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Inhibitory Effect of Naturally Occurring Flavonoids on the Formation of Advanced Glycation Endproducts

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The objective of this study was to investigate the inhibitory effect of naturally occurring flavonoids on individual stage of protein glycation in vitro using the model systems of δ-Gluconolactone assay (early stage), BSA−methylglyoxal assay (middle stage), BSA−glucose assay, and G.K. peptide−ribose assay (last stage). In the early stage of protein glycation, luteolin, qucertin, and rutin exhibited significant inhibitory activity on HbA_{1C} formation ($p < 0.01$), which were more effective than that of aminoguanidine (AG, 10 mM), a well-known inhibitor for advanced glycation endproducts (AGEs). For the middle stage, luteolin and rutin developed more significant inhibitory effect on methylglyoxal-medicated protein modification, and the IC_{50}'s were 66.1 and 71.8 μM, respectively. In the last stage of glycation, luteolin was found to be potent inhibitors of both the AGEs formation and the subsequent cross-linking of proteins. In addition, phenyl-*tert*-butyl-nitron served as a spin-trapping agent, and electron spin resonance (ESR) was used to explore the possible mechanism of the inhibitory effect of flavonoids on glycation. The results indicated that protein glycation was accompanied by oxidative reactions, as the ESR spectra showed a clear-cut radical signal. Statistical analysis showed that inhibitory capability of flavonoids against protein glycation was remarkably related to the scavenging free radicals derived from glycoxidation process ($r = 0.79$, $p < 0.01$). Consequently, the inhibitory mechanism of flavonoids against glycation was, at least partly, due to their antioxidant properties.

KEYWORDS: Antioxidant; ESR; flavonoids; glycation; reactive oxygen species

INTRODUCTION

Hyperglycemia is a well-recognized pathogenic factor of long-term complications in diabetes mellitus (*1*, *2*). Elevated levels of glucose in the blood or other body fluids are known to cause an oxidative damage, followed by an imbalance between the productions of reactive oxygen species (ROS) and the antioxidant defense mechanisms present in biological systems. It has been suggested that free radicals and oxidation reaction are directly involved in glucose-mediated modification of proteins (*3*). Diabetic patients are more susceptible to oxidative attack than normal subjects owing to their higher production of ROS (*4*) and lower content of antioxidants (GSH, vitamins C and E) (*5*). Several mechanisms appear to be involved in hyperglycemia-mediated oxidative stress, such as glucose autoxidation, protein glycation, and the formation of advanced glycation endproducts (AGEs) (*3*). Glycation is a major source of ROS and reactive carbonyl species (RCS) that are generated by both oxidative (glycoxidative) and non-oxidative pathways (*6*). In addition, glucose itself can auto-oxidize to form hydrogen peroxide and keto aldehydes in the presence of transition metal ions (*4*, *7*), and subsequently accelerate the formation of AGEs and oxidative DNA damage (*8*).

It is noted that no AGE product is solely derived from glucose. RCS such as 3-deoxyglucosone, glyoxal, and methylglyoxal (MGO), are critical intermediates formed during glycation of proteins by glucose (*9*). They were all identified as the important precursors of AGEs in vivo (*9*, *10*). MGO can readily bind to amino groups, thereby modifying biological molecules to form covalently cross-linked aggregates (*11*). Increased levels of MGO are found in the blood of diabetic patients and streptozotocin-induced diabetic rats (*12*, *13*). Similar to the glycation of proteins by glucose, the generation of superoxide anion has been documented during the glycation of amino acids by MGO (*14*). Thus, studies aimed at minimizing the undesired consequences of protein glycation should include MGO as a target.

In consideration of the significance of glycooxidative stress to diabetic pathology, a supplement of antioxidants in response to the inhibition of protein modification should be a theoretical strategy for preventing diabetic complications (*3*, *6*). This hypothesis has been supported by the clinical results which indicated that the development of Type 2 diabetes may be reduced by the intake of antioxidants in diets (*15*). Fruits, vegetables, and beverages are important dietary sources of flavonoids (*16*, *17*). It has been reported that the human intake of flavonoids from diets is about 1 g/day (*18*). Flavonoids are of current interest in research due to their important biological

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and pharmacological properties attributed to their antioxidant properties (*19*). Nevertheless, the literature data concerning the effect of flavonoids in preventing glycoxidative modification of proteins is limited. Given the link mentioned above, we hypothesized that antioxidant flavonoids might possess significant antiglycoxidation activities as well. In this study, BSA−glucose assay (early stage), BSA−methylglyoxal assay (middle stage), and G.K. peptide−ribose assay (last stage) were utilized to investigate the inhibitory effect of flavonoids on each stage of protein glycation. This study will underline the importance of naturally occurring flavonoids in the prevention of hyperglycemia-mediated protein modification.

MATERIALS AND METHODS

Chemicals. Catechin, epicatechin (EC), epicatechchin gallate (ECG) epigallocatechin (EGC), epigallocatechin gallate (EGCG), kaempferol, luteolin, quercertin, naringenin, rutin, *N*-acetylglycyl-lysine methyl ester (G.K.) peptide, aminoguanidine (AG), bovine serum albumin (BSA) (fraction V, essentially fatty acid free), D-glusose, δ gluconolactone (δ-Glu), mannitol, methylglyoxal (MGO) (40% aqueous solution), phenyl-*tert*-butyl-nitron (PBN), and ribose were purchased from Sigma Chemical Co. (St. Louis, MO).

Hemoglobin−δ-Gluconolactone (δ-Glu) Assay. Evaluation of early stage of protein glycation was determined by δ-Glu assay (*20*). This method is specific for investigation of inhibitors on early glycation products (Amadori) formation. In short, samples were prepared by mixing 200 μL of fresh human blood with 40 μL of PBS (pH 7.4) as the blank or 40 μL of δ-Glu (50 mM) as the δ-Glu control. Test samples each contained 200 μL of blood plus 40 μL of δ-Glu and 10 μL of indicated flavonoids in a final concentration of 100 μM. After incubation at 37 °C for 16 h, the percentage of glycated hemoglobin present was determined using a dedicated ion-exchange HPLC system (BIORAD DIASTAT). Blood samples were analyzed in triplicates. The % inhibition of HbA_{1C} formation = [(HbA_{1C} of the δ-Glu control − HbA_{1C} of the test group)/(HbA_{1C} of the δ-Glu control − HbA_{1C} of the blank)] × 100%. AG was used at a final concentration of 10 mM as a positive control (*21*).

BSA−MGO Assay. The BSA−MGO assay is a specific method for investigation of inhibitors on middle stage of the glycation of protein and was measured according to the method of Lee et al. (*22*). BSA (50 mg/mL) was incubated with 100 mM MGO under sterile conditions in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 9 days. In certain experiments, the indicated flavonoids were added to the model system in the concentration range of 0−300 μM. Fluorescence of samples was measured at the excitation and emission maxima of 330 and 410 nm, respectively, versus an unincubated blank containing the protein, MGO, and inhibitors. The % inhibition of AGEs formation = [1 − (fluorescence of the test group/fluorescence of the control group)] × 100% (*23*). The IC_{50} values of samples were evaluated from the dose−response curves of each experiment using Microsoft-Excel computer software. AG (10 mM) was used as a positive control.

BSA−MGO Modification as Determined by SDS−PAGE. BSA was reacted with methylglyoxal in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 9 days in the absence (control) and presence of each sample (100 μM), as described for the BSA−MGO assay (*22*). After modification, samples were repeatedly filtered though PM-10 ultrafiltration membrane (Amicon) using 20 mM phosphate buffer (pH 7.4) and further desalted with Fast Desalting Column H10/10 (Amersham Pharmacia Biotech, Uppsala, Sweden). The effect of MGO modification on the cross-linking and aggregation of BSA was investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) under reducing conditions using a 4% stacking and 10% separating gel. Protein map was visualized by Coomassie blue stain. AG (10 mM) was used as a positive control.

BSA−Glucose Assay. The assay is used to evaluate the ability of naturally occurring flavonoids to inhibit the glucose-mediated protein glycation, and the development of fluorescence of BSA was measurement according to the method of Rahbar et al. (*20*). Briefly, BSA (50 mg/mL) was nonenzymatically glycated by incubation under sterile conditions in 1.5 M phosphate buffer (pH 7.4) at 37 °C for 7 days in the presence of 0.8 M glucose. In certain experiments, the indicated flavonoids were added to the model system in the concentration range of 0−300 μM. Fluorescence of samples was measured at the excitation and emission maxima of 330 and 410 nm, respectively, versus an unincubated blank containing the protein, glucose, and inhibitors. The % inhibition by different concentrations of inhibitor was calculated as described above. AG (10 mM) was used as a positive control.

G.K. Peptide−Ribose Assay. This test is used to evaluate the ability of flavonoids to inhibit the cross-linking of G.K. peptide (last glycation products) in the presence of ribose using the method described by Nagaraj et al. (*24*) and Rahbar et al. (*20*). G.K. peptide (80 mg/mL) was incubated with 0.8 M ribose under sterile conditions in 0.5 M sodium phosphate buffer (pH 7.4) at 37 °C for 24 h. The flavonoids were added to the final concentration of 0−300 μM, respectively. At the end of the incubation period, samples were analyzed for specific fluorescence (excitation, 340 nm; emission, 420 nm). The % inhibition by different concentrations of inhibitor was calculated as described above.

Determination of Free Radical Generation by ESR. Free radical generation was measured according to the method of Finotti et al. (*11*). ESR spectra were measured on glycated samples (BSA−glucose assay) with a reaction mixture containing 0.2 mM PBN. The ESR spectra were recorded on a Burker (EMX-10/12) spectrometer (Karlsruhe, Germany) under the following conditions: incident microwave power, 20.117 mW; frequency, 9.722 GHz; modulation amplitude, 1.50 G; modulation frequency, 100.00 kHz; time constant, 163.84 ms; sweep time, 167.77 s; receiver gain, 1.00×10^{-5}; sweep width, 100.00 G; field center, 3300.00 G. No ESR signals were detected in any of the reagents used in ESR analysis. All spectra were recorded at room temperature.

Statistical Analysis. Results are expressed as the mean ± SD for the number of separate experiments indicated. Data were tested by one-way ANOVA. $p < 0.01$ was assumed to be statistically significant.

RESULTS

Effect of Flavonoids on the Early Stage of Potein Glycation. Figure 1 shows the inhibitory effect of 10 flavonoids (1 flavone, 3 flavonols, 5 flavanols, and 1 flavanone) on the early stage of protein glycation (δ-Glu assay). Previous study has evidenced that δ-Glu is a potent glycating agent of human hemoglobin in vitro (*20*). Co-incubation of human hemoglobin with δ-Glu for 16 h increased of 5.6% glycated hemoglobin over the baseline control (4.3%). The result indicated that luteolin, qucertin, and rutin had the most potent inhibitory effect of 53.6%, 55.4%, and 66.1%, respectively, at a concentration of 100 μM, which more effective than that of AG (25%), indicating that flavonoids are effective in the prevention of HbA_{1C} formation.

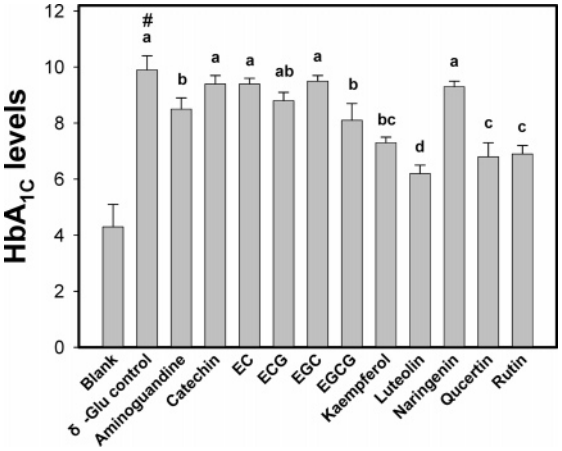

Figure 1. Inhibitory effect of flavonoids on early stage of protein glycation (δ-Glu assay). Whole blood (200 μL) was incubated with 40 μL of δ-Glu (50 mM) in PBS (pH 7.4) at 37 °C for 16 h in the absence (δ-Glu control) and presence of each sample (100 μM). Aminoguandine was used as a positive control at a final concentration of 10 mM. Results are means ± SD for $n = 3$. $p < 0.01$ compared with blank (unglycated, without δ-Glu and sample). Groups with different letters superscripts are significantly different ($p < 0.01$).

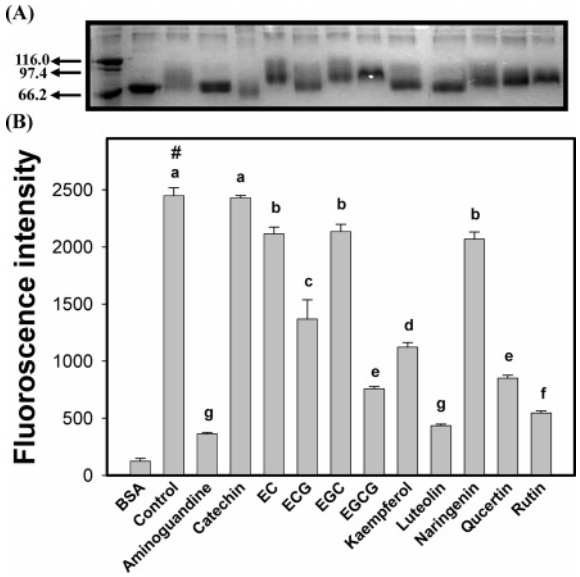

Figure 2. Inhibitory effect of flavonoids on middle stage of protein glycation. (A) The modulation of MGO-mediated protein modification as revealed by SDS–PAGE. BSA (50 mg/mL) was incubated with MGO (0.3 M) under sterile conditions for 9 days in the absence (control) and presence of each sample (100 μM). The first left lane is molecular mass standard (kDa). (B) BSA–MGO assay. Samples were treated as described in Material and Method. Fluorescence of samples was measured at Ex 330 nm and Em 420 nm. Results are means ± SD for $n = 5$. $p < 0.01$ compared with BSA (unglycated). Groups with different letters superscripts are significantly different ($p < 0.01$). AG was used as a positive control at a final concentration of 10 mM.

Effect of Flavonoids on the Middle Stage of Protein Glycation. The inhibition of MGO-mediated protein glycation by flavonoids was determined by protein electrophoresis (**Figure 2A**) and fluorescence formation (**Figure 2B**). MGO readily reacts with protein lysine and arginine residues to produce high molecular weight, cross-linked, products (*22*, *24*). This was demonstrated by protein map of MGO-treated BSA under denaturing conditions using SDS−PAGE. The result showed that in the absence of flavonoids, there was a decrease in the

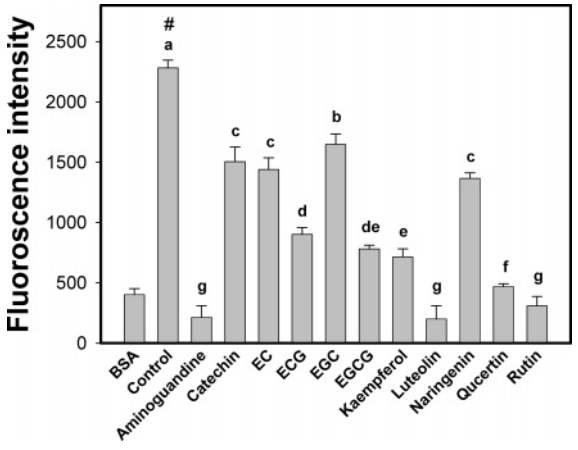

Figure 3. Inhibitory effect of flavonoids on glucose-mediated development of fluorescence of AGEs (BSA–glucose assay). BSA (50 mg/mL) was incubated with glucose (0.8 M) in 1.5 M phosphate buffer (pH 7.4) at 37 °C for 7 days in the absence (control) and presence of each sample (100 μM). Aminoguandine (10 mM) was used as a positive control. Fluorescence of samples was measured at excitation 330 nm and emission 420 nm versus an unincubated blank containing the protein, glucose, and inhibitors. Results are means ± SD for $n = 5$. $p < 0.01$ compared with BSA (unglycated). Groups with different letters superscripts are significantly different ($p < 0.01$).

detectable amount of BSA in its usual position toward the bottom of the gel together with the less resolution and spreading of bands compared to untreated protein. This result was similar to MGO-treated BSA and ovalbumin as described by Lee et al. (*22*). However, when flavonoids (100 μM) were present in the incubation mixture, especially luteolin, rutin, EGCG, and qucertin, both loss of BSA and formation of the high molecular weight protein were inhibited. This was an indication of protection against cross-linking by naturally occurring flavonoids. As for MGO−BSA assay (**Figure 2B**), luteolin, rutin, EGCG, and qucertin exhibited significant inhibition by 82.2%, 77.7%, 69.1%, and 65.3%, respectively, while the other flavonoids showed a 13−54% suppressing effect at a concentration of 100 μM. The inhibition effect of luteolin was similar to that of AG (85.2%). As mentioned above, AG has been demonstrated as an antioxidant and nucleophilic agent, possessing potent scavenging effect on highly reactive carbonyl species (*21*). However, catechin, a tea polyphenol, had no significantly inhibitory effect against MGO-mediated glycation of proteins ($p > 0.01$).

Effect of Flavonoids on the Last Stage of Protein Glycation. Protein cross-linking and fluorescence formation are the major end results of the Maillard reaction (*24*). In the method adopted in this study, BSA was chosen as the model protein and glucose was used as the glycated agent. The products were characterized by their intrinsic fluorescence after an incubation period of 7 days. As shown in **Figure 3**, the result suggested that flavone compound, luteolin, had the most potent inhibitory effect of 91.2% at a concentration of 100 μM, which was approximately equivalent to that of AG (90.6%). At the same concentration, rutin, qucertin, kaempferol, and EGCG exhibited 86.4%, 79.5%, 68.7%, and 65.8% of inhibitory activity, respectively, indicating that flavonoids are effective in the prevention of high glucose-mediated protein modification. In addition, a synthetic peptide (G.K. peptide**)** containing a lysine residue was incubated with ribose for 24 h. This procedure was expected to generate peptides with advanced Maillard reaction product with dimerization through lysine-lysine cross-linking (*24*). Rahbar et al. (*20*) pointed out that co-incubation of G.K. peptide with ribose increased the late glycation products

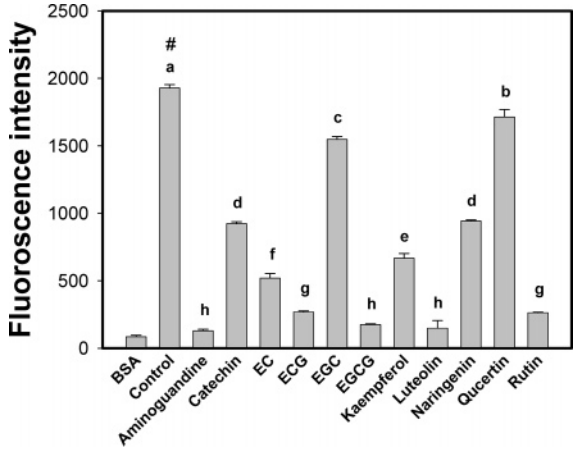

Figure 4. Inhibitory effect of flavonoids on the last stage of protein glycation (G.K. peptide–ribose assay). G.K. peptide (80 mg/mL) was incubated with ribose (0.8 M) for 24 h in the absence (control) and presence of each sample (100 μM). Aminoguandine (10 mM) was used as a positive control. Fluorescence of samples was measured at excitation 340 nm and emission 420 nm versus an unincubated blank containing the protein, glucose, and inhibitors. Results are means ± SD for $n = 5$. $p < 0.01$ compared with BSA (unglycated). Groups with different letters superscripts are significantly different ($p < 0.01$).

Table 1. Summarized Data Obtained by Three Different Methods on Flavonoids Using AG as a Positive Control

	IC_{50} (μM)[a]		
compounds	BSA–MG assay	BSA–glucose assay	G.K. peptide–ribose assay
aminoguandine[b]	5.0 ± 0.8	4.1 ± 1.1	4.8 ± 0.6
catechin	293.1 ± 6.2	147.8 ± 7.3	105.9 ± 11.2
EC	227.4 ± 20.7	141.3 ± 5.2	40.5 ± 6.3
ECG	138.5 ± 14.4	67.5 ± 4.4	27.7 ± 3.8
EGC	214.8 ± 11.4	160.0 ± 8.7	249.9 ± 21.5
EGCG	80.9 ± 4.1	62.0 ± 2.0	26.7 ± 1.6
kaempferol	136.5 ± 4.6	59.6 ± 4.0	69.8 ± 3.0
luteolin	66.1 ± 4.2	16.0 ± 0.9	25.1 ± 5.2
naringenin	198.2 ± 5.8	132.4 ± 11.1	94.7 ± 5.5
qucertin	86.4 ± 9.2	43.0 ± 2.5	186.5 ± 11.8
rutin	71.8 ± 3.4	41.9 ± 2.3	36.7 ± 8.4

[a] The IC_{50} was defined as the concentration of the 50% inhibition. Results are means ± SD for $n = 5$. [b] The IC_{50} concentration of aminoguandine was expressed as mM.

formation. On the basis of these interactions, we have used this model system to evaluate the inhibitory effect of flavonoids on protein cross-linking. As shown in **Figure 4**, four out of 10 flavonoids (ECG, EGCG, kaempferol and luteolin) exhibited substantial anti-cross-linking activities. At a concentration of 100 μM, the inhibitory effects of EGCG and luteolin were all over 85% which approximately equivalent to that of AG (93.3%). ECG and rutin also had 73.1 and 65.4% of inhibitory effect, respectively. The result demonstrated the protection of flavonoids against AGEs formation.

Table 1 summarizes the data obtained by the above assays on 10 flavonoids using AG as a positive control, and the IC_{50} was defined as the concentration of the 50% inhibition of glycation. The results showed that, on each stage of protein glycation, luteolin and rutin had the most potent suppressing effect among all flavonoids with IC_{50} values of 16.0, 66.1, and 25.1 μM for luteolin and 41.9, 71.8, and 36.7 μM for rutin, respectively. These observations suggested the potential inhibition of luteolin on the progression of glycoxidative modification of proteins.

Table 2. Effect of Flavonoids on ESR Spectrum Signal Intensity of PBN Spin Adducts Generated by Protein Glycation

compound	% radical remaining[a]
control	100.0[a]
aminoguandine	16.6 ± 2.9[b]
kaempferol	35.7 ± 4.2[cd]
luteolin	19.5 ± 1.1[b]
naringenin	68.2 ± 9.2[e]
qucertin	29.0 ± 2.4d[b]
rutin	22.1 ± 3.3[b,d]
ECG	67.5 ± 11.4[e]
EGCG	47.7 ± 4.7[c]

[a] Relative ESR signal intensity (%) = $\{[h\Delta H^2(\text{sample})/h\Delta H^2(\text{dpph})]/[h\,\Delta H^2(\text{control})/\,h\Delta H^2(\text{dpph})]\}$*100. h = height of the peak. ΔH = width of the peak. [a–e] Values in column with different letters were significantly different ($p < 0.01$). Data are presented as means ± SD of triplicates.

Scavenging Activity of Flavonoids on the Glycation-Derived Free Radicals. Many studies have indicated that ROS were involved in the Maillard reaction and that the formation of free radicals occurred on the early stage of protein glycation (*25*, *26*). On the basis of the significant inhibitory effect of flavonoids against glycation in vitro **(Figures 1−4)**, ESR spectrometer was applied to investigate the scavenging effect of flavonoids on glycation-derived radicals. The ESR spectra showed that glycation of protein lead to increased free radicals production (data not shown). As shown in **Table 2**, the addition of indicated flavonoids to the reaction system caused a significant decrease in the ESR signal intensity ($p < 0.01$). The scavenging effect of flavonoids on glycation-mediated radical formation was in the order of luteolin (84.4%) > rutin (77.9%) > qucertin (71.0%) > kaempferol (64.3%) > EGCG (52.3%) > ECG (32.5%) > naringenin (31.8%), at a concentration of 100 μM. This trend is also in agreement with the above result of BSA−glucose assay.

Correlation Analysis. Linear regression analysis of data concerning about the flavonoid compounds in the present study showed a strong and positive correlation ($r = 0.79$, $p < 0.01$) between the free radicals scavenging activity and the inhibition of protein glycation (data not shown).

DISCUSSION

In this study, the phenomenon of protein glycation was demonstrated in the reaction mixtures of albumin with sugar by several model systems in vitro. Sugars (glucose, δ-Glu and ribose) and dicarbonyl compound (MGO) are used as glycated agents, which are commonly adopted in many Maillard reaction studies (*9*, *27*−*29*). Human hemoglobin, BSA, and G.K. peptide representing the amine sources could serve as targets for glycated agent, although G.K. peptide is not found in physiology or food systems (*20*, *24*). These experimental methods can uniquely differentiate between specific inhibitors of the early stage (Amadori products), middle stage (RCS), and the last stage of glycation (AGEs formation and cross-linking) (*20*, *29*). The degrees of glycation are analyzed for the development of specific fluorescence, and AG is used as a positive control. Ten flavonoids were investigated, including flavanols (catechin, EC, ECG, EGC, and EGCG), flavone (luteolin), flavonols (kaempferol, qucertin, and rutin), and flavanone (naringenin). They belong to different classes of flavonoids distinguished by their structural features. Our results revealed that 10 flavonoids were able to inhibit fluorescence. A marked reduction on each stage of glycation was observed in the luteolin treatment (**Figures 1−4** and **Table 1**). Moreover, ESR spectra demonstrated that luteolin

significantly inhibited free radicals generation during glycoxidative process (**Table 2**). A highly significant correlation ($r = 0.79$, $p < 0.01$) was observed between antioxidant and antiglycated activity (data not shown).

In the initial stage, glucose reacts with an amine group to form a labile Schiff base that rearranges to the Amadori product. The Schiff base is highly prone to oxidation and free radical generation, which leads to the formation of RCS such as glyoxal (*9*). Given the link mentioned above between glycation and oxidation, we hypothesized that antioxidant flavonoids might possess antiglycoxidative activities. Strong evidence supported the view that glycated albumin is a potential target of therapy in the treatment of diabetic complications (*30*). For human serum albumin in blood plasma, the concentration ranges of Schiff's base adduct and AGEs were 1−5% and 6−15%, respectively (*9*, *31*). This proportion typically increased between 2−3-fold in hyperglycemia (*32*). Having available clinical and experimental information, it could thus be of great interest to propose that administrations of naturally occurring flavonoids are beneficial for the prevention of protein glycation. Daflon 500, a clinical drug which is made up of the flavonoids, had attenuated effect on HbA_{1C} and protein glycation in a group of 28 Type 1 diabetic patients (*33*). Phytoestrogenic isoflavonoids, such as daidzein, genistein, and resveratrol, have been shown to interfere with AGEs-mediated oxidative DNA damage in hypertensive rats, which were appeared to be attributed to direct scavenging action on AGEs-derived radicals (*34*). Recently, Suzuki et al. (*28*) isolated two flavone c-glycosides from the style of *Zea mays*, which exhibited in vitro glycation inhibitory activity (53−64%) similar to that of AG at 1 mM. Antiglycated agents such as green tea extract (*27*) and tomato paste fraction (*29*) were related to their flavonoid contents in a similar system.

Attention has been focused on preventing protein glycation by antioxidants (*3*, *6*). The inhibition of free radicals generation derived form glycation process and subsequent inhibition of the protein modification was considered to be one of the mechanisms of anti-glycation effect. Many data have shown that typical antioxidants/nutrients such as vitamin B1 (thiamine pyrophosphate), B6 (pyridoxamine), C, E, niacinamide, carnosine, and sodium selenite inhibited the in vivo and in vitro AGEs formation (*35*−*37*). The present study showed that flavonoids suppressed fluorescence in a similar model system and approximately followed the order of flavone > flavonol > flavanol > flavanone, with some exceptions (ECG and EGCG). It was found that when protein was subjected to sugar-mediated modification, the addition of flavonoids decreased the HbA_{1C} level (**Figure 1**), the fluorescence intensity of glycation reaction (**Figures 2-4**), and the radical signals of ESR spectra (**Table 2**). Most of these effects were concentration dependent, especially for luteolin (**Table 1**), a natural antioxidative flavone. In addition, the effectiveness of preventing protein glycation by flavonoids examined in this study was related to the structure of these compounds. Luteolin, with hydroxyl groups at the C-5, 7 and 3′, 4′ positions, was the most effective compound on each stage of protein glycation in our study. It indicated that hydroxyl group at the C-3 position would not be the necessary functional group for inhibition. Naringenin, lacking the C-3′hydroxyl group and 2,3-double bond in conjugation with the 4-oxo functional group in the C ring of luteolin, was the less effective as a antiglycated agent. This suggested that the hydroxyl group at the C-3′ position contributed to the AGEs formation inhibitory activity of these compounds. As in flavanols, the presence of the hydroxyl group at the C-5′ and 3′ position in the B ring affected their inhibitory activity against glycoxidative reaction. These results were in agreement with other study that has suggested the possible contribution of the single hydroxyl group at position 5′ of the B ring to the inhibition of glycation (*27*).

RCS are important precursors in the formation of AGEs in vivo (*9*). In a glycation reaction, RCS compounds such as 3-deoxyglucosone, MGO, and GO are more reactive than the parent sugars with respect to their abilities to react with amino groups of proteins to form inter- and intramolecular cross-links of proteins. Another preventive or treatment method is to develop nucleophilic molecules with RCS such as AG, pydidoxamine, OPB-9195, or metformin, etc. (*6*). Such molecules could inhibit AGEs, remove RCS, and prevent AGEs from acting with cells, and compete against AGEs−RAGEs (receptor of AGEs). However, some problems of toxicity have been encountered in phase III clinical trial with AG, so this drug should be considered to be a prototype for many new molecules which are being synthesized and examined in vitro at present (*6*, *20*). Pharmacological intervention to scavenge RCS compounds is likely to be an effective strategy to inhibit the formation of AGEs and prevent AGEs-mediated disease. Therefore, research and development on the scavenging of RCS should be another direction of controlling diabetic complications. MGO has been identified as an intermediate in nonenzymatic glycation, and increased levels of MGO have been reported in patients with diabetes (*9*). This highly reactive speciesis able to induce oxidative degradation of protein in vitro (*38*). Findings from the present study indicated that luteolin and rutin had potential ability in preventing glycation by MGO (**Figure 2**). Although the anti-glycated ability of flavonoids were observed in **Table 1** without distinction in chemical structure, luteolin showed the highest ability, implying flavone molecule may possibly exhibit a MGO-scavenging effect.

Another part of this experiment worthy of notice is that most flavonoids showed anti-cross-linking activity in G.K. peptide−ribose assay. Protein cross-linking is a major end result of the Maillard reaction. In the experiment, a synthetic peptide containing a lysine residue (G.K. peptide**)** was incubated with ribose for 24 h. This procedure was expected to generate peptides with advanced Maillard reaction product with dimerization through lysine-lysine cross-linking (*24*). Rahbar et al. (*20*) pointed out that co-incubation of G.K. peptide with ribose increased the formation of late glycation products. On the basis of these interactions, flavonoids were studied with an assay using the G.K. peptide−ribose system to evaluate their ability in inhibition of protein cross-linking. The results suggested that the inhibitory activity of flavonoids were not only due to their antioxidant properties, but also some other mechanisms needed to be clarified in future.

Recent studies in diabetic patients and rats have stated that vitamin E supplementation can reduce glycated hemoglobin levels in blood. The mechanism by which vitamin E suppresses glycation of proteins is still not known (*36*). Yim et al. (*39*) indicated that glycation of proteins generated some active centers for catalyzing one-electron oxidation−reduction reactions, which mimics the characteristics of the metal-catalyzed oxidation system. In addition, glycated proteins accumulated in vivo may provide stable active sites for catalyzing the formation of free radicals. Results from Jiang et al. (*7*) and Sakurai and Tsuchiya (*40*) also demonstrated that ROS such as hydrogen peroxide and superoxide anion were generated during glycation process. Moreover, diabetic patients exhibited elevated levels of iron and copper ions that, in the presence of glycated proteins, have been shown in vitro to generate free radicals (*4*, *40*). Wolff et al. (*4*) indicated that glucose itself could auto-oxidize to form hydrogen peroxide and keto aldehydes in the presence of transition metal

ions, and subsequently accelerated the formation of AGEs and oxidative DNA damage (*8*). In this study, we have provided evidence that glycated BSA in the BSA−glucose assay, showed significant effect on free radicals generation. These results were in agreement with other study that has suggested the glycation of albumin leading to the increased production of ROS (*11*). Protein glycation and oxidation could be inhibited effectively when flavonoids were added during the glycoxidative process. Thus, it could be concluded that the antioxidant activity of flavonoids was possibly correlated with their abilities to scavenge radicals, especially for luteolin and rutin.

In conclusion, the results obtained in the present study shown that luteolin has the most potent inhibitory effects on each stage of protein glycation. The scavenging of free radicals derived from glycation plays an important role in this reaction. This mechanism may help to provide a protective effect against hyperglycemia-mediated protein damage.

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