metal interaction, or the good intercalation with DNA, or the change in the membrane fluidity [159, 202].

There are numerous anticipated mechanisms by which flavonoids and metal ions interaction can induce anticancer activity [1]. Some flavonoids can inhibit cell proliferation via activation of signal transduction pathways and cell cycle arrest, such as hesperetin which stimulates the tumor suppressor protein P53 in breast cancer cells, and arrests cancer cells in G2-M phase [203-205], and quercetin which showed inhibition to Epidermal Growth Factor Receptor tyrosine kinase activity [206], and was the first flavonoid tested in clinical trials [207].

Metals ions in flavonoid metal complex

The human body uses bulk and trace minerals for different functions [1]. Bulk minerals such as calcium, magnesium, potassium, and phosphorus are required and used in large amounts in the body to achieve different biological functions [2]. Conversely, trace minerals are needed in small amounts to maintain a balanced diet. The most important trace metals are copper, iron, and zinc. Several trace metals were studied in flavonoid metal interaction, some of which boost the production of reactive species, so may play an important role in oxygen metabolism. For example, Fe^{3+} or $Cu^{2+}$are trace metals that efficiently chelate flavonoids.

Copper (Cu)

More attention has been paid to the study of flavonoids with complexes of copper (Cu) and iron (Fe) since these metals play an important biological role in enzymes of reactive oxygen species in living organisms.

Copper is one of the most important trace elements. It is found in two oxidative conditions, $Cu^{1+}$and Cu^{2+}. It participates in different biochemical reactions; Cu is required along with amino

acids and vitamins for normal metabolic pathways [208]. Also, Cu is essential and many enzymes such as cytochrome c-oxidase require Cu [209, 210]. Cu is essential in various biological systems [211].

Many studies refer to the role of Fenton-like reactions in inducing DNA breaks [212-215]. In general, a Fenton-like reaction produces oxygen free radicals in the presence of transited metal and hydrogen peroxide. Although iron is more abundant in biological processes in the body than Cu, Cu is more active in Fenton like reactions [216], and DNA damage formed in the presence of Cu is more extensive than DNA breaks formed by iron [217, 218]. The previous paper demonstrated that Cu catalyzes the conversion of H_2O_2 to •OH *in vitro*, and consequently suggests that oxidative damage could be a significant factor in Cu toxicity and inducing DNA damage and certain types of cancers.

Flavonoids bind more tightly with Cu^{2+}, and the stability of the flavonoid metal complex with Cu^{2+} is higher than the stability of a complex with Cu^{1+}. This is attributed to the presence of a short bond between Cu and oxygen in Cu^{2+} [5]. As an example, 3-hydroxy flavone (3HF) binds more strongly to Cu^{2+}. Furthermore, there is evidence shows flavonoids such as quercetin form a complex with $Cu^{2+}$and result in the formation of wide-ranging oxidative damage to DNA due to the formation of 8-oxoguanine [160, 219].

The former paper showed that copper accumulated in cancer more than normal cells and can kill cancer cells and induce apoptosis [220], and this has been attributed to the production of •OH which leads to DNA damage by both $Cu^{1+}$and Cu^{2+}. The presence of Cu^{1+} can directly induce DNA breaks by free radicals in cancer cells via Fenton-type reactions.

Application fields of flavonoids-metal interaction

Flavonoids with metal ions demonstrate different characteristics compared with parental flavonoids. There is evidence that shows that some flavonoids in the presence of metal ions have more beneficial effects on biological and pharmaceutical activities. Some flavonoids with metal ions exhibit more antimicrobial activity compared to parent flavonoid. This alteration in the effect could be attributed to the interaction of metal ions binding to their preferred binding sites within the enzymes [20]. Another cause might be the binding of metal ions to the cell membrane which leads to a change in cell signaling [221]. Other proposed mechanisms that occur through gene expression alteration happen when metal ions intercalate with DNA in a non-specific way [187].

Furthermore, it is important to understand the mechanism of action and determine the stability of flavonoid metal ions interaction, since the interaction between flavonoids and metal ions could work in many biological processes that chelate metal ions. The complex could be used to provide the body with essential minerals or to eliminate minerals in minerals overloads disorders.

Objectives and Research Questions

Flavonoids are a large group of polyphenolic compounds found in most plant families. Flavonoids are commonly used by humans in traditional and recent medicine. Flavonoids serve a significant role in animal and human health due to being used in a variety of pharmaceutical activities. There is a huge number of flavonoids. More than 8000 different flavonoids have been identified.

Flavonoids are represented in a large percent of our diet, different vegetables and fruits contain flavonoids for instance. More attention among scientists has been given to using herbal plants to prevent or cure different diseases. Flavonoids possess many nutritional and medical

properties that allow them to play different roles such as anti-inflammatory, anticancer, anti-diabetic…etc. Many studies have been done to understand the biological roles of flavonoids and the various modes of action by which flavonoids can act as an antioxidant or prooxidant in presence of metal ions.

Flavonoids reportedly have two conflicting working mechanisms. They play an antioxidant role and protect the body against oxidative stress via scavenging the free radicals or can produce radicals via acting as a prooxidant in presence of transit metals. The prooxidant mechanism of flavonoids is an area of interest; scientists were also trying to elucidate this mechanism to use flavonoids safely and synthesize flavonoids by adding some more functional groups that may enhance the nutritional and the biological or pharmacological behavior in presence of metals.

Synthesis of flavonoids in recent years has seen more attention to increase the commercial quantities. Also, scientists continue to work on adding some functional groups to flavonoids to introduce changes in their biological properties and increase their bioavailability and solubility in water. This project is an attempt to provide further understanding of the flavonoid-metal complex prooxidant property, to clarify and investigate their mechanism of action to break DNA, and their anti-cancer action. We hypothesized that the presence of specific hydroxyl groups at specific rings in flavonoids will induce free radicals that causes DNA damage in presence of cupric ions

Experiments were designed to investigate the following specific aims:

1) The first aim was to investigate the effect of hydroxyl position on inducing single strand breaks in the presence of copper.

2) The second aim was to compare the effect of flavonoids possessing an OH group on position 3 on the C ring with different OH distribution on B ring on inducing ssDNA damage and to determine the most potent flavonoid among tested flavonoids that induce the highest ssDNA damage in the presence of cupric ions.

3) The third aim was to determine if the DNA damage induced by tested flavonoids in the presence of cupric ions is p53 dependent or not.

Also, several specific Research Questions were addressed:

1) Does the temperature have an impact on flavonoid metal complex reaction?

2) Is there any difference of prooxidant activity between the natural and synthetic flavonoids in their ability to enhance ssDNA breaks?

3) What is the relationship between the number of hydroxyl groups of flavonoids and their prooxidant activity?

4) Is there a relationship between flavonoid structure and the growth inhibition of cells carry loss of function mutations in P53?

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CHAPTER 2/CHARACTERIZATION OF THE MECHANISM OF SSDNA DAMAGE INDUCED BY THE FLAVONOID QUERCETIN IN PRESENCE OF CUPRIC IONS

SYNOPSIS

Background: Quercetin has been demonstrated to produce DNA damage in the presence of metal ions. In the present study, seven natural and five semi-synthetic glycosylated flavonoids were utilized to investigate cupric ion [Cu^{2+}] dependent DNA damage in *vitro.*

Methods*:* The reaction mixture, containing single-stranded DNA, different concentrations of flavonoids and Cu^{2+}in the buffer, was incubated at three different temperatures. DNA damage was then assessed by gel electrophoresis followed by densitometric analysis. Each reaction mixture was at different temperatures; 4, 20 and 54˚C. Moreover, Cu^{2+}chelating capacity was also assessed with spectroscopic analysis.

Results*:* The reaction mixture with quercetin at 4, 20 and 54˚C induced DNA damage in a concentration- and temperature-dependent manner. Furthermore, only the reaction at 54˚C resulted in DNA damage in flavonoids with glucosyl substitutions of the hydroxyl group at the 3-position on the C ring in quercetin. In contrast, loss of the hydroxyl group at the 3-position on the C ring, or at the 3'- or 4'-position on the B ring of quercetin, did not induce DNA damage formation at the investigated experimental temperatures. In addition, the experimental results suggested that the hydroxyl group at the 3-position on the C ring produced the strongest capability to induce DNA damage in the presence of Cu^{2+}. Furthermore, hydroxyl groups at the 3'- or 4'-position on the B ring were only able to induce DNA damage at higher temperatures and were less efficient in comparison with the hydroxyl group at the 3-position on the C ring.

Conclusions*:* Our data suggest that the 3-position hydroxyl group of the C ring is required to induce DNA damage at low temperatures. Furthermore, the presence of Cu^{2+} decreased the activity of the glycosylated quercetins, in the terms of their ability to induce DNA damage.

2.1 Introduction

Flavonoids are naturally occurring polyphenolic metabolites in plants that serve significant roles in traditional medicine and as food additives [1-3]. Glycosylated forms of numerous flavonoids are produced in order to alter their chemical properties, such as their bioavailability and water solubility [4]. Flavonoids without glycosylation are known as aglycones, which are often more reactive forms of flavonoids and present greater antioxidant capacity, as well as cellular toxicity, compared with the glycosylated flavonoids [5, 6]. Both aglycone and glycosylated forms of flavonoids are recognized to be beneficial regarding anti-tumorigenicity and anti-mutagenicity, due to their antioxidant and radical scavenging effects.

Numerous flavonoids, including quercetin, have been previously reported to induce mutations in bacterial systems and chromosome aberrations in mammalian cells [7-10]. This mutagenic potential may be associated with the ability of flavonoids to produce hydroxyl radicals, resulting in DNA breaks [11, 12]. In the presence of specific metal ions, flavonoids can cause DNA scission, but rarely DNA double-strand breaks, resulting from the interaction of hydrogen peroxide with metal ions [12]. This hydroxyl radical production is coupled with the reduction of cupric ions to cuprous ions [from Cu^{2+} to Cu^{1+}].

Quercetin forms chelating complexes with cupric ions and exhibits broad biological activities [13-16]. This cupric ion and the quercetin complex interact at the 3' and 4' hydroxyl groups on the B ring, and the 3-hydroxyl group and 4-oxygen residue on the A ring [17]. Furthermore, the ability to produce hydroxyl radicals and to induce DNA breaking activity has also been reported for other compounds that are structurally associated with quercetin, including fisetin, baicalein, taxifolin and curcumin with cupric ions [18,19], as well as epigallocatechin

gallate and ferrous ions [20]. However, the exact mechanisms underlying the formation of hydroxyl radicals from quercetin and its effects on glycosylation of flavonoids remain unclear.

The present study utilized a total of 7 natural and 5 recently semi‑synthesized novel flavonoids to identify the specific molecular mechanisms required to induce DNA scission in the presence of cupric ions. To analyze DNA scission formation, in an *in vitro* gel electrophoresis system, three aglycones and their glycosylated flavonoids were reacted in the presence of cupric ions at different temperatures.

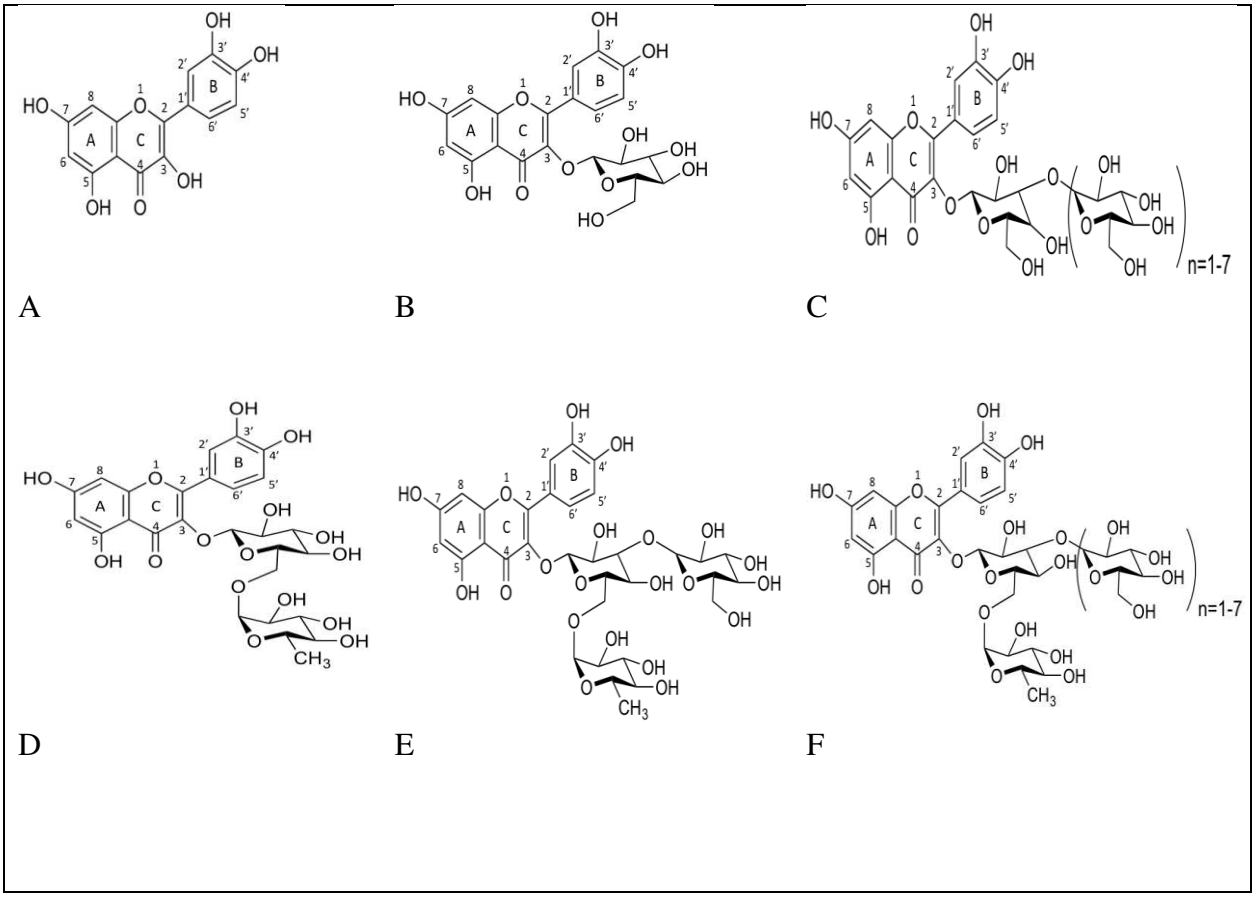

Figure 2. 1A. Structure of quercetin and quercetin glycosylated flavonoids. [A] quercetin, [B] isoquercetin, [C] rutin, [D] monoglucosyl‑rutin, [E] maltooligosyl‑isoquercetin, [F] maltooligosyl rutin.

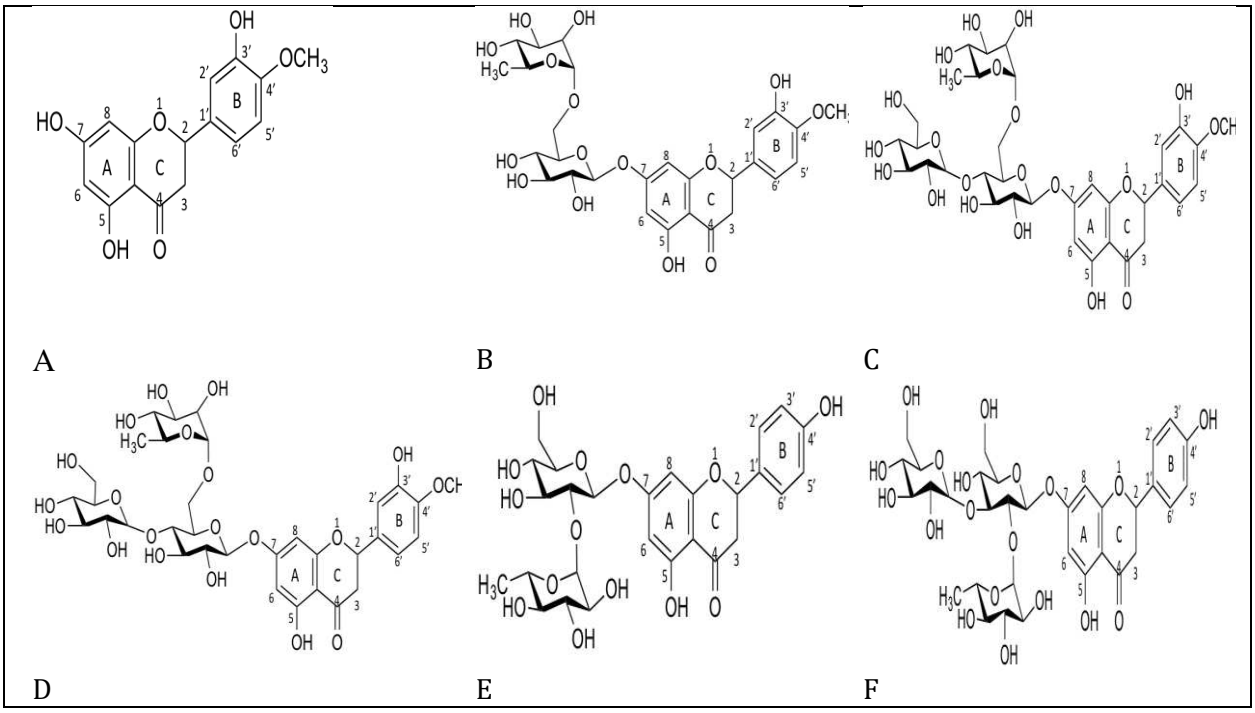

Figure 2. 1B. Hespertin and naringenin groups. [G] hesperetin, [H] hesperidin, [I] monoglucosyl hesperidin, [J] naringenin, [K] naringin, and [L] monoglucosyl naringin.

2.2 Materials and methods

2.2.1 Chemicals

All natural and synthetic flavonoids were obtained from Tokyo Sugar Refining Co., Ltd. [Tokyo, Japan]. The tested compounds, including three aglycone flavonoids [quercetin, naringenin and hesperetin] and their glycosylated flavonoids are summarized in Figs. 1A and 1 B. Quercetin is an active aglycone form of isoquercetin, rutin, maltooligosyl-isoquercetin, monoglucosyl-rutin and maltooligosyl-rutin. The glycosylated form of quercetin loses the 3-position hydroxyl group on the B ring by glycosylation. Compared with quercetin, naringenin does not contain a hydroxyl group at the 3-position on the B ring nor at the 3'-position on the C ring. In addition, compared with quercetin, hesperetin does not contain a hydroxyl group at the 3-position on the C ring nor at the 4'‑position on the C ring. Glycosylated naringenin and hesperetin lost the hydroxyl group at the 7-position on the A ring were prepared by dissolving in dimethyl sulfoxide [Thermo Fisher

Scientific, Inc., Waltham, MA, USA] at the concentration by glycosylation and are chemically inactive. All flavonoids were prepared as a 1 mM stock solution. M13mp18 single‑stranded DNA [250 μg/mL] was purchased from New England BioLabs, Inc. [Ipswich, MA, USA]. Double stranded lambda phage DNA [0.46 μg/μL] was purchased from Nippon Gene Co., Ltd. [Tokyo, Japan]. Copper chloride dehydrate was purchased from Sigma‑Aldrich [Merck KGaA, Darmstadt, Germany].

2.2.3 DNA scission reaction

A total of 20 μL reaction solution was used, containing 1.5 ng/μL single‑stranded DNA or 1.25 ng/μL of double stranded DNA, 10 mM Tris‑HCl, 0.2 mM $CuCl_2$ and various concentrations of flavonoids [0.1, 1, 10 and 100 μM]. The reaction mixtures were incubated at different temperatures [4˚C, 20˚C, 37˚C or 54˚C] for 1 h. Next, 4 μL of 6X loading dye, containing 15% Ficoll, 10% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol in water, was added to the mixture and electrophoresis was conducted on 1% agarose gel stained with ethidium bromide with 1X Tris‑acetate‑EDTA buffer at 100 V for 1 h. Subsequent to electrophoresis, destaining of ethidium bromide was performed, and a gel image was obtained using a ChemiDoc XRS system [Bio Rad Laboratories, Inc., Hercules, CA, USA] via Image Lab software [Bio‑Rad Laboratories, Inc.]. Intact M13mp18 single‑stranded DNA presented two bands, indicative of the circular and linear form of DNA. The DNA intensity of the circular form was obtained with Image Lab, while the fraction of intact DNA was calculated as follows:

$$Fraction\ of\ intact\ DNA = \frac{Amount\ of\ circular\ DNA\ with\ flavonoids}{Amount\ of\ circular\ DNA\ without\ flavonoids}$$

Half maximal inhibitory concentration [IC50] values, which refer to the specific concentrations required to induce 50% of DNA breaks, were obtained from sigmoidal regression curves using Prism 6 software [GraphPad Software, Inc., La Jolla, CA, USA].

2.2.4 Absorption spectrum analysis

In order to investigate the cupric ion chelating capability of flavonoids, the flavonoids and cupric ions were mixed at rations of 1:2, 1:1 and 2:1, and absorbance values from 230 to 430 nm were obtained via a Nanodrop spectrophotometer [Thermo Fisher Scientific, Inc.]. According to the peak shifting and the reduction of peak height, the chelating capacity of cupric ions was estimated [14, 15, and 17].

2.2.4 Oxidation of luminol

In order to investigate the potential mechanisms underlying the induction of DNA damage, oxidative capacity analysis was conducted. In total, 100 μL of reaction solution, including 20 μL enhanced chemiluminescence solution [Thermo Fisher Scientific, Inc.], 2 μL of 100 μM flavonoids and 2 μL of 100 μM $CuCl_2$, were mixed. Subsequently, arbitrary relative luminescence units [RLU] were measured immediately until 3 min after mixing via a Lumat LB9507 luminometer [Berthold Technologies, Oak Ridge, TN, USA]. RLU values were plotted with the exponential decay model, and half-time values were obtained.

2.2.5 Statistical analysis

Statistical calculations were performed using Prism 6 software. The data was normally distributing because the Shapiro-Wilk Test was greater than 0.05. Two-way analysis of variance [ANOVA] and Student's unpaired t-test were conducted to assets whether the means of variables were significant different or not. P‑values of <0.05 were considered to indicate differences that were statistically significant. Further Bonferroni pairwise comparisons was followed ANOVA.

2.3 Results

2.3.1 DNA damage formation

Fig. 2 demonstrates the DNA damage subsequent to reaction with quercetin in the absence or presence of cupric ions at 37˚C. Without cupric ions in the reaction mixture, DNA damage was not detected from single-stranded or double-stranded DNA. Upon the incorporation of cupric ions in the reaction mixture, single-stranded DNA was degraded, whereas double-stranded DNA was not affected. Therefore, quercetin without cupric ions did not produce DNA damage. In addition, DNA damage produced by quercetin and cupric ions mainly involved single‑strand breaks rather than double-strand breaks.

Fig. 3 shows the fraction of intact DNA following reaction with glycosylated flavonoids in the presence of cupric ions in the reaction mixture. In the presence of 0.2 mM $CuCl_2$ in the reaction mixture, quercetin and its glycosylated flavonoids induced DNA breaks in a concentration and temperature dependent manner. Less intact DNA was observed following reaction with high concentrations of flavonoids, and high temperature efficiently induced DNA damage.

Quercetin was the only flavonoid capable of inducing DNA breaks in reactions performed at 4 and 20˚C. Furthermore, the reaction at 20˚C induced a greater degree of DNA damage as compared with the reaction at 4˚C. The IC50 values of quercetin at 4 and 20˚C were 47.7 and 20.5 μM, respectively, while the reaction at 54˚C reduced the IC50 value to 2.6 μM. By contrast, all quercetin glycosides, including isoquercetin, rutin, monoglucosyl rutin, maltooligosyl-rutin and maltooligosyl-isoquercetin, failed to induce any DNA damage following 4 and 20˚C reactions under the tested conditions. However, these glucosyl flavonoids did induce DNA damage when reactions were conducted at 54˚C for 1 h. The IC50 values were 7.7 μM for isoquercetin, 3.4 μM for rutin, 9.5 μM for monoglucosyl rutin, 4.5 μM for maltooligosyl rutin and 12.1 μM for

maltooligosyl isoquercetin. This indicates that glycosyl modifications at the 3-position on the C ring of quercetin suppressed the DNA scission ability of quercetin at 4 and 20˚C. However, DNA scission capacity was not altered with glycosylation at the 3-position on the C ring of quercetin at 54˚C. Therefore, increased glucosyl modifications did not contribute toward DNA degradation. In other words, the hydroxyl groups of glucosyl residues did not contribute to DNA damage.

In order to clarify which position of the hydroxyl group is responsible for inducing DNA damage, two other flavone aglycones, hesperetin and naringenin, and their glycosylated flavonoids were also tested in the same system to assess hydroxyl radical formation by DNA damage observation (Fig. 4). Hesperetin, naringenin and their glycosylated flavonoids, which do not have hydroxyl groups at the 3‑position on the C ring nor at the 3'- or 4'-position on the B ring, were incapable of producing any detectable single-stranded DNA damage in the reaction at any tested temperature. Therefore, this indicates that the presence of hydroxyl groups at the 3'- and 4'-positions is required for the induction of DNA damage at 54˚C. Furthermore, flavone glycosides did not cause any DNA damage, confirming that the hydroxyl groups of glucosyl residue have no effect on DNA damage.

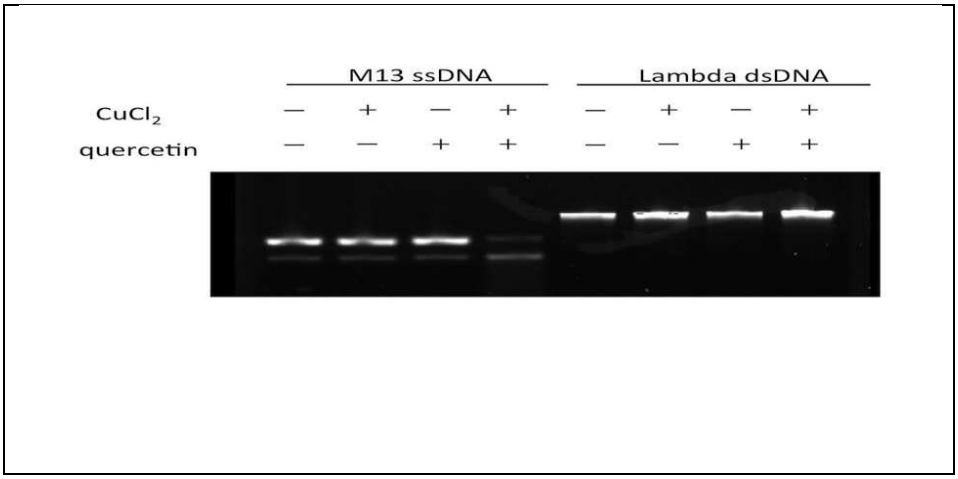

Figure 2. 2. Gel image indicating DNA damage after 1‑h reaction at 37˚C. M13 ssDNA [upper bands indicate circular intact DNA and lower bands indicate linear DNA] and lambda dsDNA

were treated with or without 0.2 mM $CuCl_2$ and 100 μM quercetin in 10 mM Tris ‑HCl solution. ss, single ‑stranded; ds, double-stranded.

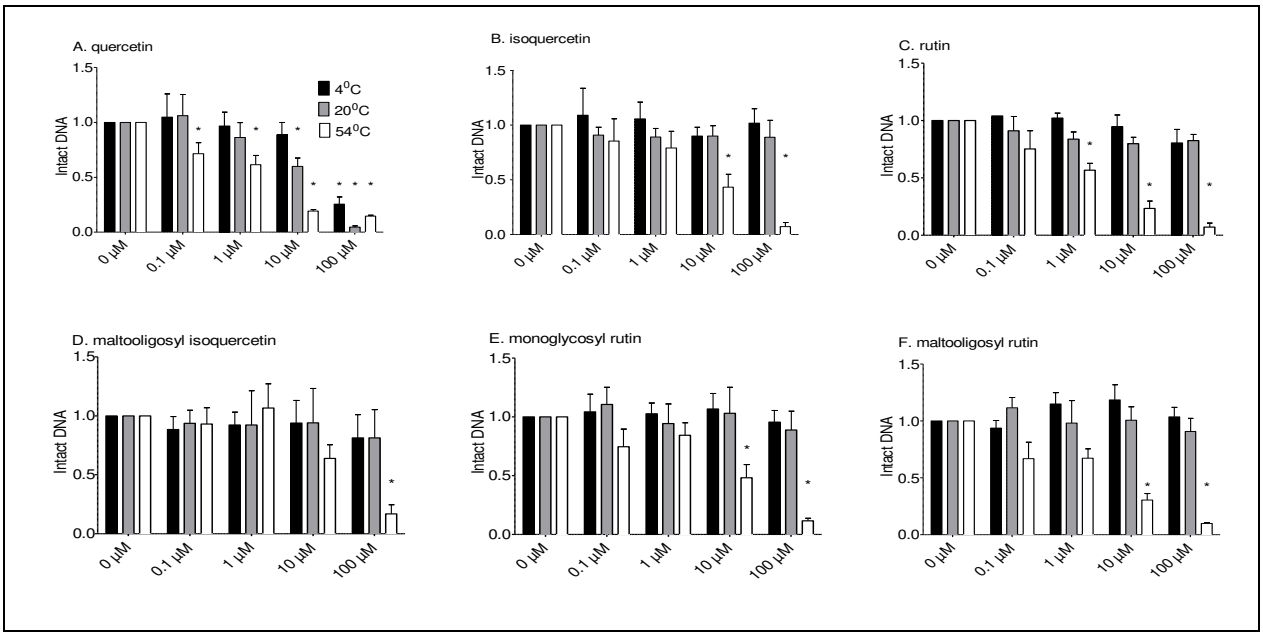

Figure2. 3. Fraction of intact DNA after 1 ‑h reaction at 4, 20 and 54˚C for quercetin and its glucosides. [A] Quercetin, [B] isoquercetin, [C] rutin, [D] maltooligosyl isoquercetin, [E] monoglucosyl rutin, and [F] maltooligosyl rutin. Error bars indicate standard error of the means. Three independent experiments were performed.

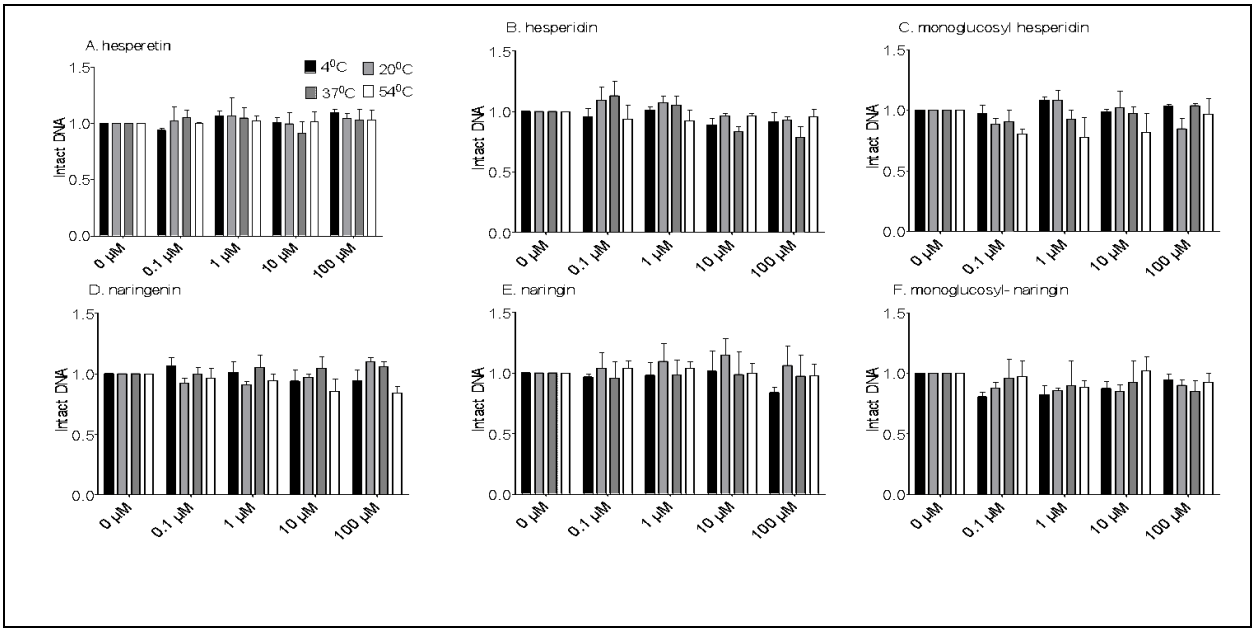

Figure2.4. Fraction of intact DNA after 1 ‑h reaction at 4, 20 and 54˚C for hesperetin, naringenin and their glucosides. Error bars indicate the standard error of the mean. Three independent experiments were performed. [A] Hesperetin, [B] hesperidin, [C] monoglucosyl hesperidin, [D] naringenin, [E] naringin and [F] monoglucosyl naringin.

2.3.2 Chelating capacity

Flavonoids are considered natural chelators; they have strong ability to chelate metal ions such as Cu^{2+} or Fe that are involved in radical formation. This chelation is crucial in prevention of production of radicals. Studying and comparing the prooxidant activity of flavonoids requires studying their metal chelating and radical scavenging ability.

In general, benzoyl and cinnamoyl bands are the two main absorption bands of most flavonoid [25]. When metal ions bind to flavonoids, the change in absorption bands will occur. The MOMO-LUMO gap in the flavonoid will decline and leads to a shift in the absorption bands of the flavonoid metal complex [26].

Absorption spectrum alterations, shown in Fig. 5A, from A to L flavonoids, occurred when flavonoids and cupric ions were mixed in different ratios [1:2, 1:1 and 2:1]. Quercetin and its glycosides displayed peaks at ~250 and 370 nm, as previously described [14- 15, 17]. Upon addition of cupric ions, the first peak at 250 nm skewed to the left and the size of the second peak was reduced.

The reduction in the second peak was the greatest for quercetin. Naringenin and hesperetin and their glycosides presented the first peak at 250 nm and the second peak at 290 nm. As observed in quercetin and its glycosides, the first peak was shifted toward the left and the second peak size was decreased in the presence of cupric ions. The reduction ratio of the second peak was used for assessment of cupric ion chelating capacity, and the results are summarized in Figure. 5B.

Quercetin demonstrated a >6 folds reduction in the absorbance peak height in the presence of cupric ions (Fig. 5B). The peak heights of other flavonoids were reduced by 2‑4‑fold. Furthermore, quercetin showed statistically significant differences compared with the other flavonoids [ANOVA; $P<0.0001$].

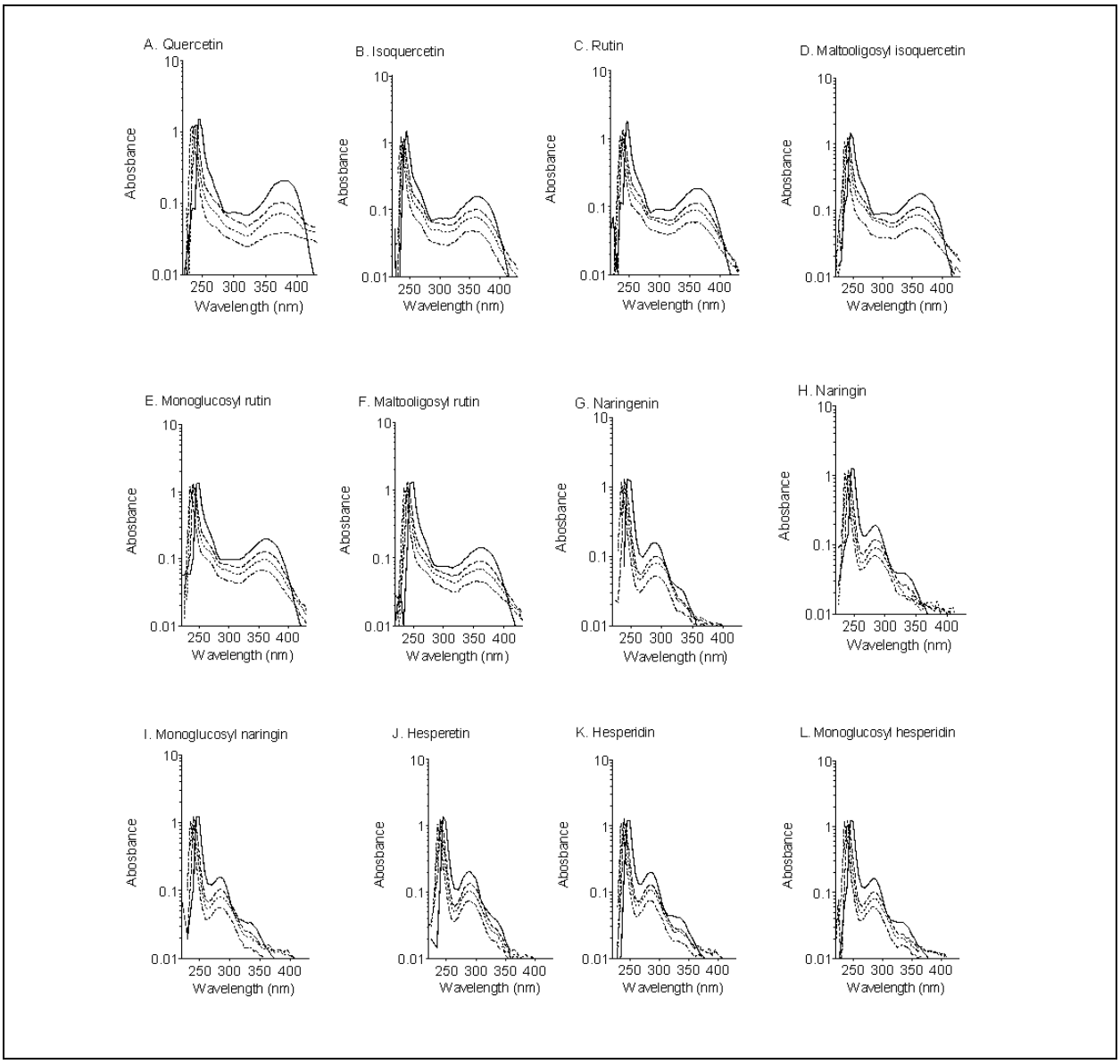

Figure 2. 5A. Absorption spectrum changes in the presence of cupric ions. Solid lines indicate the flavonoid only, and the dashed, dotted and dash¯dotted lines indicate a flavonoid and cupric ion mixture at a ratio of 2:1, 1:1 and 1:2, respectively. [A] Quercetin, [B] isoquercetin, [C] rutin, [D] maltooligosyl isoquercetin, [E] monoglucosyl rutin, [F] maltooligosyl rutin, [G] naringenin, [H] naringin, [I] monoglucosyl naringin, [J] hesperetin, [K] hesperidin and [L] monoglucosyl hesperidin spectrum changes are shown.

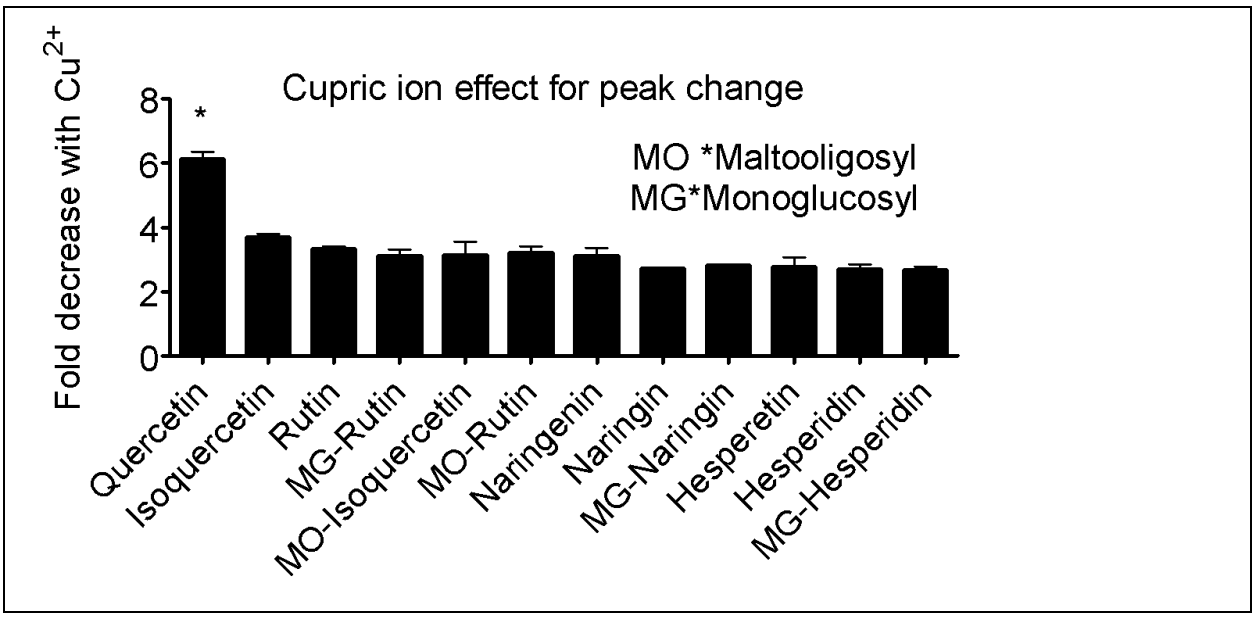

*Figure 2. 5B. The assessment of the cupric ion chelating capacity by measuring the reduction of the second peak. *P<0.05 vs. other flavonoids. Error bars indicate the standard error of the mean. Three independent experiments were performed.*

2.3.3 Oxidation of luminol

In addition to the interaction between flavonoids and cupric ions, oxidative capacity analysis was conducted with luminol as a substrate in the presence of flavonoids and cupric ions. Subsequent to mixing, the reduction of the luminescence signal was observed (Fig. 6). Emitted luminescence signals are associated with not only cupric ion induction, but also generated hydrogen peroxide by flavonoids in the presence of cupric ions. The luminescence signal [arbitrary RLU] was rapidly decreased with an exponentially decreased model for all flavonoids. Quercetin and isoquercetin exhibited the fastest reduction of RLU with a half-life of 0.22 min. In addition, rutin, monoglucosyl-rutin, maltooligosyl-rutin and maltooligosyl-isoquercetin presented intermediate reduction in kinetics with a half‑life between 0.28‑0.33 min. Naringenin, naringin, monoglucosyl-naringin, hesperetin, hesperidin and monoglucosyl-hesperidin also displayed the slowest kinetics with a half-life of 0.35-0.45 min.

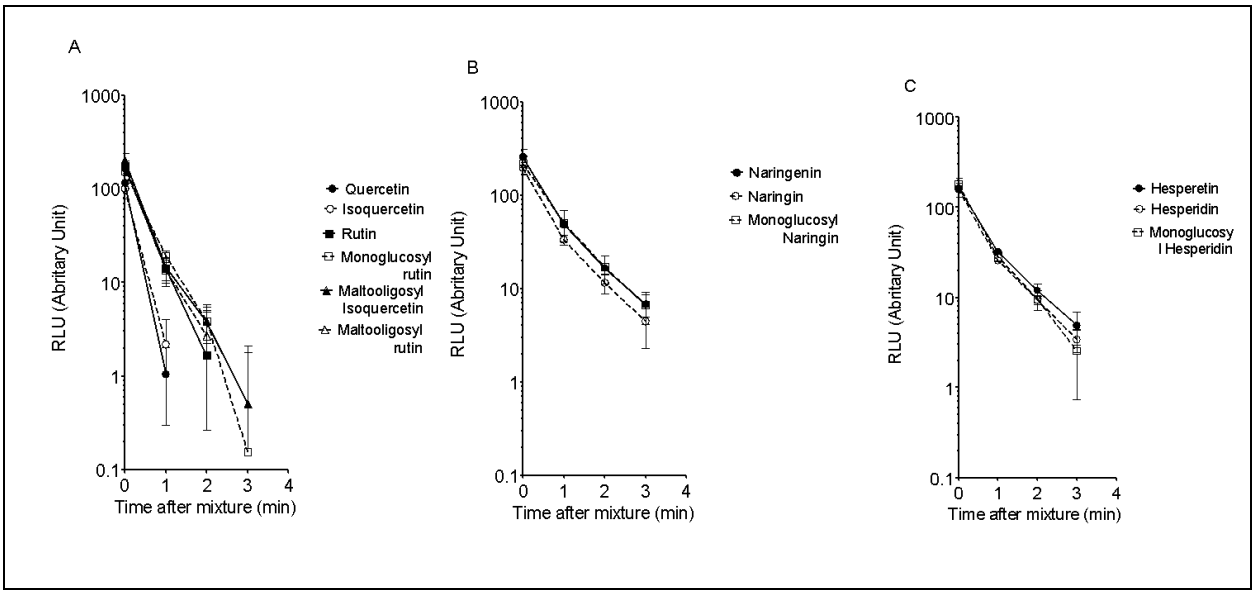

Figure2. 6. Oxidation of luminol in the presence of hydrogen peroxide, $CuCl_2$ and flavonoids. [A] Quercetin, isoquercetin, rutin, maltooligosyl isoquercetin, monoglucosyl rutin, and maltooligosyl rutin. [B] Naringenin, naringin, and monoglucosyl naringin. [C] Hesperetin, hesperidin, and monoglucosyl hesperidin. Error bars indicate the standard error of the mean values. Three independent experiments were performed. MO, maltooligosyl; MG, monoglucosyl.

2.4. Discussion

Flavonoids with a variety of chemical structures with modified residues exist; for instance, a number of hydroxyl groups are attached to the different positions of benzene rings [21]. Depending on the hydroxyl groups present at specific locations, metal ions can bind to flavonoids and induce the Fenton-like reaction, resulting in DNA damage [11, 12]. DNA double-strand breaks are rare in this event, but single-strand scission and oxidative damage are induced [11, 22]. This DNA damage may be associated with mutagenesis in the cell culture system after flavonoids reacted with cells in the presence of metals [23].

The present study clearly demonstrated the importance of the specific positions of hydroxyl groups on the flavonoids to induce DNA damage. The results revealed that quercetin was the only flavonoid capable of inducing DNA damage at any of the tested experimental temperatures (Fig. 3). Naringenin and hesperetin, which do not possess a hydroxyl group at the specific positions [the

3‑position on the C ring, and the 3'- and 4'-positions on the B ring], were incapable of inducing DNA damage (Fig. 4). Furthermore, at low temperatures, the glycosylated flavonoids of quercetin were incapable of inducing DNA breaks (Fig. 3). This suggests that a hydroxyl group at the 3-position on the C ring of quercetin is the most reactive, inducing DNA damage in a Fenton‑like reaction with cupric ions. These findings are in agreement with the previous studies that refer to the possibly significant role of this location [11] in forming a complex with cupric ions to induce DNA damage. It is also worth noting that the hydroxyl group at the 3-position on the C ring of quercetin has the ability to scavenge radicals [24]. The high cupric ion chelating capacity of quercetin (Fig. 5B) and fast oxidation of luminol (Fig. 6) were also in agreement with these previous findings.

The structural differences among quercetin, naringenin and hesperetin helped to investigate the impact of hydroxyl groups at the 3'- and 4'-positions on the B ring in the induction of DNA damage. With two hydroxyl groups at the 3'‑ and 4'‑positions on the B ring, flavonoids induced DNA damage at a high temperature, as observed with glycosylated quercetins [including isoquercetin, rutin, maltooligosyl‑isoquercetin, monoglucosyl‑rutin and maltooligosyl‑rutin] (Fig. 3). When one of the two hydroxyl groups were replaced with other residues, high temperature‑specific DNA damage was not observed in naringenin and hesperetin. Additionally, hydroxyl groups at the 7-positions on the A ring did not contribute to DNA damage induction based on the observation of the glycosylated naringenin and hesperetin. Previously, it has been reported that the hydroxyl groups of quercetin at the 3'- and 4'-positions on the B ring have higher radical scavenging effects in comparison with the hydroxyl group at the 3‑position on the A ring [24]. This may be associated with the temperature-dependent pro and antioxidant properties of quercetin.

The DNA scission observed in the present study is a result of radical formation from a Fenton-like reaction between specific hydroxyl groups and cupric ions. Temperature-dependent DNA damage between a hydroxyl group at the 3-position on the C ring and the hydroxyl groups at the 3'- and 4'-positions on the B ring may be associated with the amount of radical formation at the different temperatures. In this case, the hydroxyl group at the 3‑positions on the C ring can efficiently produce more radicals compared with the hydroxyl groups at 3'- and 4'-positions on the B ring in the presence of cupric ions. Another possible mechanism of differential DNA damage is an interaction between the quercetin-cupric ion complex and DNA. The proposed models of the quercetin-cupric ion complex involve a hydroxyl group at the 3‑position on the C ring and hydroxyl groups at the 3'- and 4'-positions on the B ring. It is possible that the complex formation between quercetin-cupric ion at the 3'- and 4'-positions on the B ring is temperature-dependent.

In conclusion, the present study reported that quercetin induces DNA scissions in the presence of cupric ions in a broad range of temperatures. The hydroxyl group at the 3-position on the C ring contributes to the temperature-independent DNA scission due to high chelating capacity and oxidative reaction. In addition, the hydroxyl groups at the 3'- and 4'-positions on the B ring contribute to DNA scission formation in the presence of cupric ions at high temperature.

In conclusion, the advantageous health effects of Flavonoid attract consumers and food industries. The safe usage of flavonoids is very important topic for the human health. Increase copper levels is associated with some pathological conditions. Therefore, it is key to study the Flavonoid metal ions interaction at different temperatures. Our data suggested that high temperature could increase the cupric ions binding and accelerate DNA damage in Quercetin and its glycosylated form. Low temperature did not show significant effect on interaction of flavonoids and cupric ions.

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CHAPTER 3/IDENTIFYING THE MECHANISM OF THE MOST POTENT FLAVONOIDS THAT INDUCE SSDNA DAMAGE MEDIATED BY CUPRIC IONS

SYNOPSIS

Background: Flavonoids such as quercetin have been demonstrated to produce DNA scission in the presence of metal ions. In the present study, quercetin and six other flavonoids that have a hydroxyl group at position 3 of the C ring were utilized to investigate the cupric ion-dependent DNA damage mechanism in vitro.

Methods: Single-stranded DNA, flavonoids, and cupric ions were mixed in buffer. The mixtures were incubated at 4, 20 or 37˚C for 1 hour to induce DNA scission. DNA damage was assessed by gel electrophoresis. The absorption spectra of flavonoid-copper complex were determined with a spectrometer analysis. The free radicals scavenging ability of each flavonoid with was measured by using the DPPH assay in presence of cupric ions. Finally, chemical molecular polarity of hydroxyl position in flavonoids was investigated.

Results: Kaempferol, quercetin, and myricetin induced significantly higher DNA damage than other structurally similar flavonoids. Among them, the pyrogallol flavonoid myricetin possessed the strongest DNA scission activity. However, myricetin did not show higher copper chelating activity or radical scavenging activity. Myricetin's ability to induce DNA scission may be associated with low molecular polarity at Ring C, hydroxyl residue. Loss of this hydroxyl residue, stereocenters, or non-catechol structure inhibited DNA scission activity in different manners.

Discussions: Alteration of the number or location of a single hydroxyl residue severely affected DNA scission activity of flavonoids cupric ions. Low molecular polarity at Ring C may be the best indicator of DNA scission activity with cupric ion

3.1 Introduction

Flavonoids are natural polyphenolic secondary metabolites of plants, found in many fruits, vegetables, grains, flowers, and beverages such as tea and wine [1,2]. Recently, flavonoids have become an area of for research due to their beneficial properties as they pertain to human and animal health [3-6]. One of the most important of these properties is flavonoids' tendency to act as an antioxidant [7-9]. Previous papers have demonstrated that flavonoids' anti-oxidant effects can play different therapeutic roles: they can act as anti-inflammatory, anti-mutagenic and anti-carcinogenic agents [9-13]. This diverse range of therapeutic roles has allowed some of these chemicals to be used in herbal, cosmetic and pharmaceutical medicine for human health benefits [14].

Although flavonoids are well known for their ability to act as antioxidants and scavengers of free radicals, they can also behave as pro-oxidants and produce radicals under certain conditions [15,16]. This lesser known property is dependent upon three different factors: flavonoid concentrations, the number of hydroxyl groups [17], and the presence of transit metals, such as ferrous ion or copper [16]. Chemically, flavonoids are composed of 3 rings; (A) and (B) are benzene rings, connected by a heterocyclic pyrane ring (C). Flavonoids have structural properties that allow them to interact with transition metal ions, such as copper and iron [17, 18]. Prior studies have investigated the ability of flavonoids to chelate transition metals at specific hydroxyl groups on the (A) and (C) rings. Literature has referred to the role of Fenton-like reactions in inducing DNA damage, such as dsDNA and ssDNA breaks [19-22]. In general, the Fenton-like reaction produces oxygen free radicals in the presence of transition metals and hydrogen peroxide. Although iron is more widely employed in biological processes than copper, copper is more active in Fenton-like reactions [23].

Quercetin is one of the flavonoids known to produce dsDNA and ssDNA breaks in the presence of cupric ions [22, 24]. A prior study that compared quercetin, hesperidin and naringin groups demonstrated the tendency of quercetin to produce radicals that induce ssDNA breaks at different temperatures. In agreement with previous studies, this paper concluded that the presence of the hydroxyl group in position 3 on ring (C) is crucial for producing radicals that cause DNA breaks over a range of temperatures (tested at 4, 20, and 54°C), while the presence of catechol hydroxyl groups on ring B produced ssDNA breaks only at high temperatures (54^0C) [22].

In this study, we focused on studying seven flavonoids: quercetin, myricetin, Kaempferol, luteolin, galangin, taxifolin, and morin (Fig. 1). Each of these chemicals except luteolin have a hydroxyl group at position 3 of the C ring but have different distributions of hydroxyl groups on the B ring: phenolic, catechol, and pyrogallol structures. Phenolic structures have one hydroxyl group attached to the B ring, as seen in Kaempferol molecules. Catecholic structures have two hydroxyl groups on the B ring. In order to investigate the impact of the normal catechol structure, and catechol found in flavonoids with conformation alterations, we compared quercetin and taxifolin. Each of these flavonoids has same number and position of hydroxyl groups, but they differ in structure - taxifolin has two stereocenters on the C ring while quercetin doesn't have any. Flavonoids with pyrogallol rings have three hydroxyl groups attached to the B ring as seen in myricetin molecules. We used galangin and morin to test flavonoids with a non-catechol structure on the B ring. Galangin was used to examine the influence of the hydroxyl group in position 3 on the C ring without any of the aforementioned structures, and morin was used to test the effect of a non-catecholic structure. All targeted flavonoids were compared to quercetin in order to identify which chemical is most adept at producing ssDNA damage, with the additional aim of understanding the mechanisms of DNA damage in the presence of cupric ions.

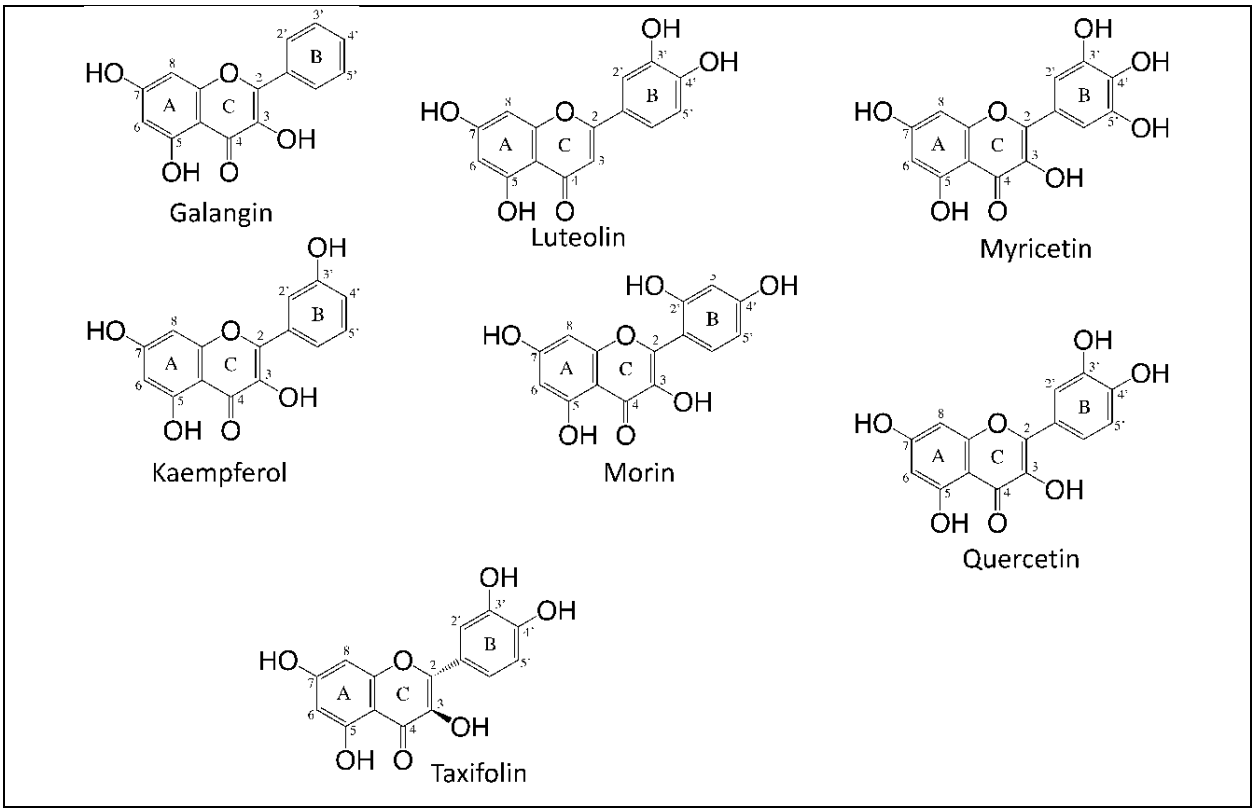

Figure 3. 1. Chemical structures of tested flavonoids

3.2 Methods and Materials

3.2.1 Chemicals

M13mp18 single-stranded DNA (250 µg/mL) was obtained from New England BioLabs, Inc. (Ipswich, MA). 2, 2-Diphenyl-1-picrylhydrazyl (1, 1-Diphenyl-2-picrylhydrazyl radical, 2, 2-Diphenyl-1- "2, 4, 6-trinitrophenyl" hydrazyl, DPPH) was purchased from Sigma-Aldrich (St. Louis, MO) and prepared by dissolving in 100% ethanol. Copper chloride was obtained from Sigma-Aldrich.

The seven flavonoids were obtained from Tokyo Sugar Refining Co., Ltd. (Tokyo, Japan). 100 µM stock solutions of each flavonoid were prepared under sterilizing conditions by using dimethyl sulfoxide (DMSO) as the solvent (Thermo Fisher Scientific, Inc., Waltham, MA). The chemicals were chosen according to the number of hydroxyl groups attached to ring B in the flavonoids (Fig.

1). **Galangin** is the only flavonoid without any OH groups attached to B ring. **Kaempferol** has only one OH group on the targeted ring (phenyl ring). **Quercetin, taxifolin, and luteolin** have two OH groups (Catechol). **Myricetin** has three OH groups on ring B (Pyrogallol), **morin** was used to test the discern of the effect of the presentence of two hydroxyl groups on the B ring in non-catechol structure in presence of cupric ions. The chemical structures were drawn with ChemDraw 19.0 (Perkin Elmer, Waltham, MA). Molecular electrostatic potential maps (MEP-MAPS) was used to study polarity. MEP-MAPS was computed using Gauss with software, the calculation was performed with Gaussian 98 program package

3.2.3 DNA scission analysis with electrophoresis

In order to investigate the capability of cupric and flavonoid complex interactions to induce DNA damage, master mix reactions were used in a total volume of 20 µL in Eppendorf tubes; we used various concentrations of each flavonoid (1, 10, 100 and 1000 µM) in this reaction which contains 1.5 ng/µL single-stranded DNA, 10 mM Tris-HCl, 0.2 mM $CuCl_2$ and water. The reactions with various concentrations were then incubated for one hour at different temperatures (4, 20 and 37°C).

Electrophoresis was carried out at 100 V for one hour using 1% agarose and stained with ethidium bromide with 1X Tris-acetate-EDTA buffer. Reaction solutions were mixed with 6X loading dye containing 15% Ficoll, 10% glycerol, 0.25% Bromophenol blue and xylene cyanol in water. A measurement of the intact DNA density was conducted using Image Lab software with the ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA). The fraction of intact DNA was calculated by dividing the amount of circular DNA by the flavonoid by the average of the corresponding control group. The IC_{50} value was defined as the concentration of flavonoids required to induce DNA damage in 50% of the intact DNA. Three independent experiments were carried out.

3.2.4 Absorption spectrum analysis

Flavonoids have aromatic conjugated systems that absorb UV-Vis light. The ultraviolet–visible (UV–VIS) absorption spectrum can be measured with UV-Vis detectors. Flavonoids have two absorption bands in UV spectra areas. The first band (band I), and second band (band II), are both associated with the ring structures, arising from B ring and A ring of respectively. Band I is thought to arise from the cinnamoyl system, with absorbance between 300-400 nm, while band II exists between 240-285 nm.

The absorption spectra of the different ratios of the flavonoid copper complex were determined by spectrophotometric analysis. The cupric ions' ability to chelate flavonoids was measured by observing the absorption activity of 2 µL of reaction mixtures containing different ratios of 100 µM cupric ion solutions and 100 µM solutions of each flavonoid. Four mixtures of flavonoid and cupric ion solutions were created with molar ratios of 1:0, 1:2, 1:1 and 2:1 respectively. Absorbance values of these mixtures were measured with a Nanodrop Spectrophotometer (Thermo Fisher, Inc.) from 230 to 500 nm. The reduction ratio of the second band peak was used for the assessment of cupric ion chelating capacity. Each data point was produced from the means of three independent experiments.

3.2.5 DPPH Antioxidant Assay

The 1 mM DDPH stock solution was prepared by dissolving 3.9 mg of DPPH in 10 mL of ethanol. The working solution for the DPPH assay was prepared by diluting in ethanol to 100 µM. Flavonoids at 50, 500 and 5000 µM were prepared in DMSO as stock solutions. 5 µL of each of these dilutions were used to prepare 100 µL of three solutions with concentrations of 1, 10, and 100 µM respectively. DMSO was used as a control. The scavenging ability of each flavonoid compound was conducted as previously described [26]. In which we use a 96–well plate, the

radical scavenging activity of each flavonoid was measured by mixing 20 µL of 1, 10 and 100 µM of flavonoid with 100 µL of DPPH working solution, 80 µL of 100% ethanol, and one of two concentrations of $CuCl_2$:10 or 30 µM. The solutions were mixed vigorously and kept in darkness for 30 minutes. 16 µL of DMSO were added to 4 µL of DPPH working solution as a blank control. Next, the absorbance was measured at 490 nm using the ELISA Benchmark microplate Reader (Bio-Rad Laboratories, Inc, CA, USA). DPPH becomes discolored when it deprotonates a flavonoid hydroxyl group, this color change allows the ELISA Microplate Reader to measure the absorbance of the solutions. Three independent experiments were carried out. The flavonoid concentrations antioxidant capacity was plotted to obtain IC50 at 490 nm, The IC_{50} value was defined as the concentration of flavonoids required to decrease the activity of DPPH by 50%.

3.2.6 Total antioxidant capacity (TAC) assays

The TAC assay is colorimetric; it measures the total antioxidant capacity based on reduction of Cu^{2+} to Cu^{1+}. The assay reaction starts by adding 1.2 µL of Cu^{2+} working solution (Catalog Number MAK187A) and add 58.8 µL of assay diluent (Catalog Number MAK187B) to a tube to create a 1:50 part dilution and set out to reach room temperature. Into different wells on 96-well plates we added 2 µL of 1:50 diluted Cu^{2+} solution into each well, and 7µL of each flavonoid, and mixed each solution. The 96 well plates were incubated for 90 minutes at different temperatures; in the dark at room temperature, 37^0C, and 4^0C. The absorbance at 570 nm was then measured by Nanodrop.

3.2.7 Statistical analysis

Graph Prism 6 (Graph Pad Software, Inc. La Jolla, CA, and USA) was used to calculate statistical significance with a one-way ANOVA test. Statistical significance was indicated by $P<0.05$. Error bars indicate the standard error of the mean.

3.3 Results

3.3.1 DNA damage assessment

DNA damage was induced with temperature and flavonoid concentration-dependent manners (Fig. 2). Fig. 3 shows that myricetin, quercetin, Kaempferol, and luteolin were the flavonoids most potent at inducing ssDNA damage, decreasing in potency in the order in which they are listed. Galangin, morin, and taxifolin demonstrated minor DNA damage (Fig. 4).

In order to investigate the impact of hydroxyl residue number on DNA damage, galangin, Kaempferol, quercetin, and myricetin were compared first. Myricetin has a pyrogallol structure and induced total DNA degradation with a concentration of 10 μM at 4°C. On the other hand, quercetin has a catechol structure on the B ring and needs a concentration of 100 μM at 37°C to achieve the same result. Comparatively, Kaempferol with its phenolic ring on the B ring shows DNA damage a concentration of 1000 μM at room temperature. Galangin has no hydroxyl groups on B ring and did not induce significant DNA damage under any conditions. Therefore, the number of hydroxyl residues on ring B appears to be a determining factor for DNA damage induced by flavonoids. However, morin has non catecholic structure of two OH on the B ring and induced non-significantly less DNA damage compared to quercetin. Hence, the location of the hydroxyl groups is also important for DNA damage.

In order to compare the effects of hydroxyl groups in position 3 on the C ring, luteolin and quercetin were compared. Luteolin and quercetin showed similar DNA damage induction at 4°C and 20°C. However, at 37°C quercetin produced more DNA damage than luteolin. Therefore, the hydroxyl residue in position 3 on C ring may be involved in temperature dependent radical formation. Taxifolin has two stereocenters on the C ring while quercetin has an achiral C ring.

Because taxifolin did not induce any DNA damage, it can be concluded that stereocenters on the C ring inhibit DNA scissions even if the B ring has two hydroxyl residues.

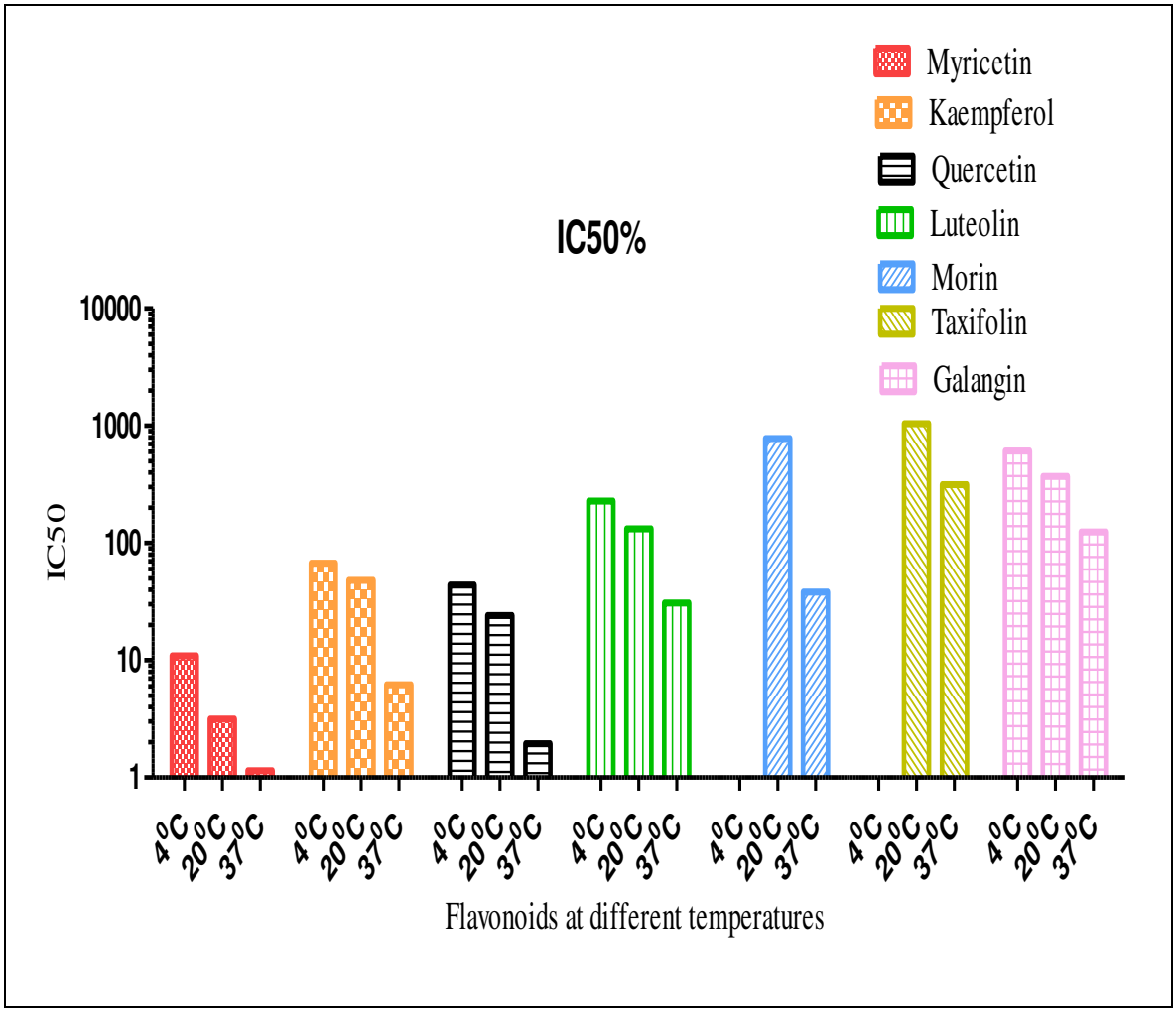

Figure 3. 2. 50% ssDNA damage induced by flavonoids in concentration and temperature dependent.

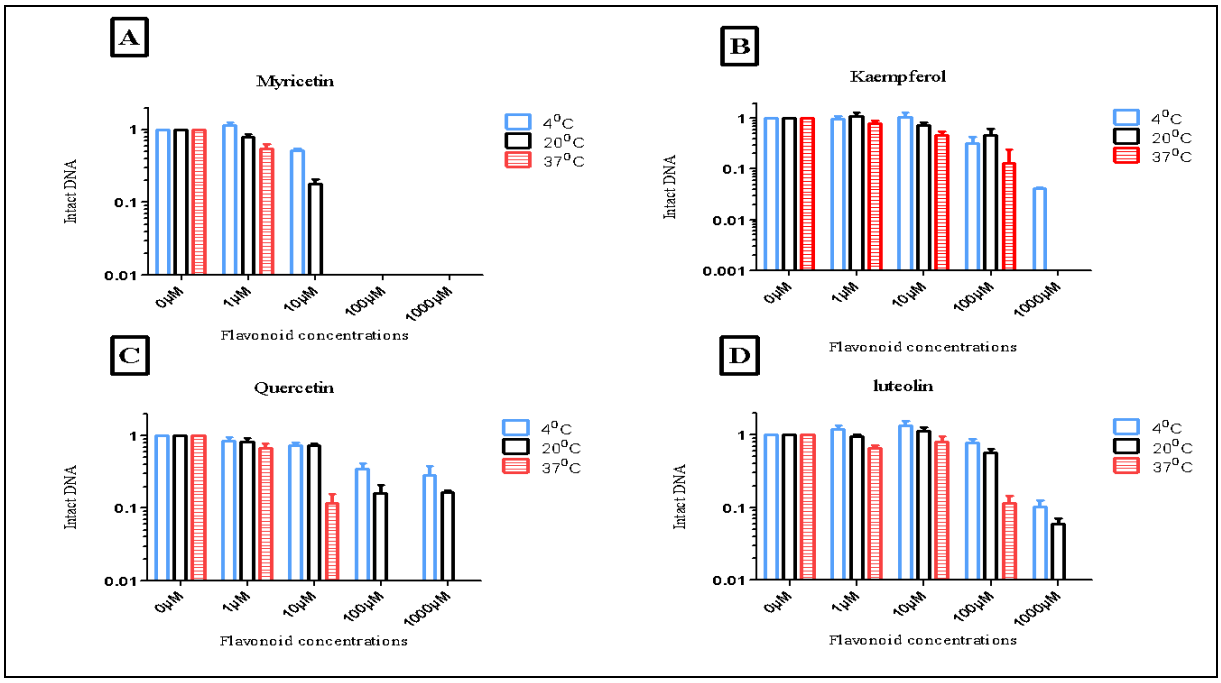

Figure 3. 3. Fraction of intact DNA after 1-h reaction at 4, 20 and 37˚C for flavonoids showed ssDNA damage which are (A) Myricetin, (B) Kaempferol, (C) Quercetin, (D) Luteolin. Error bars indicate the standard error of the mean. Three independent experiments were performed.

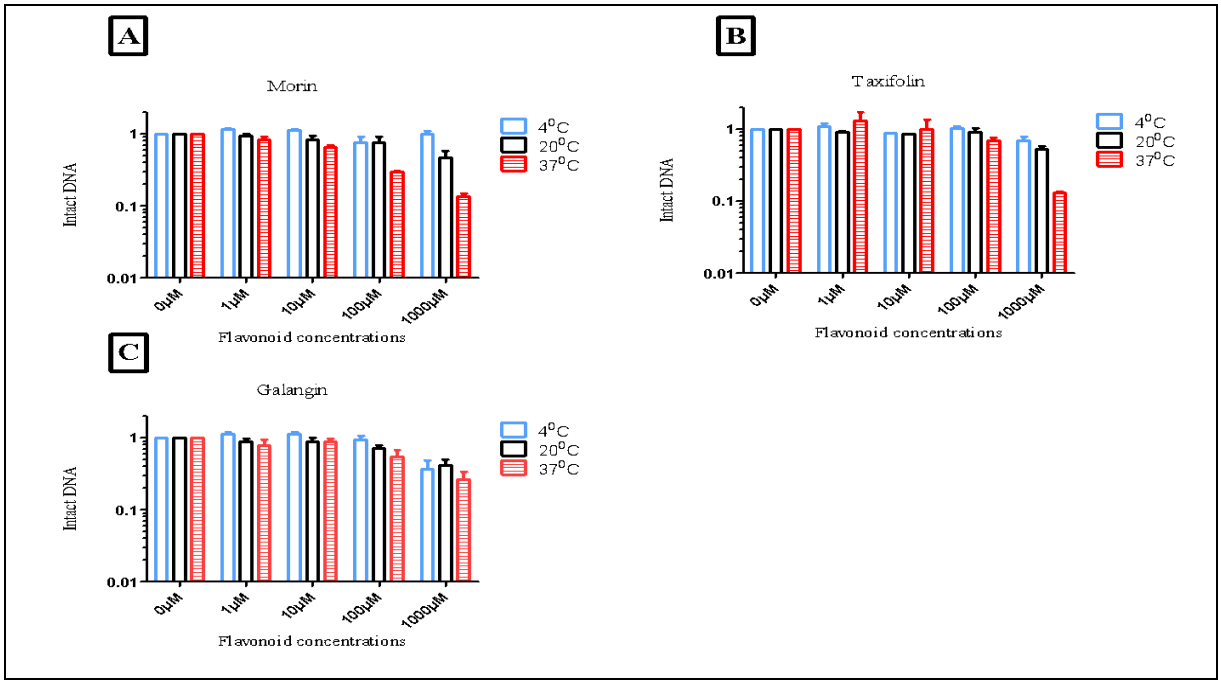

Figure3. 4. Fraction of intact DNA after 1-h reaction at 4, 20 and 37˚C for flavonoids which did not showed ssDNA damage which are: (A) Morin, (B) Taxifolin, (C) Galangin. Error bars indicate the standard error of the mean. Three independent experiments were performed.

3.3.2 Cupric chelating

Comparing the change of the two absorption bands of flavonoids in the presence of cupric ions was investigated with a spectrometer to assess the cupric ion chelating capacity of flavonoids. $CuCl_2$ was mixed with flavonoids in 1:2, 1:1, and 2:1 ratios. All seven flavonoids exhibited two major absorption bands represented in two absorption spectra peaks, ranging between ~250 and 370 nm wavelengths. The first peak showed at ~245 nm and shifted left in the presence of cupric ions and the second peak appeared at ~385 nm. The more the concentrations of cupric ions were increased, the greater reduction in the second peak observed. The reduction ratio of the second peak was used to assess the ability of flavonoids to chelate the cupric ions. Fig. *5* demonstrates the difference in chelating capacity among targeted flavonoids. Kaempferol had the greatest cupric chelating represented by the greatest reduction of the second peak from 0.194 to 0.053 at 375 nm, and then quercetin and galangin which displayed high reduction in the second peak from 0.158 to 0.045 at 380 nm. Flavonoids that didn't show any DNA breaks such as taxifolin and morin displayed lower second absorbance peak reductions in the presence of cupric ions. Taxifolin demonstrated the lowest reduction. Surprisingly, the flavonoids that have pyrogallol structures, like myricetin, displayed lower cupric chelating than other tested flavonoids. Flavonoids with catechol structures such as quercetin and luteolin showed higher capability in chelating capacity compared to the other flavonoids such as myricetin which showed highest ssDNA damage.

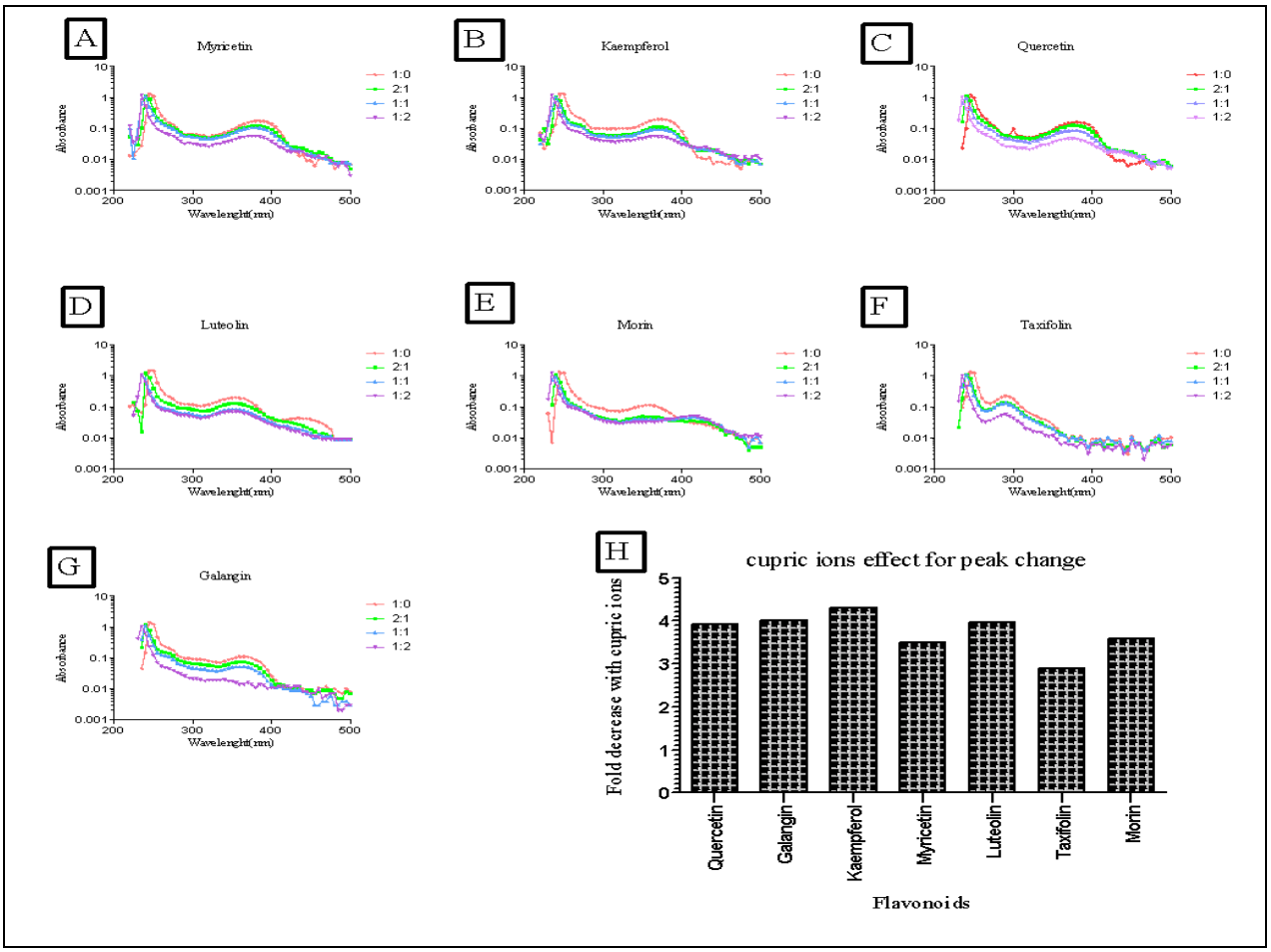

Figure3. 5. Absorption spectrum changes of flavonoids in the presence of cupric ions tested at 4 different ratios of flavonoid to cupric ions (1:2, 1:1, and 2:1 ratios). Red lines indicate the absorbance of flavonoid only, and the green line indicates the ratio of two flavonoids to one copper, the blue line indicates equal ratios of flavonoids and copper, and the purple line indicates one flavonoid to two coppers ratio. (A) Myricetin, (B) Kaempferol, (C) Quercetin, (D) Luteolin, (E) Morin, (F) Taxifolin, (G) Galangin, (H) Effect of cupric ion on peak changes is displayed.

3.3.3 DPPH free radicals scavenging capacity

There are different methods to measure free radical scavenging capacity [27, 28]. The DPPH method is a commonly used assay [29]. DPPH can generate free radicals in ethanolic solutions; when flavonoids are added to this solution, it decolorizes the purple color of DPPH [30]. The ability of flavonoids to act as antioxidants to scavenge DPPH radicals is related to their hydrogen donating ability. Fig. *6* presents the scavenging capacity of flavonoids measured by DPPH assay. The scavenging ability of flavonoids was measured at different concentrations (1,

10, and 100μM), and each flavonoid concentration was tested at two different cupric ion concentrations (10 and 30 μM).

Flavonoids having catechol structures with non-steric configurations that cause DNA damage such as quercetin exhibited the highest scavenging activity, followed by flavonoids having a phenolic structure at the B ring (Kaempferol). Among all flavonoids, myricetin with pyrogallol structures showed weaker activity than other structures mentioned above. Although this result was not compatible with the results of DNA formation with the flavonoid pyrogallol structure, they point to a particular relationship between the scavenging activity of flavonoids having phenolic and catecholic structures (Kaempferol, quercetin) at the B ring and inducing DNA damage.

Free radical scavenging activity could be attributed to the specific structure of flavonoids, not the number of OH. A previous study demonstrated that the free radical scavenging potential of flavonoids closely depends on the particular substitution pattern of free hydroxyl groups on the flavonoid skeleton; the highly active flavonoids possess a 3', 4'-dihydroxy occupied B ring and/or 3-OH group [31]. In other flavonoids possessing catechol structures such as luteolin and taxifolin, a slight scavenging activity only at high concentration of flavonoids and copper was observed.

Besides the effect of flavonoid structure, the scavenging capacity of flavonoids seems to be concentration dependent for both copper and flavonoid. Myricetin showed small scavenging ability at high concentration100 μM, and at 30μM cupric ion concentrations. Flavonoids with catechol structures such as taxifolin and luteolin showed slight scavenging ability at high copper and flavonoid concentrations.

phenolic compounds such as Kaempferol, the scavenging capability was higher in a low cupric ion concentration of 10 μM, while flavonoids with no hydroxyl group in the B ring such as galangin showed slightly more scavenging activity at 10 μM compared to 30 μM concentration of

cupric ions. Overall, the specific catechol structure of (3', 4'-dihydroxy occupied B ring) and 3-OH group at C ring of the flavonoid results in the greatest free radical scavenging capability, dependent on both flavonoid and cupric ion concentrations. However, flavonoids with phenolic structures affected by the copper concentration, for example, Kaempferol which displays greater scavenging at lower cupric ions concentration. On contrast, the flavonoid possesses pyrogallol structure has shown that its scavenging activity is affected by the flavonoid concentrations not cupric concentrations, Myricetin shows slight scavenging activity only at low concentration of flavonoids.

3.3. 4 Total antioxidant capacity (TAC) assay

This assay showed that flavonoids having catecholic structures with non-steric configuration and that caused DNA damage such as quercetin exhibited the highest scavenging activity (Fig. *7*). However, in contrast to the DPPH assay, it showed that myricetin (pyrogallol structure) produced more copper reduction than Kaempferol (phenolic structure). Surprisingly, flavonoids that induced minor DNA breaks such as morin and taxifolin displayed more copper reduction at all tested temperatures than Kaempferol, which displayed high DNA damage.

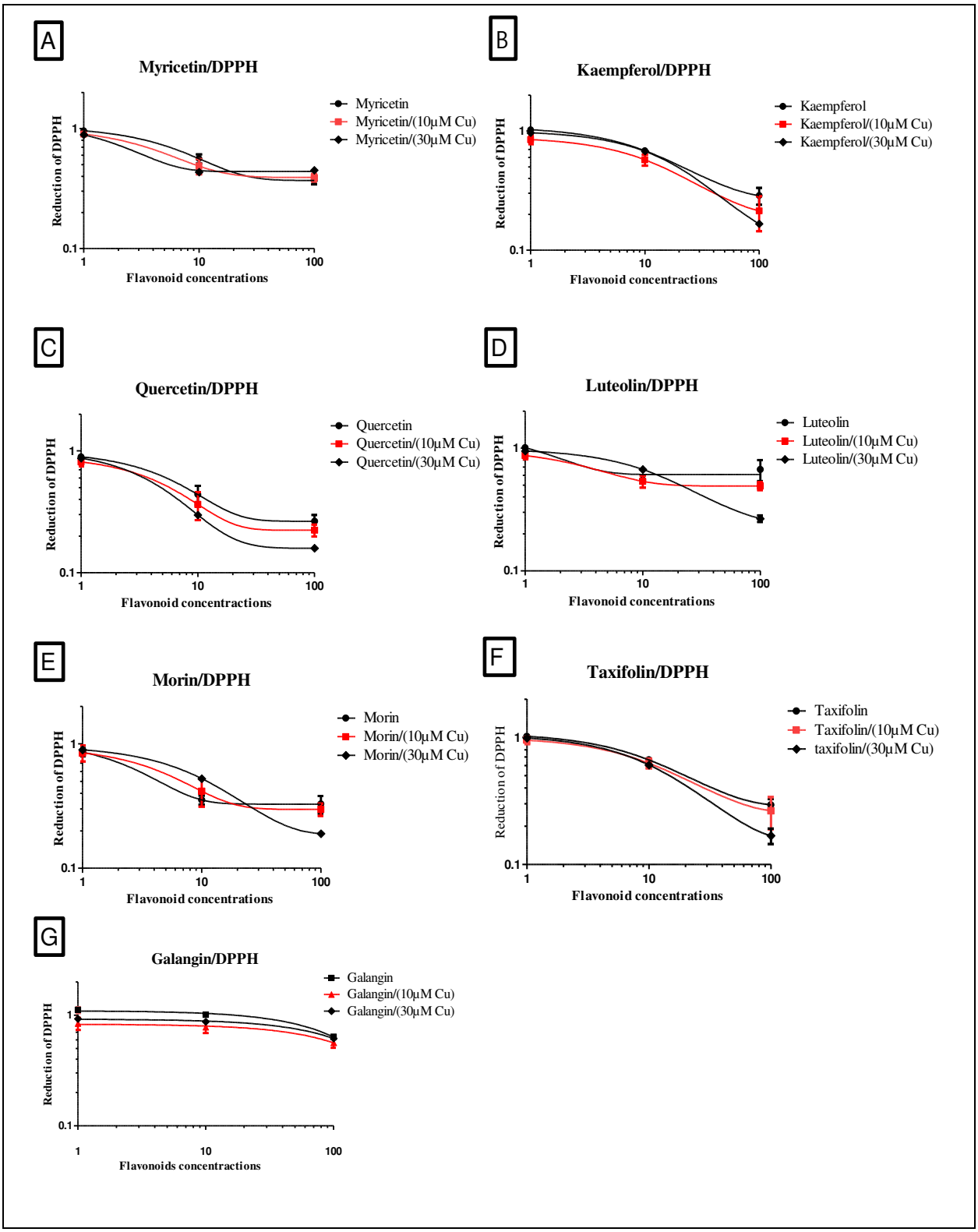

Figure 3. 6. DPPH radical scavenging capacity of the seven flavonoids at 10 and 30 µM $CuCl_2$. Data represent means and standard error of the means. ($P < 0.05$). (A) Myricetin, (B) Kaempferol, (C) Quercetin, (D) Luteolin, (E) Morin, (F)Taxifolin, (G) Galangin.

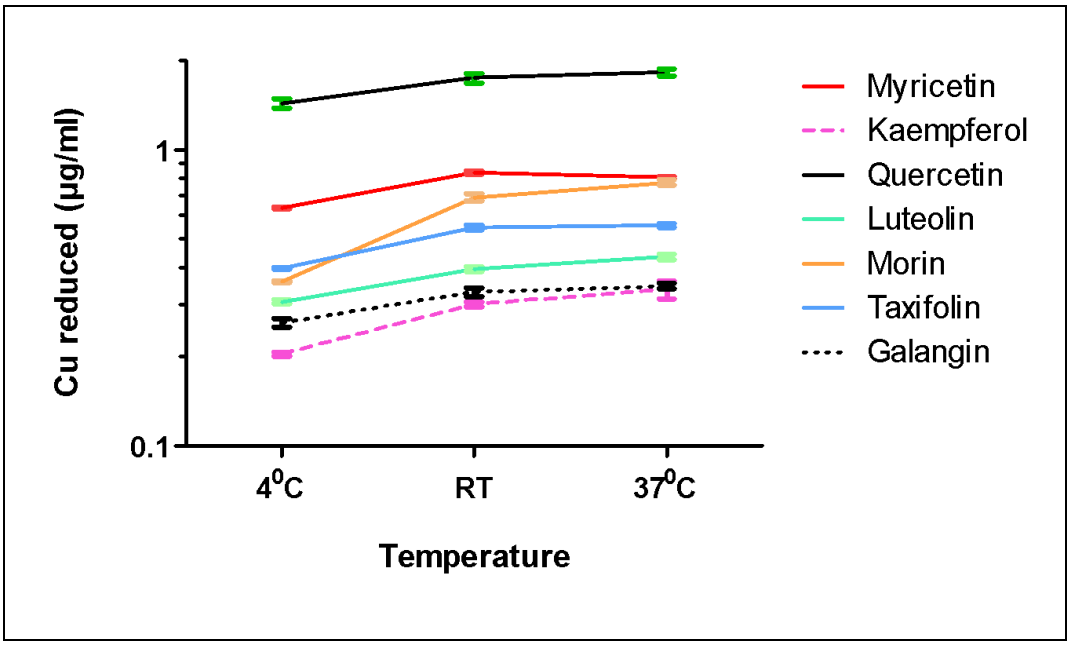

Figure3. 7. Total antioxidant capacity (TAC) assay at different temperatures.

Molecular Polarity

To get deeper insight into the effect of hydroxyl group distribution of flavonoids on inducing ssDNA damage in the presence of cupric ions, a computational theoretical comparison of hydroxyl group polarity on several flavonoids was performed by MEP maps.

The low polarity at C ring seem to be important, it was found that the flavonoids that caused highest ssDNA damage such as; myricetin, kaempferol and quercetin, possess less polarity in position 3 and 4 at the C ring (Black box at Fig. 8) compared to control group (naringenin and hesperetin), and compared to those flavonoids that hadn't shown ssDNA damage such as morin and taxifolin with the exception of galangin (Green box at Fig. 8).

Furthermore, the low polarity at position 4 of the B ring appeared to be crucial in inducing DNA damage. For instance, quercetin (show ssDNA damage) and taxifolin (no ssDNA damage) have same number and distribution of OH groups. Quercetin has mild polarity at position 4 of the B ring while taxifolin has low polarity at the same site. The presence of the stereocenters

configuration in taxifolin alters the OH polarity at position 4 of the B ring from mild to low polarity (showed inside the red box in Fig. 8), this change in polarity of OH group at position 4 of ring B may have an effect on the properties of taxifolin and its ability to cause DNA damage.

In general, flavonoids that have the low polarity at both positions together; the less polarity in OH groups at position 3, 4 at the C ring, and at position 4 at the B ring showed more ability to break DNA, it appears that the change of polarity in those sites lead to changes in the ability of flavonoids to induce DNA damage. Moreover, presence of the low polarity of the OH in one position is not enough to induce DNA damage. For example, morin did not show ssDNA damage, it has low polarity at position 4 of the B ring which seems important to induce DNA damage. And on the contrary, morin has high polarity at C ring, it seems that the change of the polarity at C ring affect the ability of morin to induce DNA damage, and also the presence of the low polarity in one site is not enough to enhance the flavonoids ability to induce DNA damage.

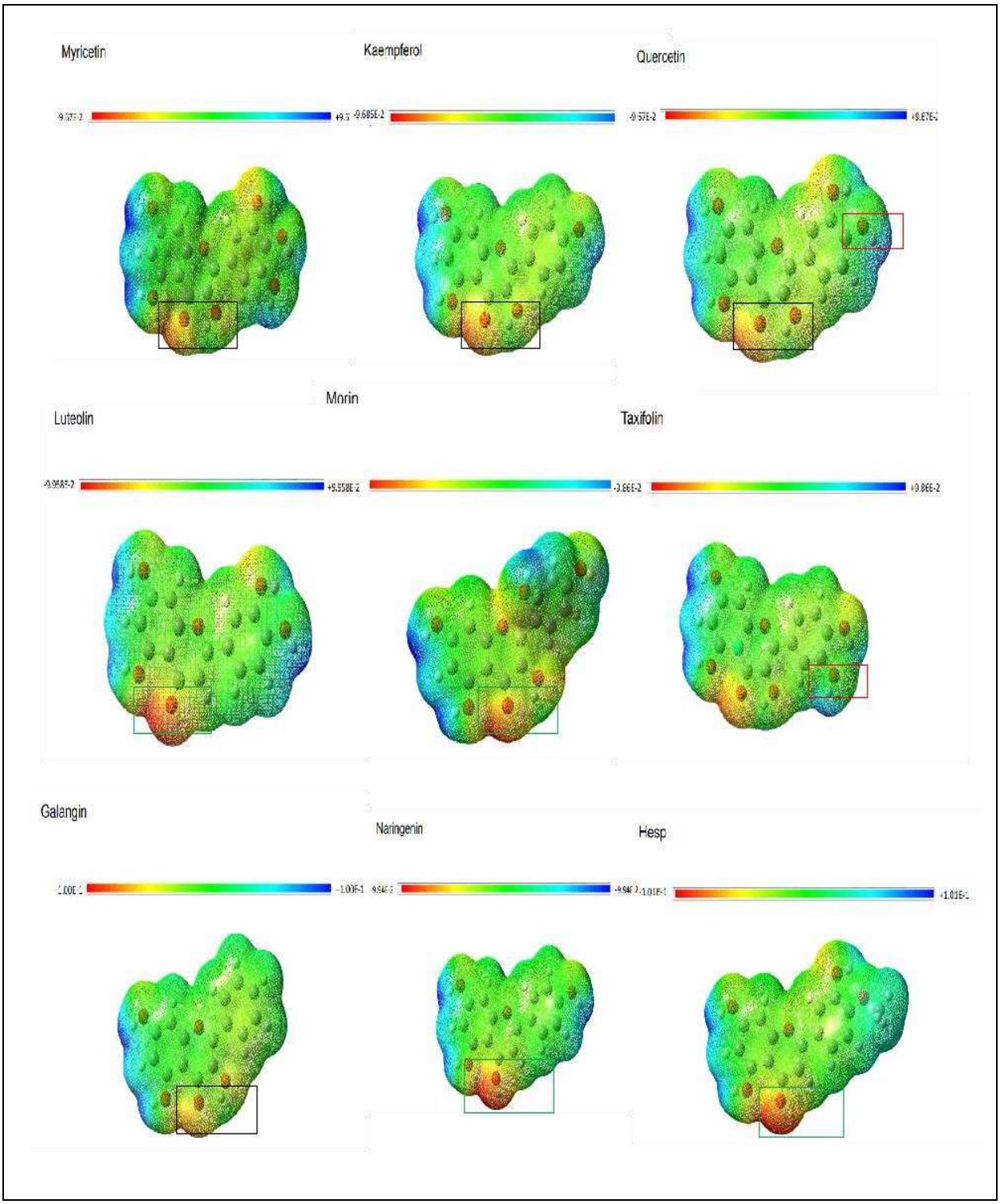

Figure.3. 8. Theoretical polarity difference between hydroxyl groups of tested flavonoids. Red box refers to the polarity of OH at position 4 of the B ring, black box indicates the low polarity at the C ring, and the green box refers to OH which has high polarity. The computational theoretical

comparison of hydroxyl groups on tested flavonoids was studied by molecular electrostatic potential maps.

3.4. Discussion

Previous studies with quercetin have suggested that flavonoids with a hydroxyl group at position 3 on the C ring can induce high DNA damage in the presence of cupric ions [22]. First, this study focused on the flavonoids with a hydroxyl group at position 3 on the C ring and different numbers of hydroxyl residues on ring B. Then, flavonoids with catechol structures on ring B like quercetin, taxifolin and luteolin were studied. The DNA damage results showed that myricetin, which has a pyrogallol structure on the B ring, induced ssDNA damage most efficiently of the tested flavonoids (Fig. 3.A). This can be explained by the fact that myricetin can form a more stable complex with cupric ions, allowing this complex to produce radicals via the Fenton reaction, which interacts with DNA leading to DNA damage [32, 33]. This is also in agreement with a previous study that showed the ability of myricetin to cause DNA damage in the presence of cupric ions, and the switch from an antioxidant to a prooxidant activity depends on myricetin dose, stability of metal-flavonoid complex and number of hydroxyl groups [32]

Reduced DNA scission activity of tested flavonoids compared to quercetin was observed for several reasons. Luteolin and Kaempferol, representative of flavonoids with zero or one hydroxyl group on the B ring respectively, showed reduced DNA scission activity (Fig. 3 B&D). Taxifolin represents an example to study the effect of stereocenters on DNA scission activity. Taxifolin showed reduction in DNA scission activity (Fig. 4). The presence of a hydroxyl groups on position 3' and 4' at ring B, as seen in Luteolin, presented as a unique temperature dependent reduction of DNA scission (Fig. 2). This result was in strong agreement with previous outcomes

that suggested the importance of hydroxyl group in the 3'- and 4'-positions on the B ring on inducing DNA damage at high temperatures [22].

Although Kaempferol and quercetin demonstrated a positive relationship between DNA scission and chelating/radical scavenging activity, myricetin, the flavonoid with the strongest DNA scission capacity, did not exhibit the strongest cupric ion chelating capacity in our assay (Fig. 6.A). In order to understand the molecular mechanisms of DNA scission with metals, additional polarity analysis was carried out. It showed that flavonoids that induce high DNA damage have very low polarity in position 3 on the C ring compared to the other flavonoids that did not cause DNA damage. These latter flavonoids had relatively mild polarity in position 3 on the C ring (Fig. 8).

Based on the obtained results, we may conclude that the flavonoids with OH group at position 3 at C ring, and have the low polarity at specific OH groups; at positions 3 and 4 on the C ring, and at position 4 on the B ring seem to produce more ssDNA damage than other flavonoids. The polarity at these sites seem to be most critical to induce DNA damage. This agrees with a previous study, which showed that flavonoids with OH groups at position 3 of the C ring are less polar than other flavonoids [25].

In conclusion, this study showed that myricetin, a flavonoid with a pyrogallol structure, demonstrated the strongest DNA scission capacity among the tested flavonoids, similar to quercetin. This activity may not be solely associated with cupric ion chelating capacity - the polarity of molecules may play a role as well. Additionally, it is possible that stereo-chemical structure may modify DNA scission capacity. Finally, the hydroxyl residue in position 3 on the C ring may be central to temperature dependent DNA scission capacity.

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CHAPTER 4/PRELIMINARILY STUDIES OF COPPER AND FLAVONOIDS INDUCED CYTOTOXICITY TO CELLS WITH DIFFERENT P53 STATUS

4.1 Introduction

Normal cells can transform to cancer cells when some changes happen leading to uncontrolled growth and division of cells. According to the Centers for Disease Control and Prevention (CDC), cancer is considered the second leading cause of death in the United States of America and in many other countries [1, 2]. There are many different causes of cancer, including genetic mutations, oxidative stress, and defects in different cellular pathways that lead to the impairment of cell functions such as apoptosis.

Many studies attempt to use natural, dietary or synthetic agents to limit or stop cancer progression. Scientists have tried to test the effect of these compounds in a variety of different ways; this includes inhibition of new cancer formation and blockage of different cell signaling pathways to prevent the overgrowth of cells. Several results emphasize to the advantages of long-term consumption of natural dietary food such as fruits and vegetables, and foods rich in antioxidants that help to prevent chronic diseases and cancer progression [3-6].

Last 20 years a particularly interesting in studying cancer having p53 mutations. This type of cancer is the most common in human where half of human cancers carry p53 gene mutations [7, 8]. There are numerous anticipated mechanisms by which flavonoids and metal ions interaction can induce anticancer activity [9]. Some flavonoids can inhibit cell proliferation via activation of signal transduction pathways, and cell cycle arrest, such as hesperetin which stimulate the tumor suppressor protein P53 in breast cancer cells, and arrested cancer cells at in the phase G2-M [10-12].

Generally, flavonoids can act as antioxidants and directly protect cells against DNA oxidative damage. Flavonoids could protect against oxidative damage by inhibiting enzymes such as xanthine oxidase which is responsible for the generation of superoxide anion. Moreover, flavonoids can protect against the oxidative damage via stimulating the gene expressions involved in carcinogenic progress. Apigenin, a flavonoid belonging to the flavone class. Apigenin has shown ability to stimulate the translation of p53 leading to increase the synthesis of nascent p53 [8, 13]. Additionally, several papers have reported that flavonoids can inhibit the growth of different cancerous cell lines. For instance, quercetin has shown anti-proliferative effect in a dose dependency manner in colon cancer cells Caco-2 and HT-29, and in rat non-transformed intestinal crypt cells [14, 15].

Although most flavonoids are known for their antioxidant properties, under different circumstances these flavonoids can act as prooxidant and induce radicals. One of these conditions is the presence of metal ions such as cupric or ferrous ions. Previous studies have shown that flavonoids in the presence of metal ions display greater anticancer effects than parent flavonoid [16]. This can be attributed to their prooxidant properties and better DNA intercalation [17, 18]. Peculiarly, the prooxidant properties appear mainly in cancer cells, but more research is needed to understand the mechanism by which some flavonoids act as efficient anticancer in presence of metal ions in cancer cells.

In this work we test the effects of flavonoids in the presence of cupric ions because of the important biological role of the copper in metalloenzyme. Copper found in high levels in some diseases such as Alzheimer in which significant raise of copper has been reported in amyloid plaques. The treatment attempt based on using flavonoid demonstrated that flavonoids are capable

to chelate free copper and consequently reduced the symptoms [19]. Moreover, previous literatures documented that copper levels are significantly higher in cancer patients from two to three folds than healthy subjects [20, 21].

In presence of copper, the antioxidant properties of flavonoids will switch to prooxidant activity. The potential anticancer properties of flavonoids are attributed to their moderate prooxidant activity, which can inhibit or kill cancer cells via increase the formation of free radical. In this study we investigated the cellular toxicity of flavonoids that showed *in vitro* ssDNA damage in presence of cupric ions. We tried to identify which flavonoids could target the P53 mutated cells in presence of cupric ions in order to recognize which flavonoid could provide a promising strategy for development of anticancer drugs.

4.2 Methods and Materials

4.2.1 Cell lines and culture condition

Human B-lymphoblastoid cell lines used to understand the role of p53 because they differ in their p53 functional status [27]. TK6 (wild type), WTK1 (mutated P53), and NH32 (knockout P53) cells were kindly provided by Dr. Howard Liber. Cells were maintained in RPMI medium (Sigma, St Louis MO) with 10% FBS (Gemini Bio-Products, West Sacramento, CA), and 1% of Penicillin and Streptomycin, and Fungizone mixture (Invitrogen).

Cells were cultured in 25cm^2 flask with appropriate cell number around 100,000 cells/ml media per flask, in and grown with 5% CO_2 in an incubator at 37 ^{0}C. To investigate the growth of cells in presence of cupric ions. Copper chloride dehydrate was purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany).

4.2.2 Chemicals

Seven flavonoids were obtained from Tokyo Sugar Refining Co., Ltd. (Tokyo, Japan). 100 µM stocks solutions of all flavonoids were prepared under sterilizing conditions by using dimethyl sulfoxide (DMSO) as solvent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The chemicals were chosen according to the number of hydroxyl groups attached to ring B in the flavonoids. Galangin was the only flavonoid without any OH group attached to B ring. Kaempferol has only one OH in the targeted ring (phenyl ring). Quercetin, taxifolin, and luteolin have two OH groups (Catechol). Myricetin has three OH groups in ring B (Pyrogallol), Morin was used to testing the presentence of two hydroxyl groups in B ring in non-catechol structure.

4.2.3 Growth inhibition assay

Previous papers showed that flavonoids have either cytotoxic or cytostatic effects to cancer cells [1,10,15]. Flavonoids use different mechanism to inhibit cell growth, by disrupting various cell functions, division or cell signaling, which often results in apoptotic cell death. This interruption will result in the drop of cell number.

In order to investigate the effect of the structural diversity of hydroxyl group at B ring of tested flavonoids on p53 functional status in the presence of cupric ions, we used assay to measure the viability cell number in time manner. Generally, the ability of flavonoids in presence of cupric ions to inhibit cell growth indicate to its anticancer activity.

The growth inhibition assay of the three cell lines that differ in their p53 functional status were performed using two different concentration of each flavonoid 1 µM and 10 µM. The assay was performed in absence or presence of 1 µM $CuCl_2$, this dose was chosen after testing Cu doses range from 0 to 25µM to avoid cell death via copper toxicity (Fig. 1). 100.000 cells per ml were cultured. Cells were allowed to acclimatize at 37 ^{0}C in incubator with 5% carbon dioxide for 24 hours. The concentration of the cells in 1 ml suspension media was determined by coulter counter

every day for four days 24, 48, 72, 96 hours. In growth assay in the absence of copper, generally Control cells for all experiments were treated with a volume of 0.1% (v/v) of DMSO instead of flavonoid. While in experiments in the presence of 1 µM of $CuCl_2$ with DMSO were used as a second control.

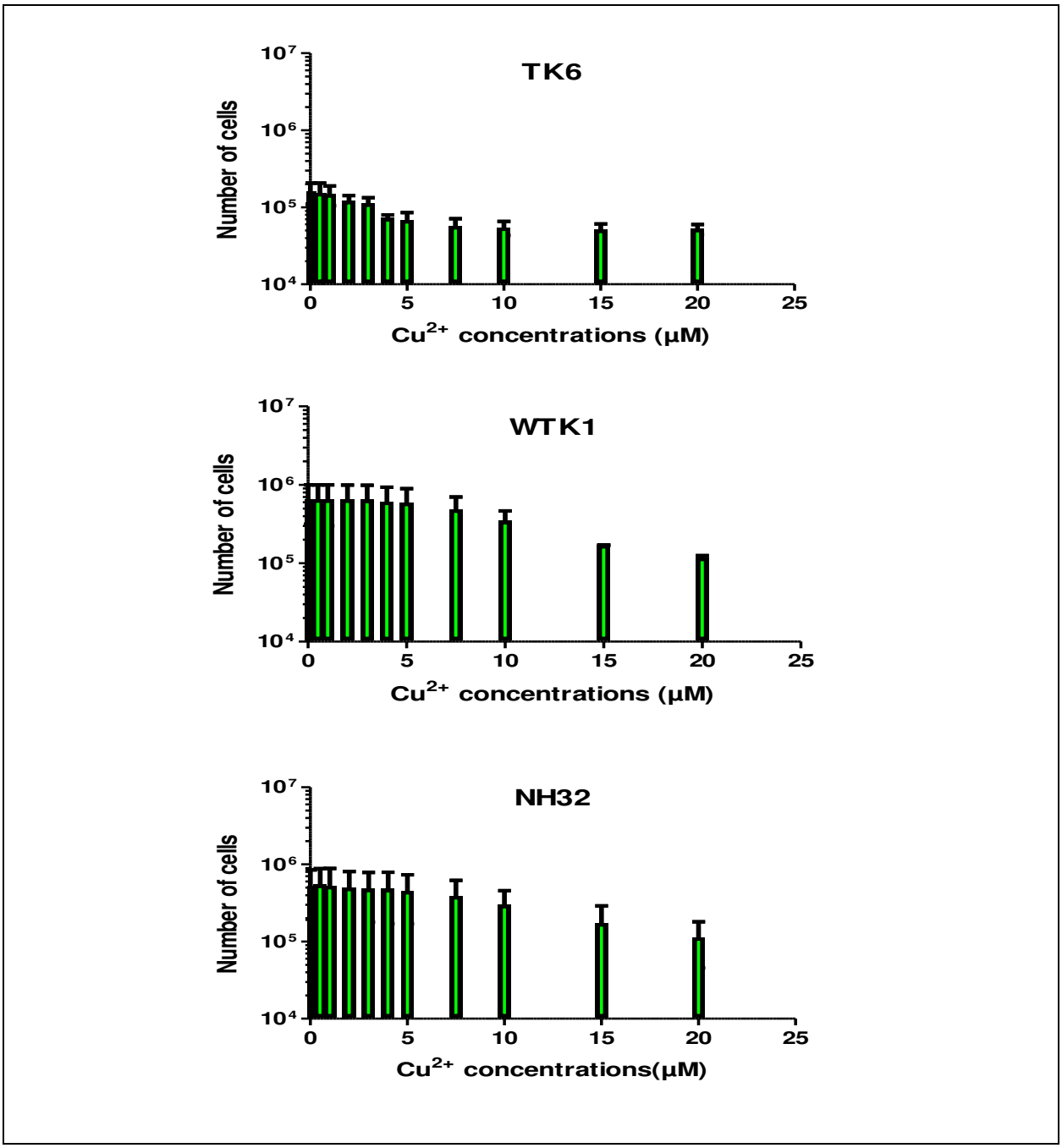

Figure. 4. 1. Toxicity of cells by copper. Error bars indicate the standard error of the mean. Three independent experiments were performed.

4.2.4 DAPI cell apoptosis assay

4′, 6-diamidino-2-phenylindole or DAPI (Sigma-Aldrich) is a fluorescent dye probe that can bind to the DNA. This dye used to detect apoptotic cells because of its ability to overpasses cell membranes of normal cells and the dye can be absorbed and apoptotic cells can be detected by the high blue fluorescence which can be seen by fluorescent microscopy.

After treat cells with 10 µM $CuCl_2$ a day before, the cells harvested after 24hrs and 48hrs from all three cells suspensions of TK6, WTK1, and NH32 cells by centrifugation at 2000 rpm for five minutes. We wash cells three times by centrifugation and breaking the pellet after discarding the media and, adding 1 ml of Methanol: Acetic acid (3:1) solution. Finally, we add 30 µl of the solution on slide and leave it to dry before adding DAPI on slide cover and investigate immediately observed at 340/380 nm of excitation wavelength by fluorescent microscopy and we count apoptosis within 100 cells.

4.2.5 Statistical Analysis

All experiments were done in triplicate. The comparison of the means value was performed using two-way analysis of variance (ANOVA) and Student's t−test with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference. Error bars indicate the standard error of the mean.

4.3. Results

To follow up with previous results, Myricetin Kaempferol, and Quercetin are the flavonoids that cause significant ssDNA damage. In cells treated with myricetin, the growth of the wild type P53 cells (TK6) was inhibited in both presence and absence of the cupric ions. The growth of mutated cells (WTK1) treated with myricetin did not showed cell growth inhibition in

presence or in absence of the cupric ions. On the other hand, myricetin showed more inhibition effect in cell growth of null P53 cells (NH32) in presence of cupric ions compared to wild type cells where the growth of TK6 was suppressed in both the presence and absence of cupric ions , it seems that the presence of the cupric ions does not increase the inhibition of the TK6 cell growth. Among all tested flavonoids only Myricetin showed mild inhibition effect on P53 knockout cells (NH32) growth in presence of 1 μM cupric ions (Fig. 2A). All other flavonoids did not show cellular toxicity in the presence of or in absence of cupric ions and seem to be that the anti-proliferation ability of these flavonoids does not enhanced in presence or absence of copper (Fig. 3).

We next investigate whether the copper toxicity induce apoptosis in absence of flavonoids. Treatment of cells by 10 μM of $CuCl_2$ result in increased apoptosis in all cells, NH32 cells affected the most where the apoptosis was 9 folds higher than untreated cells. TK6 cells also exhibit more potent apoptosis with more than 6 folds higher than cells did not exposed to copper. Although the WTK1 cells showed more apoptosis cells in absence of copper, the mutated cell showed lowest cell apoptosis than other cells in presence of copper, the apoptotic cells were only 2 folds than untreated WTK1 cells.

our results showed that myricetin in presence of the cupric ions could show minor cell growth in P53 wild type or P53 knock-out cells but not the mutated P53 cells, this could be attributed to high resistance of mutated P53 cells (WTK1).

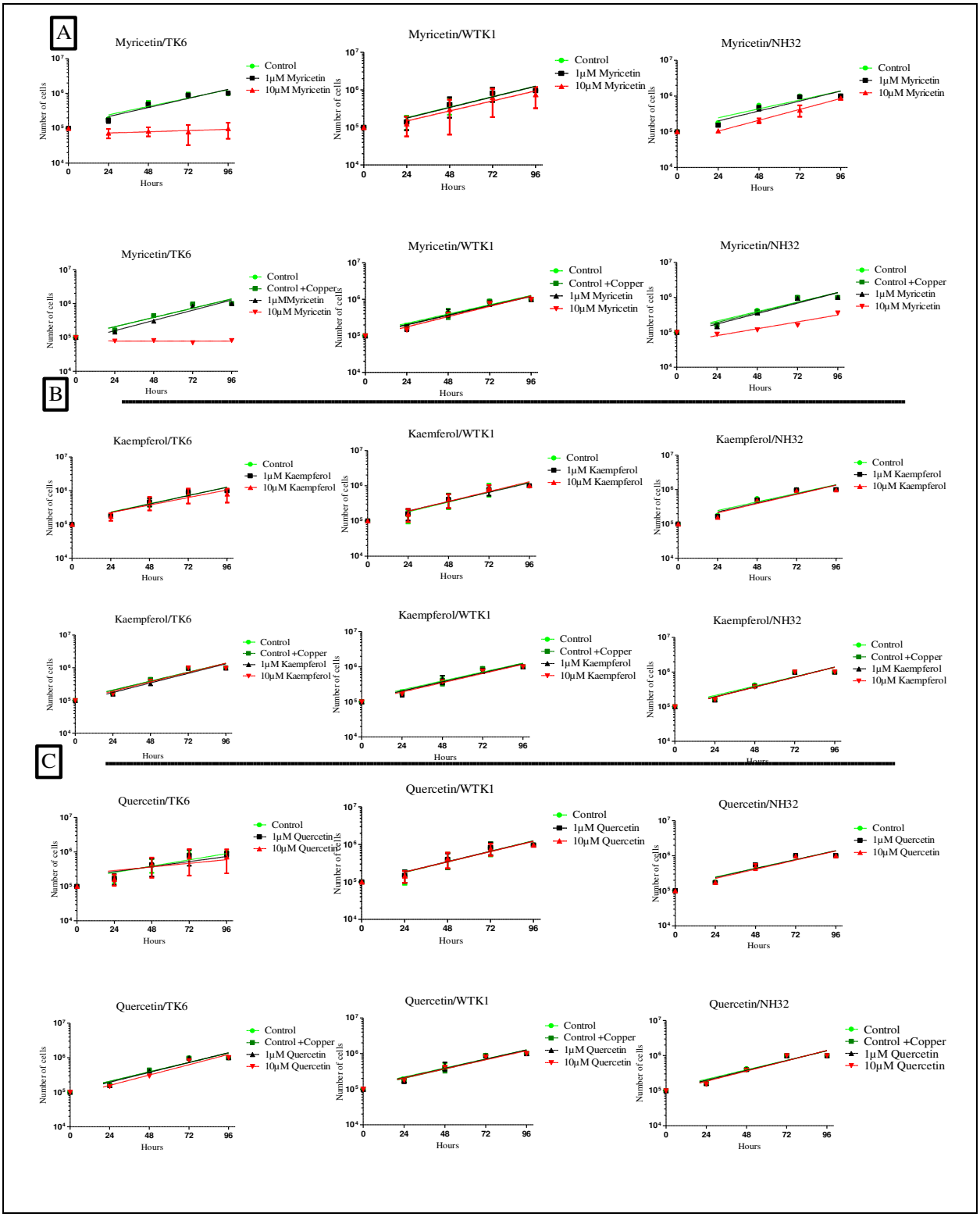

Figure.4. 2. The growth inhibition of p53 cells in different statuses by flavonoids showed highest ssDNA damage: (A) Myricetin, (B) Kaempferol, and(C) Quercetin. Error bars indicate the standard error of the mean. Three independent experiments were performed.

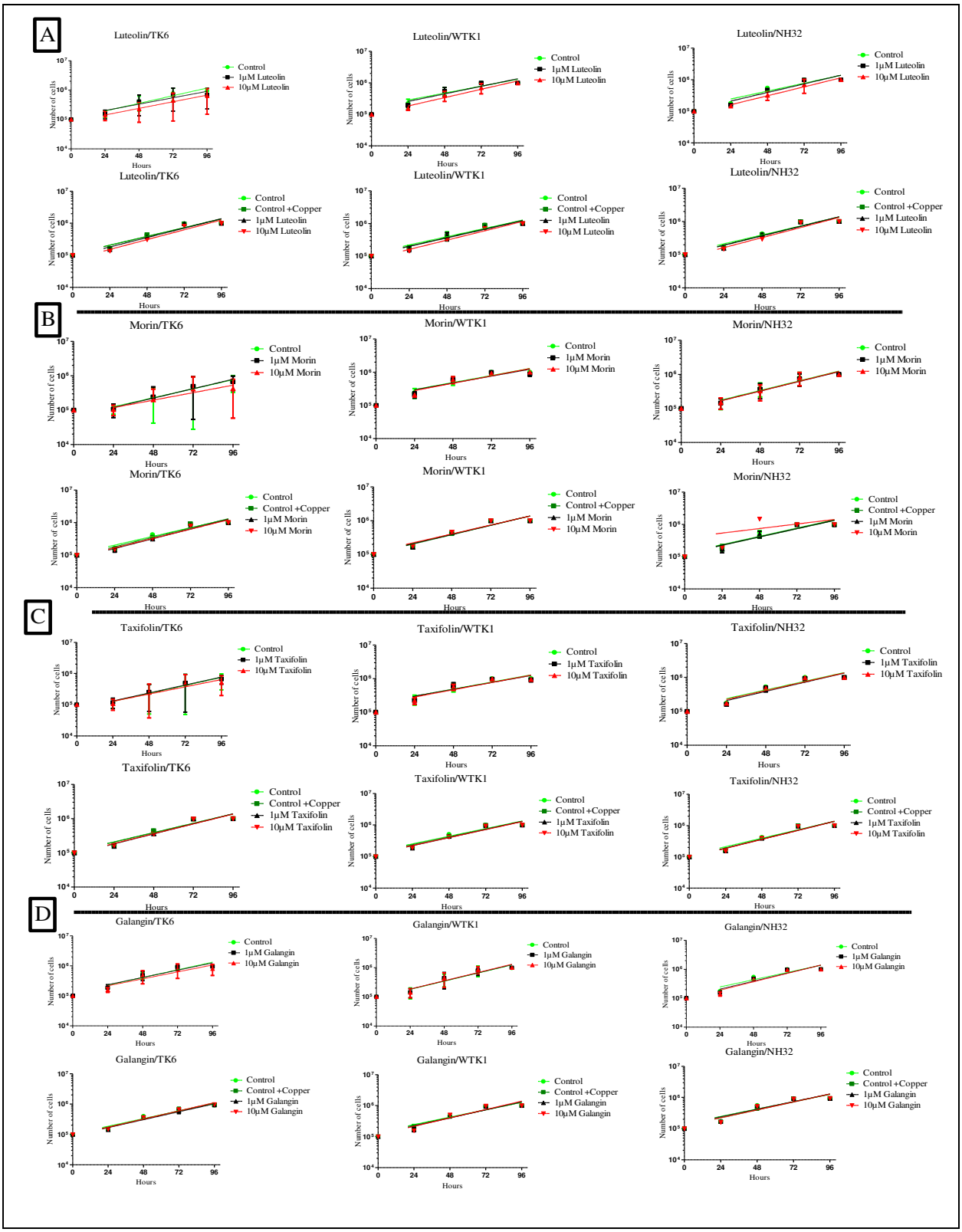

Figure.4. 3. The growth inhibition of p53 cells in different statuses by flavonoids that did not show ssDNA damage: (A) Luteolin, (B) Morin, (c) Taxifolin, and (D) Galangin. Error bars indicate the standard error of the mean. Three independent experiments were performed.

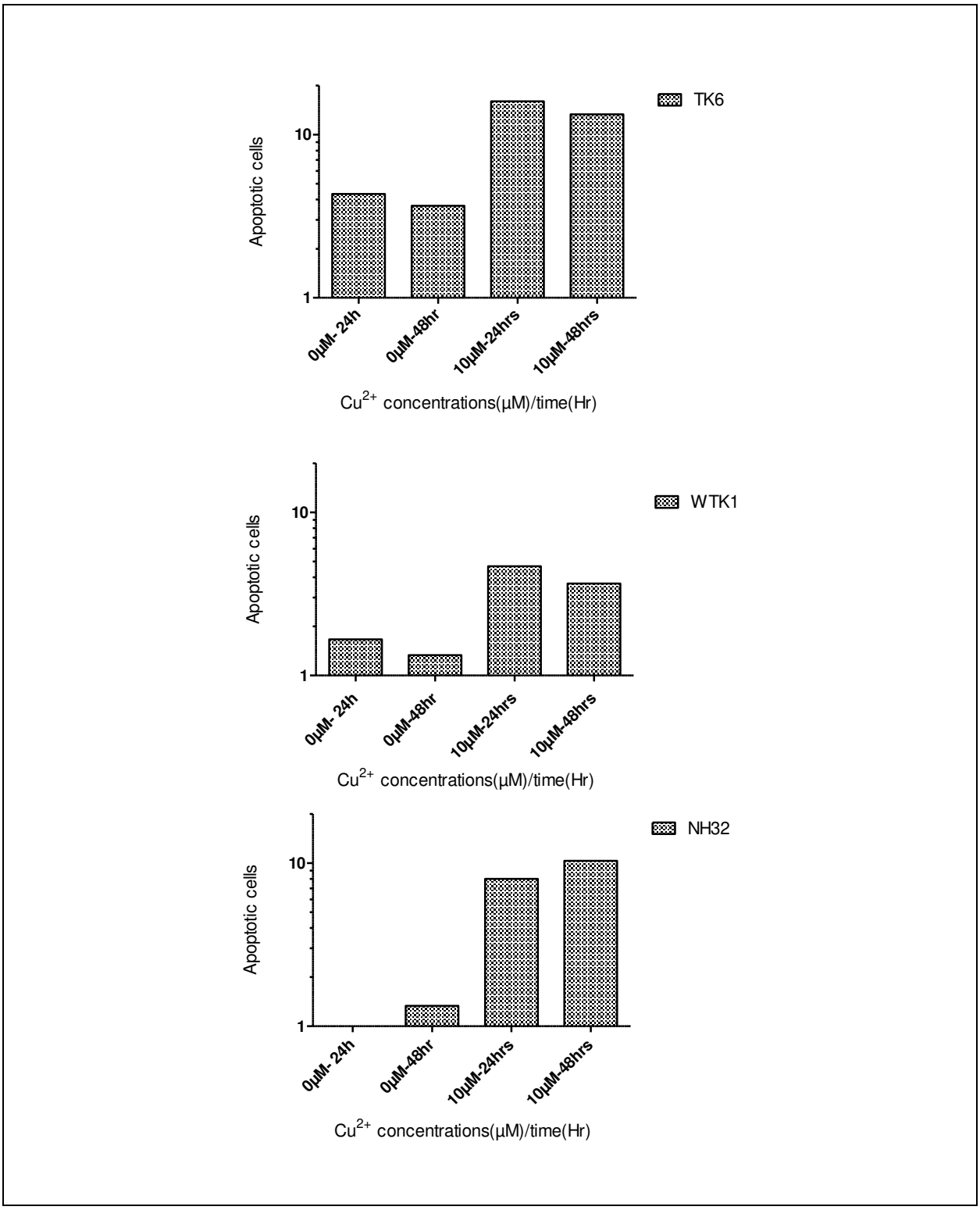

Figure.4. 4. The copper treatment of P53wild type cells (TK6), mutated P53 cells (WTK1), and P53cknocked out cells (NH32) induce apoptosis.

4.4. Discussion

Based on the obtained preliminary results of the cytotoxicity in this study, it is possible to derive a relationship between Myricetin and the cytotoxic activity of certain cancer in the presence of cupric ions. The anticancer property of this compound could be related to the formation of more free radicals in the presence of cupric ions due to its prooxidant activity. It may therefore be concluded among the tested flavonoids, the Myricetin is the only flavonoids that showed moderate cytotoxic ability against wild type P53 cells in both presence and absence of the cupric ions in contrast the mutated P53 did not showed growth inhibition in presence or in absence of cupric ions. In presence of cupric ions, the only P53 knock-out cancer cells showed slight growth inhibition. This results in agreement with previous paper referred to the high resistance of WTK1 to radiation compared to NH32 and TK6 [28].

Overall, in agreement with data obtained in third chapter of this dissertation, myricetin induce highest ssDNA damage among the seven tested flavonoids and highest growth inhibition in wild and null P53 cells. This is referring to the promising anticancer ability of this flavonoid. These results agree with previous results that showed the high ability of Myricetin to act as chemo preventive agent in breast cancer cells [22]. In presence of copper P53 knock-out cells exhibit highest sensitivity to induce apoptosis, on the contrast the mutated cells showed more sensitivity to induce apoptosis than P53 null cells in absence of copper.P53 is mutated in large fraction of human cancer, and P53 mutated cells represent the majority of cancer. The presence of copper did not enhance the inhibition of P53 mutated cells.

Generally, to confirm this results more experiments need to be done like evaluation of cell viability by MTT assay, cell apoptosis, cell cycle analysis by the flow cytometry, and studying the molecular docking of flavonoids by investigating the binding mode of flavonoids with kinases and

further comparisons between the cytotoxicity by using suitable copper concentration that are not toxic to the cell itself.

Project summary

In this project we hypothesized that the presence of specific hydroxyl groups at specific rings in flavonoids will induce free radicals that causes DNA damage in presence of cupric ions.

The antioxidant and prooxidant properties of flavonoids are affected by different features: the planarity of flavonoid surface, the position and number of hydroxyl groups, and the ability to chelate redox metal ions. The position and number of OH groups have an important role in antioxidant activity [23]. This study aimed to understand the mechanism by which flavonoids could induce DNA damage in the presence of cupric ions. This aim was studied by comparing the difference in structural features of flavonoids, particularly the position and number of hydroxyl groups on flavonoids. And we investigate the cytotoxicity induced by flavonoids in cells with different p53 status in presence of the cupric ions.

From DNA damage assessment by means of gel electrophoresis, the results clearly showed that the position 3 at C ring is critical to induce DNA breaks in the presence of cupric ions. Moreover, the degree of DNA damage is affected by the distributions of OH groups on B ring. Flavonoids have pyrogallol structure on B ring showed higher level of DNA damage, followed by quercetin which have catechol at positions 3 and 4 at B ring and Kaempferol with phenolic structure at position 4 at B ring. These results are also consistent with a study that showed that Myricetin has the greatest pronounced DNA damage in the presence of cupric ions, and this damage is attributed to the interaction between flavonoid cupric ions stable complex and the DNA molecule, leading to production of free radicals via Fenton reaction [24].

By examining the effect of different temperatures on the reaction of flavonoid cupric ions complex on inducing DNA damage, a relationship between OH group positions and temperature has been found. Generally, our results clearly showed the temperatures and concentration dependency on inducing DNA damage in the flavonoid poses OH group at position 3 of C ring.

On the other hand, flavonoids such as luteolin which characterized by having OH group on position 3 at C ring and missing the two OH groups at position 3 and 4 at B ring. These flavonoids showed high temperature dependency to induce ssDNA damage in presence of cupric ions. The comparisons of Naringenin (no OH on position 4 on B ring) and Hesperetin groups (no OH on position 3 on B ring) revealed that a presence of specific positions of OH groups at positions 3 and 4 at B ring is important to increase the capability of flavonoid cupric ions complex to induce DNA damage at high temperatures only. And this result was confirmed by using a Luteolin which has both OH groups at 3 and 4 positions at B ring. Luteolin showed DNA damage at high temperatures only.

In comparison, experiments in which we targeted the effect of the arrangement of phenolic, catecholic, and pyrogallol structures of OH groups at B ring on chelating capacity, we found that the catecholic structure at positions 3 and 4 is the identical chelation site for chelating cupric ions and in radical scavenging activity. For instance, quercetin has the highest chelating ability among other flavonoids. Data from the literature confirms our findings, these obtained results indicated that OH groups at positions 3 and 4 on B ring are the most functionally significant sites in scavenging free radicals [29,30]. Although myricetin did not show high chelating capability with cupric ions, a study has evidence that myricetin can interact with cupric ions at these sites via the donating atoms.

Overall, these results suggest that the presence of OH groups at specific positions on flavonoids (OH group at position3 of C ring and pyrogallol structure of OH groups at B ring) give flavonoids the most efficient power to produce DNA damage in presence of cupric ions. There is further indication that these kind of flavonoids in the presence of cupric ions have moderate anticancer activity, which can increase the formation of the free radicals, and consequently could act as a potential anticancer drug limited to cancers that contain to P53 knock-out cells.

Comparing both the degree of the polarity of OH groups on flavonoids and OH group positions revealed the impact of both structural features of flavonoids on affecting their prooxidant activity in presence of cupric ions. The results clearly showed that flavonoids with OH group in 3 position of C ring: Myricetin, Kaempferol, and Quercetin induced the highest DNA damage and possessed mild polarity at C ring, compared with other flavonoids which did not show DNA damage represented in Morin, Taxifolin, and Galangin. Furthermore, the low polarity at 4 position at B ring seemed to be critical on inducing DNA damage. For instance, Taxifolin (did not show DNA damage) has the same OH group's distribution as Quercetin (showed DNA damage), and Taxifolin differs in the bond connected between C2 and C3, so that it cause a different orientation of B ring and could alter the polarity at B ring and modify the interaction with DNA molecule. Taxifolin has more mild polarity at position 4 at B ring compared to lower polarity at the same position in quercetin.

Based on the obtained results on the cytotoxicity part in this study, it is possible to derive a relationship between myricetin and the cytotoxic activity in the presence of cupric ions. The anticancer property of this compound could be related to the formation of more free radicals in the presence of cupric ions due to its prooxidant activity. It may therefore be concluded among the tested flavonoids, only Myricetin possesses moderate cytotoxic ability against wild type and P53

knock-out cancer cells. These results agree with previous results that showed the high ability of Myricetin to act as chemo preventive agent in breast cancer cells.

Significance

This project aimed to gain information on the mode of action by which flavonoid can interact with DNA in presence of cupric ions, and to reveal possible impact of structural features on flavonoid. It revealed the impact of the specific hydroxyl positions on flavonoids on inducing ssDNA break in presence of cupric ions. These finding are relevant to the field of nutrition and safety consumption of flavonoids, and with the change of lifestyle and how we take care of ourselves people have been wanting to have a healthy foods, and eating food that contains flavonoids or using it as a food additive. There are some diseases such as Alzheimer and Parkinson characterized by presence of free copper, which can catalyze the formation of the free radical and consequently lead to DNA damage. To find out the safety of the flavonoid type and dosage for healthy and unhealthy people. Furthermore, it is important to understand the mechanism of action and determine the stability of flavonoid metal ions interaction, since the interaction between flavonoids metal ions could work in many biological processes that chelate metal ions. The complex could be used to provide the body with essential minerals or to eliminate minerals in minerals overloads disorders.

Moreover, this study is relevant to the field using metal – based anticancer drugs, and in particular the copper. There is growing evidence that copper level is elevated and is a hallmark to the malignancy degree in cancer patients compared to normal cells subjects [25]. Myricetin showed more cell toxicity in presence of cupric ions, this suggested that Myricetin could have higher chemo preventive cytotoxic ability among other tested flavonoid.

Finally, the difference in polarity among the most critical OH groups on inducing DNA damage may help to explain why flavonoids with same OH group's number or distribution did not show the same DNA damage degree. Data obtained from this work suggested that the structural aspect of flavonoids play important role in enhancing more DNA damage in presence of cupric ions, and this could be utilized to develop molecules with anticancer cytotoxicity.

Future directions

Flavonoids are considered a life span essential nutrient that have wide promising health and disease preventing roles in different fields such as pharmaceutical, medicinal and cosmetic. Dietary flavonoids are found in fruit, vegetables, and beverages. It is important to investigate the health benefits and potential harmful effects of flavonoids by understanding the mechanisms of scavenging and radical forming capacity. There are many epidemiological studies concerned with the study of specific flavonoids such as quercetin in healthy people, and Researchers have tried to link the relationship between daily consumption and protective effects of flavonoids [26]. The question remains of whether this data is relevant to the unhealthy people with diseases that are characterized with high levels of copper.

Copper is elevated in certain kinds of blood or solid cancers such as leukemia, Acute lymphocytic, Non-Hodgkin's lymphoma breast and ovarian cancer [25]. Myricetin showed high ssDNA damage and slight cell growth inhibition in presence in the cupric ions. More specific researches of certain flavonoids such as myricetin are needed to study the anticancer activity with certain cancers that characterized by high levels of copper such as breast, liver, Hodgkin's lymphoma, and leukemia.

There are huge number of flavonoids distributed in different groups and subgroups. It is important to make other comparisons between flavonoids that show ssDNA damage such as quercetin and myricetin with other flavonoids that have same number and distribution of OH groups. For instance, quercetin and fisetin have same catechol hydroxyl groups structure at B ring and have hydroxyl group at position 3 of C ring (Fig. 5). Quercetin did not show consistent results. It was able to induce ssDNA damage in vitro, and in contrast inside cancer cells did not show cell growth inhibition. Comparing the quercetin to fisetin where both have same number and distribution of hydroxyl groups will help to re-evaluate the flavonoids with catechol structure which showed the high ssDNA damage and cupric chelating.

Furthermore, the results of cupric ions chelating did not show agreement with the level of ssDNA damage, for instance, the cupric chelating of kaempferol was higher than myricetin, but kaempferol showed less DNA damage than myricetin. Thus, further studies to investigate the chelating capability of flavonoids by other precise methods like HPLC and assessment of the metal ions stoichiometry to investigate the stability of the flavonoid metal ion interaction has an impact on understanding the relation between flavonoid chelating capability and inducing DNA damage. Finally, I wish had the chance to do more experiment such as cell apoptosis to study the cellular toxicity of the flavonoids in presence of cupric ions.

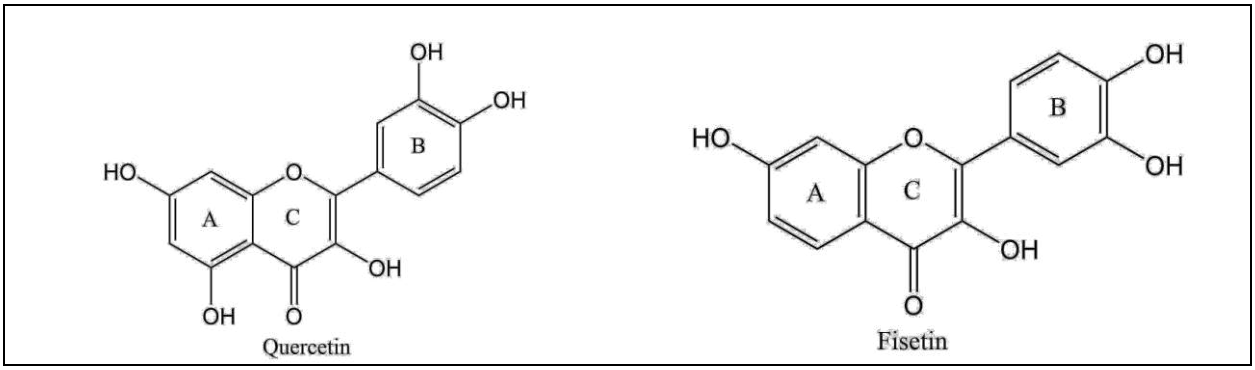

Figure. 4. 5. Chemical structure of quercetin and fisetin illustrated with ChemDraw Version pro 16.0.

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Effect of hydroxyl group position in flavonoids on inducing single-stranded DNA damage mediated by cupric ions

SUAD ELMEGERHI[1], CATHY SU[2], DYLAN JOHN BUGLEWICZ[2], YASUSHI AIZAWA[3] and TAKAMITSU A KATO[1,2]

[1]Cell and Molecular Biology Program, and [2]Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO 80523, USA; [3]Research and Development Group, Toyo Sugar Refining Co., Ltd., Tokyo 103-0046, Japan

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Abstract. Quercetin has been demonstrated to produce DNA damage in the presence of metal ions. In the present study, 7 natural and 5 semi-synthetic glycosylated flavonoids were utilized to investigate the cupric ion (Cu^{2+})-dependent DNA damage *in vitro*. The reaction mixture, containing single-stranded DNA, different concentrations of flavonoids and cupric ion in the buffer, was incubated at three different temperatures. DNA damage was then assessed by gel electrophoresis followed by densitometric analysis. The reaction mixture with quercetin at 4, 20 and 54˚C induced DNA damage in a concentration- and temperature-dependent manner. Furthermore, only the reaction at 54˚C resulted in DNA damage in flavonoids with glucosyl substitution of the hydroxyl group at the 3-position on the C ring in quercetin. By contrast, loss of the hydroxyl group at the 3-position on the C ring, or at the 3'- or 4'-position on the B ring of quercetin, did not portray DNA damage formation at the investigated experimental temperatures. In addition, the experimental results suggested that the hydroxyl group at the 3-position on the C ring produced the strongest capability to induce DNA damage in the presence of cupric ions. Furthermore, hydroxyl groups at the 3'- or 4'-position on the B ring were only able to induce DNA damage at higher temperatures, and were less efficient in comparison with the hydroxyl group at the 3-position on the C ring. Cupric ion chelating capacity was also assessed with spectroscopic analysis, and quercetin presented the largest chelating capacity among the tested flavonoids. Hydroxyl radical formation was assessed with a luminol reaction, and quercetin presented faster consumption of luminol. These results suggest that the 3-position hydroxyl group of the C ring is required to induce DNA damage at low temperatures. Furthermore, the results of the present study also indicated that the presence of cupric ions will decrease the activity of the glycosylated quercetins, in terms of their ability to induce DNA damage.

Correspondence to: Professor Takamitsu A Kato, Department of Environmental and Radiological Health Sciences, Colorado State University, 1618 Campus Delivery, Fort Collins, CO 80523, USA
E-mail: takamitsu.kato@colostate.edu

Key words: quercetin, cupric ion, DNA scission

Introduction

Flavonoids are naturally occurring polyphenolic metabolites in plants that serve significant roles in traditional medicine and as food additives (1-3). Glycosylated forms of numerous flavonoids are produced in order to alter their chemical properties, such as their bioavailability and water solubility (4). Flavonoids without glycosylation are known as aglycones, which are often more reactive forms of flavonoids and present greater antioxidant capacity, as well as cellular toxicity, compared with the glycosylated flavonoids (5,6). Both aglycone and glycosylated forms of flavonoids are recognized to be beneficial in regard to anti-tumorigenicity and anti-mutagenicity, due to their antioxidant and radical scavenging effects.

Numerous flavonoids, including quercetin, have been previously reported to induce mutation frequency in bacterial systems and chromosome aberrations in mammalian cells (7-10). This mutagenic potential may be associated with the ability of flavonoids to produce hydroxyl radicals, resulting in DNA breaks (11,12). In the presence of specific metal ions, flavonoids can cause DNA scission, but rarely DNA double-strand breaks, resulting from the interaction of hydrogen peroxide with metal ions (12). This hydroxyl radical production is coupled with the reduction of cupric ions to cuprous ions (from Cu^{2+} to Cu^{1+}). Quercetin forms chelating complexes with cupric ions and portrays broad biological activities (13-16). This cupric ion and the quercetin complex interact at the 3' and 4' hydroxyl groups on the B ring, and the 3-hydroxyl group and 4-oxygen residue on the A ring (17). Furthermore, the ability to produce hydroxyl radicals and to induce DNA breaking activity has also been reported for other compounds that are structurally associated with quercetin, including fisetin, baicalein, taxifolin and curcumin with cupric ions (18,19), as well as epigallocatechin gallate and ferrous ions (20). However, the exact mechanisms underlying the formation of hydroxyl radicals from quercetin and its effects on glycosylation of flavonoids remain unclear.

The present study utilizes a total of 7 natural and 5 recently semi-synthesized novel flavonoids to identify the specific molecular mechanisms required to induce DNA scissions in the presence of cupric ions. To analyze DNA scission formation in an *in vitro* gel electrophoresis system, three aglycones and their glycosylated flavonoids were reacted in the presence of cupric ions at different temperatures.

Materials and methods

Chemicals. All natural and synthetic flavonoids were obtained from Tokyo Sugar Refining Co., Ltd. (Tokyo, Japan). The tested compounds, including three aglycone flavonoids (quercetin, naringenin and hesperetin) and their glycosylated flavonoids are summarized in Fig. 1. Quercetin is an active aglycone form of isoquercetin, rutin, maltooligosyl-isoquercetin, monoglucosyl-rutin and maltooligosyl-rutin. The glycosylated form of quercetin loses the 3-position hydroxyl group on the B ring by glycosylation. Compared with quercetin, naringenin does not contain a hydroxyl group at the 3-position on the B ring nor at the 3'-position on the C ring. In addition, compared with quercetin, hesperetin does not contain a hydroxyl group at the 3-position on the C ring nor at the 4'-position on the C ring. Glycosylated naringenin and hesperetin lost the hydroxyl group at the 7-position on the A ring by glycosylation and are chemically inactive. All flavonoids were prepared by dissolving in dimethyl sulfoxide (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at the concentration of 1 mM as a stock solution. M13mp18 single-stranded DNA (250 μg/ml) was purchased from New England BioLabs, Inc. (Ipswich, MA, USA). Double-stranded lambda phage DNA (0.46 μg/μl) was purchased from Nippon Gene Co., Ltd. (Tokyo, Japan). Copper chloride dehydrate was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

DNA scission reaction. A total of 20 μl reaction solution was used, containing 1.5 ng/μl single-stranded DNA or 1.25 ng/μl of double-stranded DNA, 10 mM Tris-HCl, 0.2 mM $CuCl_2$ and various concentrations of flavonoids (0.1, 1, 10 and 100 μM). The reaction mixtures were incubated at different temperatures (4˚C, 20˚C, 37˚C or 54˚C) for 1 h. Next, 4 μl of 6X loading dye, containing 15% Ficoll, 10% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol in water, was added to the mixture and electrophoresis was conducted on 1% agarose gel stained with ethidium bromide with 1X Tris-acetate-EDTA buffer at 100 V for 1 h. Subsequent to electrophoresis, destaining of ethidium bromide was performed, and a gel image was obtained by ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) via Image Lab software (Bio-Rad Laboratories, Inc.). Intact M13mp18 single-stranded DNA presented two bands, indicative of the circular and linear form of DNA. The DNA intensity of the circular form was obtained by Image Lab, while the fraction of intact DNA was calculated as follows:

$$Fraction\ of\ intact\ DNA = \frac{Amount\ of\ circular\ DNA\ with\ flavonoids}{Amount\ of\ circular\ DNA\ without\ flavonoids}$$

Half maximal inhibitory concentration (IC_{50}) values, which refer to the specific concentrations required to induce 50% of DNA break, were obtained from sigmoidal regression curves obtained by Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Absorption spectrum analysis. In order to investigate the cupric ion chelating capability of flavonoids, the flavonoids and cupric ions were mixed at rations of 1:2, 1:1 and 2:1, and absorbance values from 230 to 430 nm were obtained via a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). According to the peak shifting and the reduction of peak height, the chelating capacity of cupric ions was estimated (14,15,17).

Oxidation of luminol. In order to investigate the potential mechanisms underlying the induction of DNA damages, oxidative capacity analysis was conducted. In total, 100 μl of reaction solution, including 20 μl enhanced chemiluminescence solution (Thermo Fisher Scientific, Inc.), 2 μl of 100 μM flavonoids and 2 μl of 100 μM $CuCl_2$, were mixed. Subsequently, the arbitrary relative luminescence unit (RLU) was measured immediately until 3 min after mixing via a Lumat LB9507 luminometer (Berthold Technologies, Oak Ridge, TN, USA). RLU values were plotted with the exponential decay model, and half time values were obtained.

Statistical analysis. Statistical calculations were preformed via Prism 6 software. Two-way analysis of variance (ANOVA) and Student's t-test were conducted. P-values of <0.05 were considered to indicate differences that were statistically significant.

Results

DNA damage formation. Fig. 2 demonstrates the DNA damage subsequent to reaction with quercetin in the absence or presence of cupric ions at 37˚C. Without cupric ions in the reaction mixture, DNA damage was not detected from the single-stranded and double-stranded DNA. Upon the incorporation of cupric ions in the reaction mixture, single-stranded DNA was degraded, whereas double-stranded DNA was not affected. Therefore, quercetin without cupric ions did not produce DNA damage. In addition, DNA damage produced by quercetin and cupric ions mainly involved single-strand break rather than double-strand break.

Fig. 3 shows the fraction of intact DNA following reaction with glycosylated flavonoids in the presence of cupric ions in the reaction mixture. In the presence of 0.2 mM $CuCl_2$ in the reaction mixture, quercetin and its glycosylated flavonoids induced DNA breaks in a concentration and temperature dependent manner. Less intact DNA was observed following reaction with high concentration of flavonoids, and high temperature efficiently induced DNA damages.

Quercetin was the only flavonoid capable of inducing a DNA break in reactions performed at 4 and 20˚C. Furthermore, the reaction at 20˚C induced a greater degree of DNA damage as compared with the reaction at 4˚C. The IC_{50} values of quercetin at 4 and 20˚C were 47.7 and 20.5 μM, respectively, while the reaction at 54˚C reduced the IC_{50} value to 2.6 μM. By contrast, all quercetin glycosides, including isoquercetin, rutin, monoglucosyl rutin, maltooligosyl-rutin and maltooligosyl-isoquercetin, failed to induce any DNA damage following 4 and 20˚C reactions under the tested conditions. However, these glucosyl flavonoids did induce DNA

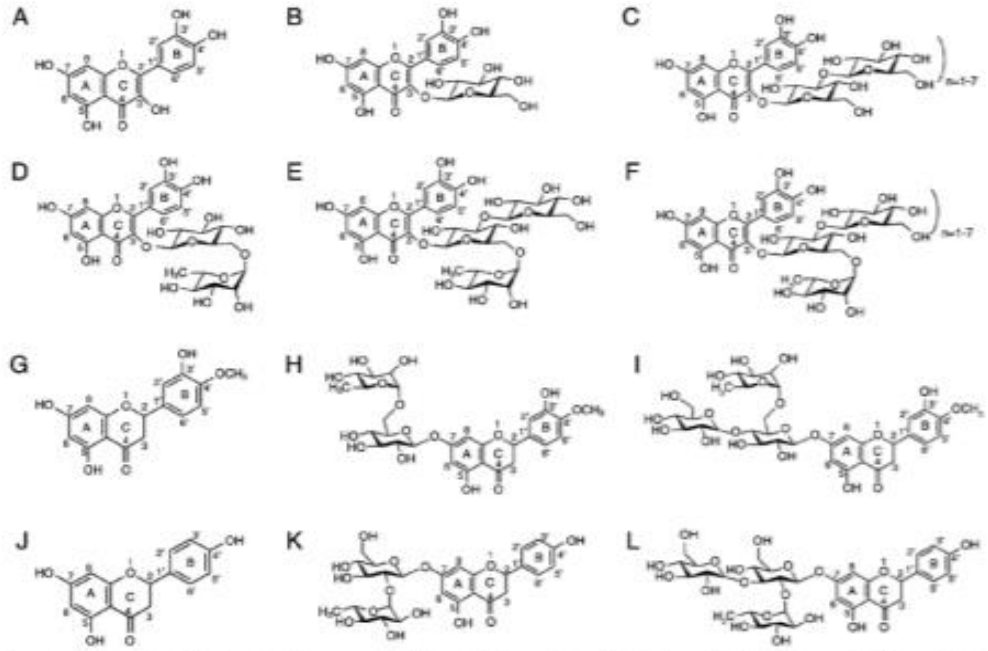

Figure 1. Structure of flavonoids. (A) Quercetin, (B) isoquercetin, (C) rutin, (D) monoglucosyl-rutin, (E) maltooligosyl-isoquercetin, (F) maltooligosyl rutin, (G) hesperetin, (H) hesperidin, (I) monoglucosyl hesperidin, (J) naringenin, (K) naringin, and (L) monoglucosyl naringin.

damage when reactions were conducted at 54˚C for 1 h. The IC_{50} values were 7.7 μM for isoquercetin, 3.4 μM for rutin, 9.5 μM for monoglucosyl rutin, 4.5 μM for maltooligosyl rutin and 12.1 μM for maltooligosyl isoquercetin. This indicates that glycosyl modifications at the 3-position on the C ring of quercetin suppressed the DNA scission ability of quercetin at 4 and 20˚C. However, DNA scission capacity was not altered with glycosylation at the 3-position on the C ring of quercetin at 54˚C. Therefore, increased glucosyl modifications did not contribute toward DNA degradation. In other words, the hydroxyl groups of glucosyl residues did not portray any contributions towards DNA damage.

In order to clarify which position of the hydroxyl group is responsible for inducing DNA damage, two other flavone aglycones, hesperetin and naringenin, and their glycosylated flavonoids were also tested in the same system to assess hydroxyl radical formation by DNA damage observation (Fig. 4). Hesperetin, naringenin and their glycosylated flavonoids, which do not have hydroxyl groups at the 3-position on the C ring nor at the 3'- or 4'-position on the B ring, were incapable of producing any detectable single-stranded DNA damage in the reaction at any tested temperature. Therefore, this indicates that the presence of hydroxyl groups at the 3'- and 4'-positions is required for the induction of DNA damage at 54˚C. Furthermore, flavone glycosides did not cause any DNA damage, confirming that the hydroxyl groups of glucosyl residue have no effect on DNA damage.

Chelating capacity. Absorption spectrum alterations, shown in Fig. 5A-L, occurred when flavonoids and cupric ions were

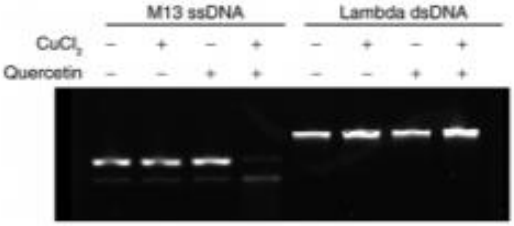

Figure 2. Gel image indicating DNA damage after 1-h reaction at 37˚C. M13 ssDNA (upper bands indicate circular intact DNA and lower bands indicate linear DNA) and lambda dsDNA were treated with or without 0.2 mM $CuCl_2$ and 100 μM quercetin in 10 mM Tris-HCl solution. ss, single-stranded; ds, double-stranded.

mixed in different ratios (1:2, 1:1 and 2:1). Quercetin and its glycosides displayed peaks at ~250 and 370 nm, as previously described (14,15,17). Upon addition of cupric ions, the first peak at 250 nm skewed to the left and the size of the second peak was reduced. The reduction in the second peak was the greatest for quercetin. Naringenin, hesperetin and their glycosides presented the first peak at 250 nm and the second peak at 290 nm. As observed in quercetin and its glycosides, the first peak was shifted toward the left and the second peak size was decreased in the presence of cupric ions. The reduction ratio of the second peak was used for assessment of cupric ion chelating capacity, and the results are summarized in Fig. 5M. Quercetin demonstrated >6-fold reduction in the absorbance peak height in the presence of cupric ions. The peak heights of

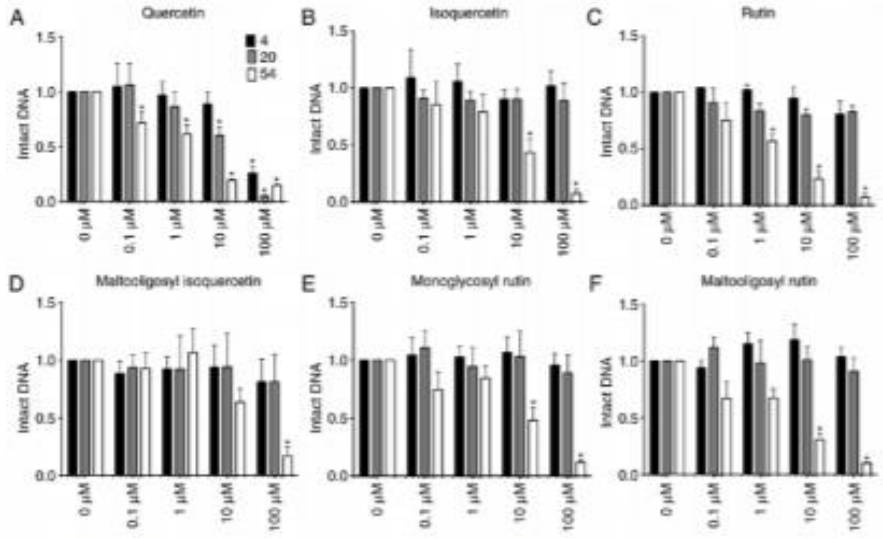

Figure 3. Fraction of intact DNA after 1-h reaction at 4, 20 and 54˚C for quercetin and its glucosides. (A) Quercetin, (B) isoquercetin, (C) rutin, (D) maltooligosyl isoquercetin, (E) monoglucosyl rutin, and (F) maltooligosyl rutin. Error bars indicate standard error of the means. Three independent experiments were performed.

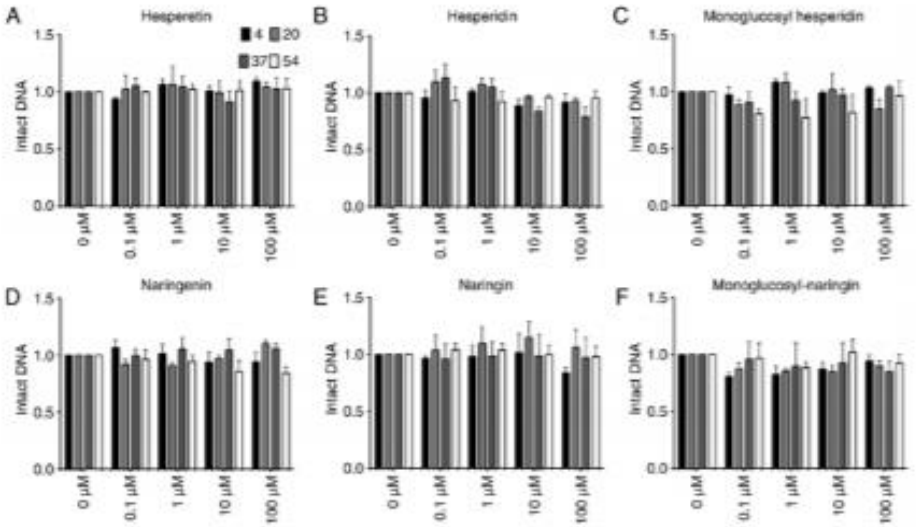

Figure 4. Fraction of intact DNA after 1-h reaction at 4, 20 and 54˚C for hesperetin, naringenin and their glucosides. Error bars indicate the standard error of the mean. Three independent experiments were performed. (A) Hesperetin, (B) hesperidin, (C) monoglucosyl hesperidin, (D) naringenin, (E) naringin and (F) monoglucosyl naringin.

other flavonoids were reduced by 2-4-fold. Furthermore, quercetin showed statistically significant differences compared with the other flavonoids (ANOVA; $P<0.0001$).

Oxidation of luminol. In addition to the interaction between flavonoids and cupric ions, oxidative capacity analysis was conducted with luminol as a substrate in the presence of flavonoids and cupric ions. Subsequent to mixing, the reduction of the luminescence signal was observed (Fig. 6). Emitted glow signals are associated with not only cupric ion induction, but also generated hydrogen peroxide by flavonoids in the presence of cupric ions. The luminescence signal (arbitrary RLU) was rapidly decreased with an exponentially decreased model for all flavonoids. Quercetin and isoquercetin exhibited the fastest reduction of RLU with a half-life of 0.22 min. In addition, rutin, monoglucosyl-rutin, maltooligosyl-rutin and maltooligosyl-isoquercetin presented intermediate reduction in kinetics with a half-life between 0.28-0.33 min. Naringenin,

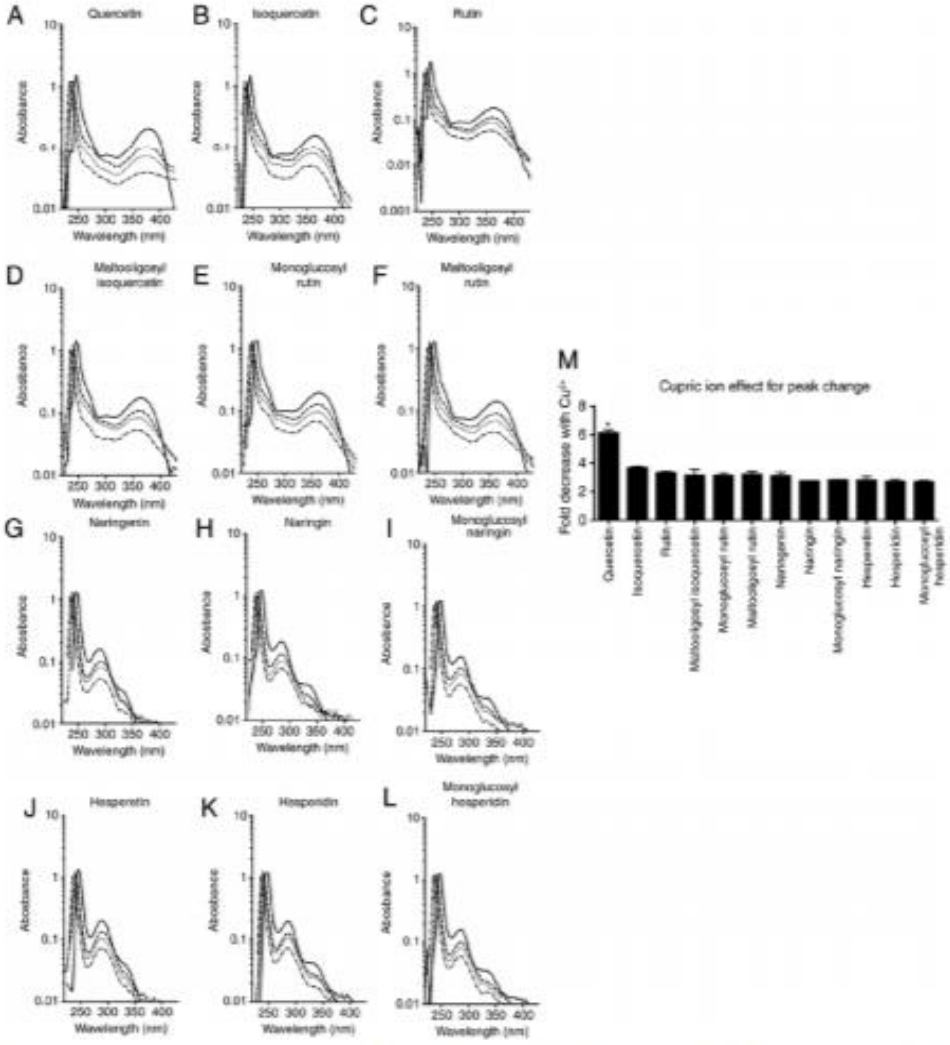

Figure 5. Absorption spectrum changes in the presence of cupric ions. Solid lines indicate the flavonoid only, and the dashed, dotted and dash-dotted lines indicate a flavonoid and cupric ion mixture at a ratio of 2:1, 1:1 and 1:2, respectively. (A) Quercetin, (B) isoquercetin, (C) rutin, (D) maltooligosyl isoquercetin, (E) monoglucosyl rutin, (F) maltooligosyl rutin, (G) naringenin, (H) naringin, (I) monoglucosyl naringin, (J) hesperetin, (K) hesperidin and (L) monoglucosyl hesperidin spectrum changes are shown. (M) Effect of cupric ion on peak changes is displayed. *P<0.05 vs. other flavonoids. Error bars indicate the standard error of the mean. Three independent experiments were performed.

naringin, monoglucosyl-naringin, hesperetin, hesperidin and monoglucosyl-hesperidin also displayed the slowest kinetics with a half-life of 0.35-0.45 min.

Discussion

Flavonoids with a variety of chemical structures with modified residues exist; for instance, a number of hydroxyl groups are attached to the different positions of benzene rings (21). Depending on the hydroxyl groups present at specific locations, metal ions can bind to flavonoids and induce the Fenton-like reaction, resulting in DNA damage (11,12). DNA double-strand breaks are rare in this event, but single-strand scission and oxidative damage are induced (11,22). This DNA damage may be associated with mutagenesis in the cell culture system following flavonoid reaction with cells in the presence of metals (23).

The present study clearly demonstrated the importance of the specific positions of hydroxyl groups on the flavonoids to induce DNA damage. The results revealed that quercetin was the only flavonoid capable of inducing DNA damage at any of the tested experimental temperatures (Fig. 3). Naringenin and hesperetin, which do not possess a hydroxyl group at the specific positions (the 3-position on the C ring, and the 3'- and 4'-positions on the B ring), were incapable of inducing DNA damage (Fig. 4). Furthermore, at low temperatures,

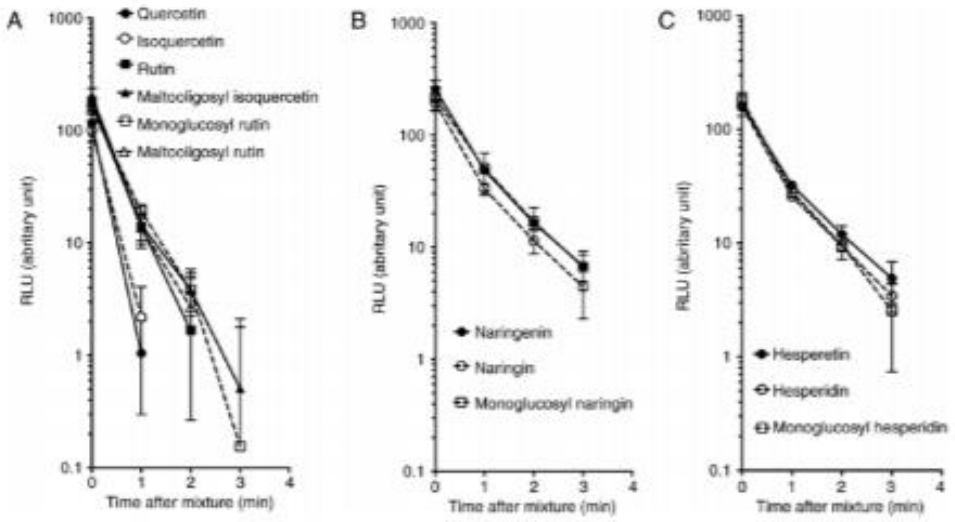

Figure 6. Oxidation of luminol in the presence of hydrogen peroxide, $CuCl_2$ and flavonoids. (A) Quercetin, isoquercetin, rutin, maltooligosyl isoquercetin, monoglucosyl rutin, and maltooligosyl rutin. (B) Naringenin, naringin, and monoglucosyl naringin. (C) Hesperetin, hesperidin, and monoglucosyl hesperidin. Error bars indicate the standard error of the mean values. Three independent experiments were performed. MO, maltooligosyl; MG, monoglucosyl.

the glycosylated flavonoids of quercetin were incapable of inducing DNA breaks (Fig. 3). This suggests that a hydroxyl group at the 3-position on the C ring of quercetin is the most reactive, inducing DNA damage in a Fenton-like reaction with cupric ions. These findings are in agreement with the previous studies that refer to the possibly significant role of this location (11) in forming a complex with cupric ions to induce DNA damage. It is also worth noting that the hydroxyl group at the 3-position on the C ring of quercetin has the ability to scavenge radicals (24). The high cupric ion chelating capacity of quercetin (Fig. 5) and fast oxidation of luminol (Fig. 6) were also in agreement with these previous findings.

The structural differences among quercetin, naringenin and hesperetin helped to investigate the impact of hydroxyl groups at the 3'- and 4'-positions on the B ring in the induction of DNA damage. With two hydroxyl groups at the 3'- and 4'-positions on the B ring, flavonoids induced DNA damage at a high temperature, as observed with glycosylated quercetins (including isoquercetin, rutin, maltooligosyl-isoquercetin, monoglucosyl-rutin and maltooligosyl-rutin) (Fig. 3). When one of the two hydroxyl groups was replaced with other residues, high temperature-specific DNA damage was not observed in naringenin and hesperetin. Additionally, hydroxyl groups at the 7-positions on the A ring did not contribute to DNA damage induction based on the observation of the glycosylated naringenin and hesperetin. Previously, it has been reported that the hydroxyl groups of quercetin at the 3'- and 4'-positions on the B ring have higher radical scavenging effects in comparison with the hydroxyl group at the 3-position on the A ring (24). This may be associated with the temperature-dependent pro- and anti-oxidant properties of quercetin.

The DNA scission observed in the present study is a result of radical formation from a Fenton-like reaction between specific hydroxyl groups and cupric ions. Temperature-dependent DNA damage between a hydroxyl group at the 3-position on the C ring and the hydroxyl groups at the 3'- and 4'-positions on the B ring may be associated with the amount of radical formation at the different temperatures. In this case, the hydroxyl group at the 3-positions on the C ring can efficiently produce more radicals compared with the hydroxyl groups at 3'- and 4'-positions on the B ring in the presence of cupric ions. Another possible mechanism of differential DNA damage is an interaction between the quercetin-cupric ion complex and DNA. The proposed models of the quercetin-cupric ion complex involve a hydroxyl group at the 3-position on the C ring and hydroxyl groups at the 3'- and 4'-positions on the B ring. It is possible that the complex formation between quercetin-cupric ion at the 3'- and 4'-positions on the B ring is temperature-dependent.

In conclusion, the present study reported that quercetin induces DNA scissions in the presence of cupric ions in a broad range of temperatures. The hydroxyl group at the 3-position on the C ring contributes to the temperature-independent DNA scission due to high chelating capacity and oxidative reaction. In addition, the hydroxyl groups at the 3'- and 4'-positions on the B ring contribute to DNA scission formation in the presence of cupric ions at high temperature.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

TAK conceived and designed the experiment. SE, CS and DJB performed experiments and wrote the manuscript. YA and TAK performed data analysis and critical revision of the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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List of Abbreviations

3HF	3-hydroxy flavone
AKT	Serine/ threonine kinase
αGH	Alpha glucosyl hesperidin
AT	Adenine/thymine-rich
ATP	Adenosine triphosphate
BAD	BCL2-antagonist of cell death
BCL2	B-cell lymphoma 2
CDC	Centers for Disease Control and Prevention
CDK1	Cyclin dependent kinase 1
CHO	Chinese hamster ovary
CoA	Coenzyme A
Cu	Copper
DMSO	Dimethyl sulfoxide
DOCA	Deoxycorticosterone acetate
DPPH	2, 2-diphenyl-1-picrylhydrazyl
dsDNA	Double strand deoxyribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regular kinase
Fe	Ferrous

GC	Guanine/cytosine-rich
GN	Galangin
HES	Hesperetin
HIV	Human immunodeficiency virus
HOMO	Highest occupied molecular orbital
HTC	Hepatoma tissue culture
IC50	Half maximal inhibitory concentration
IgE	Immunoglobulin E
IQ	Isoquercetin
JNK	c-Jun N-terminal kinase
KL	Kaempferol
LUMO	lowest unoccupied molecular orbital
MAPK	Mitogen-activated protein kinase
MG-HES	Monoglucosyl Hesperidin
MG	Monoglucosyl
MG-N	Monoglucosyl Naringin
MG-R	Monoglucosyl rutin
MMP-3	Matrix metalloproteinase-3
MN	Myricetin
MO-IQ	Maltooligosyl Isoquercetin
MO	Maltooligosyl
MO-R	Maltooligosyl rutin
NAR	Naringenin

NO	Nitric oxide syntetase
Nrf2	Nuclear factor erythroid 2- related factor 2
OH·	Hydroxyl radicals
OH	Hydroxyl group
PARP	Poly (ADP-ribose) polymerase
PI3K	Phosphoinositide 3-kinase
QN	Quercetin
RLU	Relative Luminescence Units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
R	Rutin
RSK2	Ribosomal S6 kinase2
SsDNA	Single strand Deoxyribonucleic acid
TAC	Total Antioxidant Capacity
TAX	Taxifolin
UV/VIS	Ultraviolet/visual spectra
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
XO	Xanthine oxidase