In vitro screening of radioprotective properties in the novel glucosylated flavonoids

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Abstract. Novel glucosyl flavonoids are developed by the addition of glucose to naturally occurring flavonoids. Flavonoids are known antioxidants that possess radioprotective properties. In order to investigate the radioprotective properties of novel glucosyl flavonoids, *in vitro* DNA double-strand breaks (DSBs) analysis was carried out. In the present study, Quercetin, Naringenin, and Hesperetin groups of flavonoids included in the natural and novel glucosyl 13 flavonoids were investigated. Flavonoids were mixed with Lambda DNA, and subsequently exposed to gamma-rays. Furthermore, DNA DSB yields were visualized by gel electrophoresis. Quercetin derivatives displayed reduced DNA DSB formation at 10 μM. At a high concentration, the majority of flavonoids displayed radioprotective properties as a reduction of DSB yields. Suppression of DSB formation was confirmed via the molecular combing assay for Quercetin, and three monoglucosyl flavonoids. Glucosylation showed positive effects for radioprotection and monoglucosyl-Rutin showed superior radioprotective properties when compared to monoglucosyl-Naringin and Hesperidin. In addition, Quercetin derivatives had greater total antioxidant capacities and DPPH radical scavenging ability than other flavonoid groups. Since Quercetin, Isoquercetin, and Rutin display poor water solubility, monoglucosyl-Rutin, maltooligosyl-Isoquercetin, and maltooligosyl-Rutin may be better radioprotective agents and easily bioavailable with increased water solubility.

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Introduction

A noteworthy property of ionizing radiation is its ability to produce highly reactive free radicals [hydroxyl radical (•OH)] (1). Free radicals, whether formed by indirect or direct action, eventually damage biologically important materials such as DNA and lead to radiation-induced damage formation (2). This damage may be single-strand breaks (SSBs), base damage or the most molecularly deleterious double-strand breaks (DSBs). Effectively, the harmful damage produced by strong oxidation can damage the body's tissues and cells, thereby causing chronic diseases including cancer and accelerated aging (3,4).

As with all biological systems there are methods of protection against harmful exogenous genotoxic mutagens (5,6). Flavonoids natural occur in plants and other leafy organisms (7,8). Flavonoids are also commonly regarded as antioxidant (9). New evidence suggests that these antioxidant properties may help plants protect themselves from harmful UV and ionizing radiation by absorbing harmful free radicals (10,11).

Moreover, if a flavonoid is void of a glucose entity, then they are referred to as aglycons. Naturally occurring flavonoids hold none or few glucosyl residues coinciding with an aglycon (7). Additionally, novel flavonoids are synthesized by glucosylating the original glucosyl flavonoids. Glucosylation changes many properties of a flavonoid and specifically enhances their solubility in water (12,13). Our previous study supports that glucosylation reduces cellular toxicity and genotoxicity in tissue culture systems (14). Furthermore, we suggested this may be due to a reduction of bioavailability in cells by glucosylation or a reduction in chemical properties, such as an inhibitory effect of PARP (15).

Previous findings suggest that a select few flavonoids and glucosyl flavonoids possess radioprotective properties (16). These include glucosyl chemicals such as the glucosylation of an ascorbic acid product, and ascorbic acid-2-glucoside, which did not alter the radioprotective properties of ascorbic acid (17,18). However, the effect that glucosylation has on the radioprotective properties of flavonoids remains to be determined.

Among radiation-induced DNA damage, DNA DSBs contribute the most towards radiation-induced cell death. DNA DSBs can be measured by an array of assay systems such as neutral elution (19,20), gel electrophoresis (21,22), and immunohistochemistry (22,23). For the present study 13 natural and novel synthesized flavonoids were used. Naked Lambda DNA was run through gel electrophoresis to visualize the suppression of DNA DSB formation by the chemicals. In the present study, we investigated whether glucosylation affects the antioxidant and radical scavenging properties of flavonoids. The radioprotective effects of novel glucosyl flavonoids were constructed visually via a molecular combing technique (24).

Materials and methods

Chemicals. The flavonoids were obtained from the Toyo Sugar Refining Co., Ltd. (Tokyo, Japan). Fig. 1a shows the chemical structures of natural and novel synthetic flavonoids used in the present study. The Quercetin group treatment consisted of Quercetin (302.23 g/mol), Isoquercetin (464.58 g/mol), Rutin (610.51 g/mol), monoglucosyl (MG)-Rutin (772.5 g/mol), and maltooligosyl (MO)-Isoquercetin (914.38 g/mol), MO-Rutin (1104.4 g/mol). The Naringenin group treatment consisted of Naringenin (272.257 g/mol), Naringin (580.54 g/mol), MG-Naringin (742.54 g/mol) and MO-Prunin (922.54 g/mol). The Hesperetin group treatment consisted of Hesperetin (302.28 g/mol), Hesperidin (610.57 g/mol) and MG-Heperidin (610.57 g/mol). Flavonoids were dissolved in double-distilled water. Their pH values at 100 μM solution showed slightly acidic to neutral, ranging from 6.0 to 6.9. Turbidity of solution was measured by VersaMax ELISA microplate reader with SoftMax Pro (Molecular Devices, LLC, Sunnyvale, CA, USA) software at 660 nm. Lambda DNA in Tris-EDTA was purchased from Nippon Gene Co., Ltd. (Tokyo, Japan). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and freshly prepared for each experiment in ethanol.

Irradiation. For gamma-ray irradiation, a J.L. Shepherd Model Mark I-68 nominal 6000 Ci ^{137}Cs irradiator (J.L. Shepherd and Associates, San Fernando, CA, USA) was used at room temperature (20˚C). The dose rate was 3.9 Gy/min.

Electrophoresis and DNA DSBs. Two microliters of Lambda DNA (12 μg/μl) was mixed with 18 μl of each concentration (0, 10, 100 μM) of flavonoids. Lambda DNA *Hin*dIII digest (0.2 μl) was used as a marker. A total of 20 μl of sample was exposed to gamma-rays, and run through electrophoresis after mixing DNA with 4 μl of 6X DNA loading dye. The Lambda DNA and the marker were immediately immersed in a 60˚C water bath for 5 min to allow for the denaturion of DNA and the samples were placed on ice for 3 min. The gel comprised 0.5 g agarose with 50 ml of 1X TAE buffer. Electrophoresis was carried out at 100 V (7.63 V/cm) for 1 h in 1X TAE buffer. After electrophoresis, the DNA was stained in ethidium bromide solution for at least 3 h. The gel was washed with 1X TAE buffer for 1 h. Gel images were obtained with the Molecular Imager Gel Doc XR system with Image Lab software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Single molecule observation. To evaluate the induction of DNA DSBs visually, single molecule observation was conducted using a molecular combing method (24). Before irradiation, the 60x24 mm cover glass (no. 24601; Matsunami Glass Ind., Inc., Osaka, Japan) was pretreated with 30% H_2O_2 at 4˚C for at least 4 h, and a coverslip was washed with distilled water and 100% ethanol. After irradiation, 100 μl samples were stained with 0.02 μl 1 mM YOYO-1 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), a fluorescent dye reagent mixed with 0.2 μl 14.3 M 2-mercaptoethanol and 1 μl DMSO.

The cover glass was set at 45˚ angles. A 50 μl Lambda DNA solution was dropped onto the top end of the coverslip and allowed to run down to the bottom due to gravity. Additionally, the coverslip was placed on the glass slide without a mounting agent. Images of Lambda DNA were captured using a Zeiss Axioplan microscope (Carl Zeiss AG, Oberkochen, Germany) with Q-Imaging EXi Aqua CCD camera with QCapture Pro software (QImaging, Surrey, BC, Canada). The evaluation was performed by measuring the length of Lambda DNA using QCapture Pro software. At least 50 DNA samples were scored for three independent experiments.

Total antioxidant capacity (TAC). The antioxidant activity of each chemical was measured by a Total Antioxidant Capacity kit (Sigma-MAK187) according to the manufacturer's instructions. Cu^{2+} reagent was diluted 50-fold with assay diluent. Five microliters of each concentration of flavonoid were diluted at a 1:1 ratio with 50-fold diluted Cu^{2+} reagent. The samples were mixed and incubated for 90 min at room temperature. The absorbance of each sample was measured at 570 nm with NanoDrop (Thermo Fisher Scientific, Inc.). The values obtained with double-distilled water were used as the control. Each data point was produced by mean of three replicates per experiment and three independent experiments were carried out.

DPPH antioxidant properties. DPPH analysis was performed as previously reported (25). One hundred microliters of 100 μM DPPH, 80 μl of ethanol and 20 μl each concentration of flavonoid were mixed. The mixtures were agitated vigorously and allowed to stand at room temperature for 30 min. Absorbance was measured at 517 nm using a VersaMax ELISA Microplate Reader. DPPH scavenging activity was calculated by absorbance of control minus absorbance of sample divided by absorbance of control (26). Each data point was produced by mean of triplicates per experiment and three independent experiments were carried out.

Statistical analysis. Statistical comparison of the mean values was performed using a 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.

Results

Turbidity of each flavonoid at absorbance at 660 nm. Our results indicated that natural flavonoids were water insoluble and with increased glucosylation this resulted in improved water solubility. In the Quercetin group, the natural flavonoids, Quercetin, Isoquercetin and Rutin had poor solubility in water,

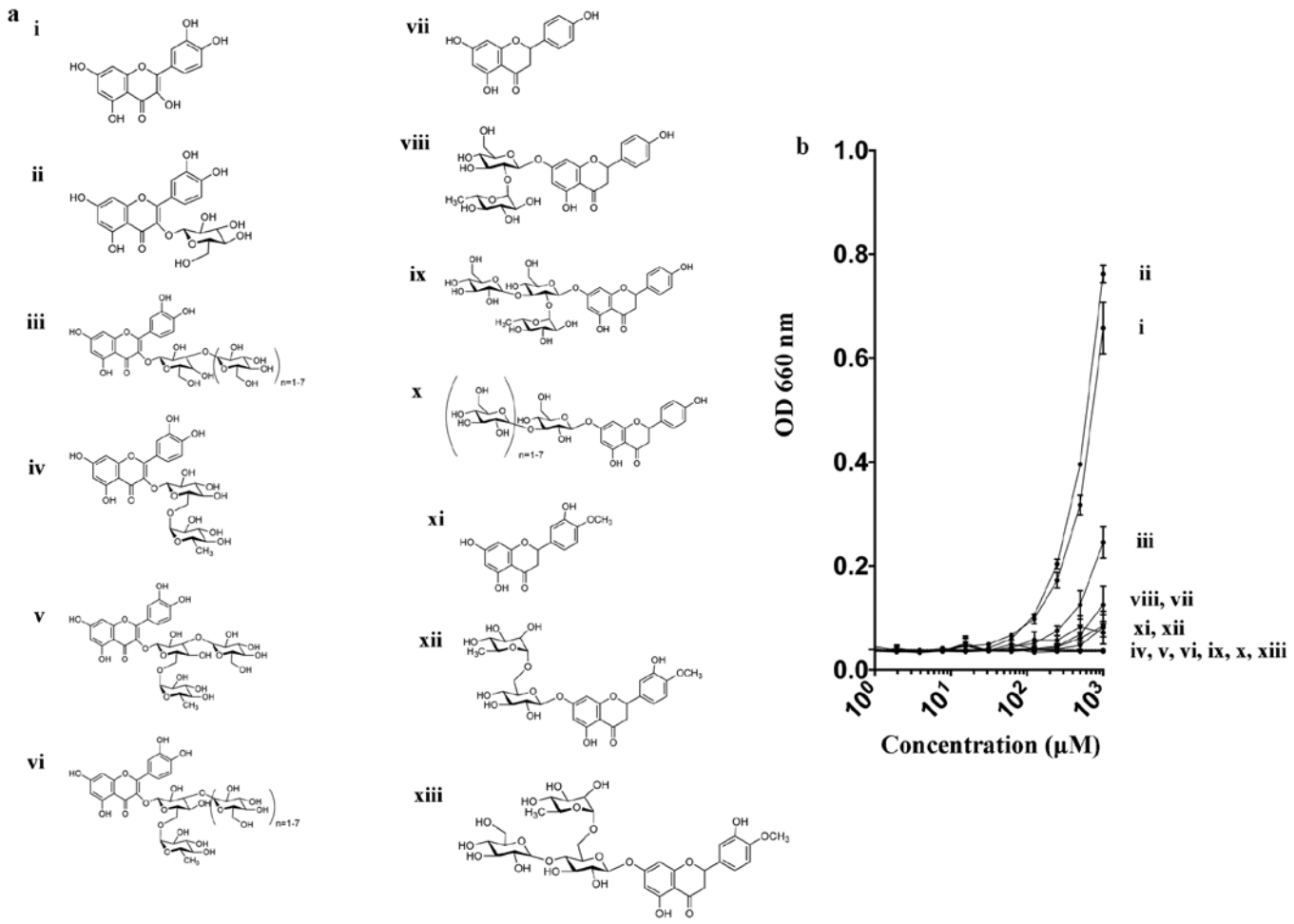

Figure 1. (a) Various chemical structures of 13 flavonoids in the present study. i) Quercetin, ii) Isoquercetin, iii) Rutin, iv) MG-Rutin, v) MO-Isoquercetin, vi) MO-Rutin, vii) Naringenin, viii) Naringin, ix) MG-Naringin, x) MO-Prunin, xi) Hesperetin, xii) Hesperidin, and xiii) MG-Hesperidin. (b) Turbidity of flavonoid solution measured at 660 nm. Dashed line indicates background water value. Error bars indicate standard error of the means.

whereas monoglucosyl and maltooligosyl flavonoids had complete solubility, even at 1 mM concentration (Fig. 1b).

Gel electrophoresis. In the gel electrophoresis assay, fragmented Lambda DNA migrated more and was observed as a smear. Fig. 2a shows the DNA DSBs were dose-dependent between 0 and 20 Gy for Lambda DNA in this assay. Fig. 2b shows that flavonoids did not produce DSBs by themselves. Fig. 2c and d shows the DSB radioprotective abilities of flavonoids at 10 and 100 μM. For 10 μM, the Quercetin group, particularly Isoquercetin, MG-Rutin, and MO-Isoquercetin MO-Rutin, displayed the strongest radioprotective abilities. All the flavonoids displayed positive radioprotective abilities at concentrations of 100 μM, with the exception of Quercetin and Hesperetin. Therefore, glucosylation enhanced the radioprotective properties observed as a reduction of DSB formation.

Molecular combing. The flavonoids Quercetin (no glucosylation, water insoluble), MG-Rutin (glucosylated water soluble), MG-Naringin (glucosylated water soluble) and MG-Hesperidin (glucosylated water soluble) were tested for molecular combing. Fig. 3 shows the result of molecular combing. Each intact Lambda DNA molecule was observed as stretched DNA, and radiation-induced fragments of Lambda DNA were observed in a single molecule (Fig. 3a and b). There was a dose-dependent DSB formation of 0 to 20 Gy. Radiation (20 Gy) produced approximately 5 DSB/Lambda DNA. After exposure to 20 Gy, the addition of 10 μM MG-Rutin showed the strongest reduction in the frequency of DSB among the four flavonoids ($p<0.05$) (Fig. 3d), and the addition of 100 μM of any MG-flavonoids greatly reduced the DSB frequency ($p<0.005$) but not Quercetin ($p=0.84$). MG-Naringin and MG-Hesperidin (10 μM) did not show statistically significant radioprotection compared to the 20 Gy-irradiated control ($p>0.1$).

TAC. Fig. 4 shows the total antioxidant capacity of 13 flavonoids, which was measured at the optical density of 570 nm. Quercetin group showed good TAC at each concentration compared with other flavonoid groups. The Quercetin groups also exhibited antioxidant properties similar to that of ascorbic acid at 10 and 100 μM, with the exception of 10 μM Quercetin. The Naringenin and Hesperetin groups were poor antioxidants compared to the ascorbic acid at the tested concentrations. No clear correlation between glucosylation and total TAC was observed.

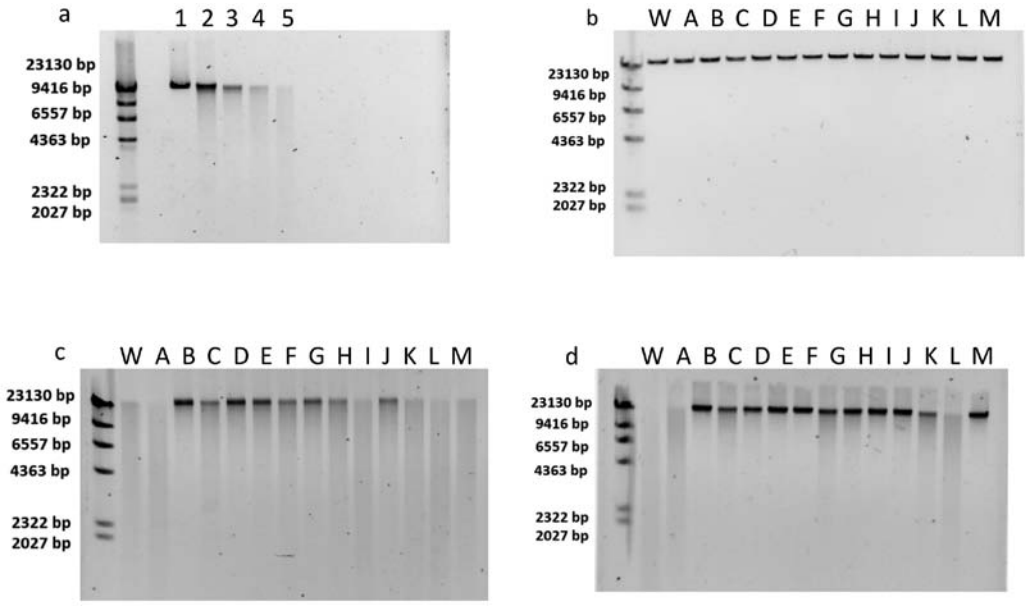

Figure 2. (a) Dose-dependent DNA double-strand breaks (DSBs). Lane 1, 0 Gy; lane 2, 5 Gy; lane 3, 10 Gy; lane 4, 15 Gy; and lane 5, 20 Gy. (b) Effect of flavonoids without irradiation. (c and d) Radioprotective effects of flavonoids at 10 μM with 20 Gy and radioprotective effects of flavonoids at 100 μM with 20 Gy. First left lane is Lambda DNA-*Hin*dIII digest ladder; lane W, is negative control with water; lane A, Quercetin; lane B, Isoquercetin; lane C, Rutin; lane D, MG-Rutin; lane E, MO-Isoquercetin; lane F, MO-Rutin; lane G, Naringenin; lane H, Naringin; lane I, MG-Naringin; lane J, MO-Prunin; lane K, Hesperetin; lane L, Hesperidin; and lane M, MG-Hesperidin.

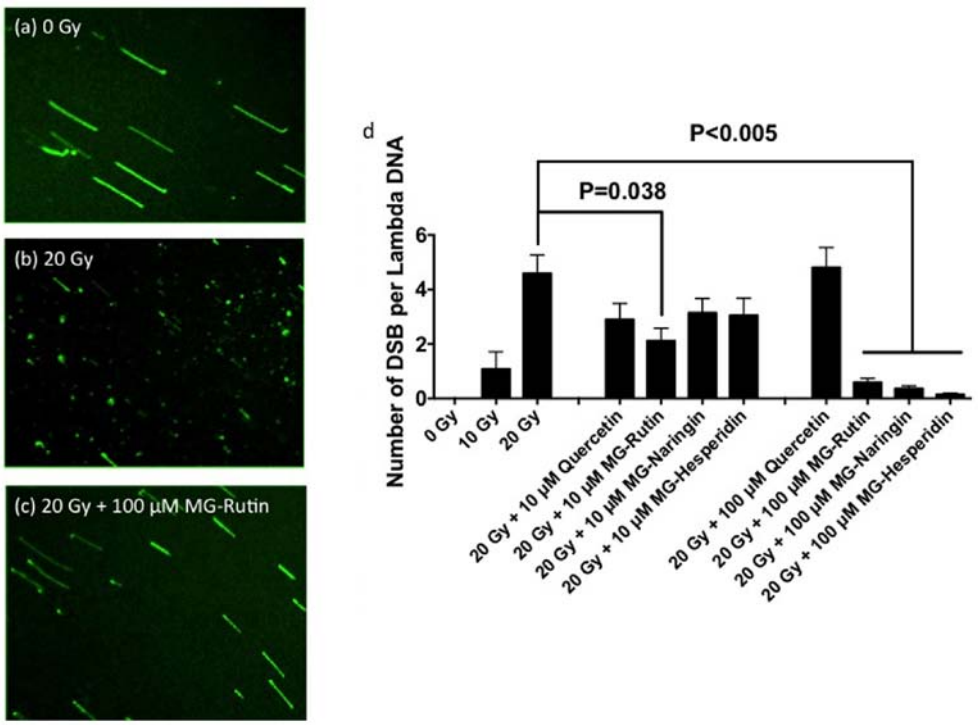

Figure 3. Molecular combing experiments. Images of molecular combing for (a) 0 Gy, (b) 20 Gy, and (c) 20 Gy + 100 μM MG-Rutin. (d) Number of DSB with radiation and flavonoids.

DPPH radical scavenging properties. DPPH is a stable free radical. Fig. 5 shows the result of DPPH radical scavenging properties. We used ascorbic acid as a control to compare the radical scavenging ability of 13 flavonoids. The Quercetin group showed much better radical scavenging ability than the other two groups. The result showed glucosylation slightly reduced free radical scavenging ability in the Quercetin group at 10 and 100 μM. The Naringenin and Hesperetin groups were poor radical scavengers at 10 and 100 μM.

Discussion

The results of the present study displayed that radioprotective properties for DSBs were dependent on flavonoid groups at

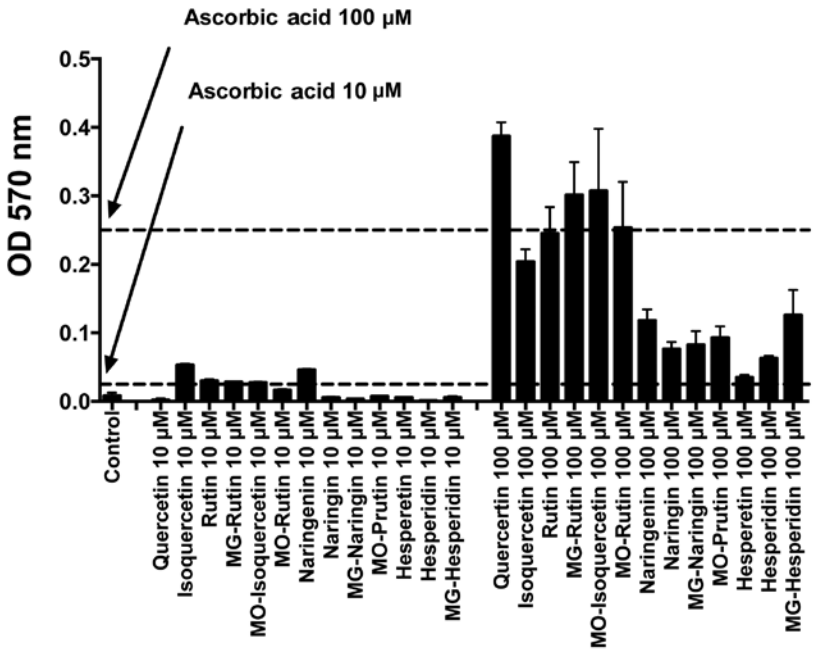

Figure 4. Total antioxidant capacity of 13 flavonoids. Dashed lines are 10 and 100 μM ascorbic acid. Error bars indicate standard error of the means.

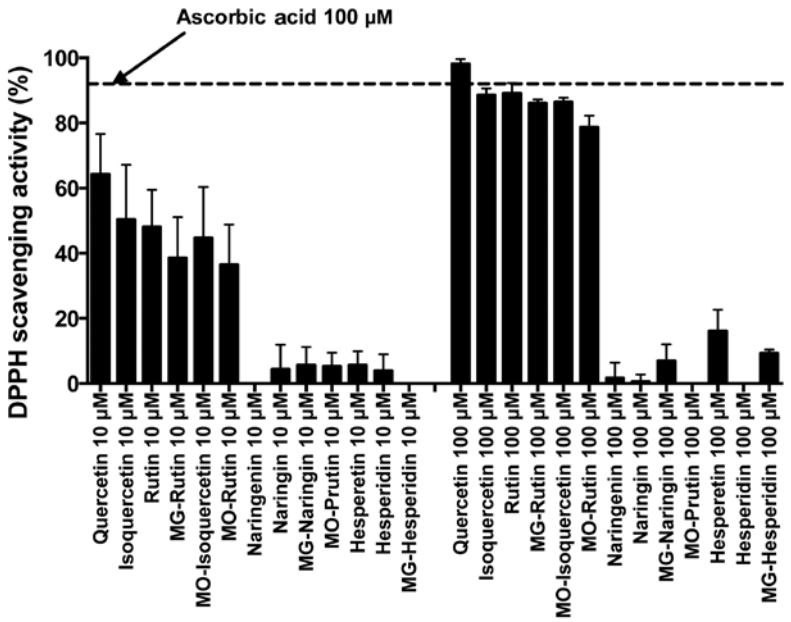

Figure 5. DPPH radical scavenging ability of 13 flavonoids. Dashed line is 100 μM ascorbic acid. Error bars indicate standard error of the means.

a low concentration. All the Quercetin derivatives showed radioprotection after 20 Gy, except natural Quercetin. On the other hand, the majority of flavonoids showed radioprotection at 100 μM. Therefore, an increased uptake of flavonoids may protect cells from DSB formation induced by radiation exposure. Better radioprotective properties in DSB reduction for the glucosylated Quercetin group were matched with total antioxidant capacity and radical scavenging ability. Although Quercetin showed strong radical scavenging and antioxidant properties, Quercetin did not show DSB reduction even at 100 μM. This may be associated with their poor water solubility or poor interaction with DNA molecules (27). Since Quercetin, Isoquercetin, and Rutin displayed poor water solubility, we suggested that MG-Rutin, MO-Isoquercetin, and MO-Rutin are better radioprotective agents and easily bioavailable with increased water solubility. Recently, enhanced solubility of Quercetin by forming composite particles with glucosyl flavonoids was reported (28). Enhanced solubility can be produced by the glucosylation of flavonoids. The formation of composite particles may be an alternative strategy to enhanced solubility and may lead to radioprotection.

Furthermore, lambda DNA is a double-stranded DNA with 48,502 base pairs. This size is separable with simple gel electrophoresis and is readily obsevable miscroscopically when using the molecular combing method. Therefore, Lambda DNA is a good test subject for screening purposes such as that

identified in the present study. Moreover, DNA DSBs lead to serious biological consequences, including mutations, carcinogenesis, or apoptosis (29). We used two assays to measure DSBs. The gel electrophoresis assay is convenient and can be applied with multiple samples. On the other hand, the molecular combing method is time-consuming and labor intense. However, in the molecular combing method one can visualize individual DNA DSB in a single molecule. DNA fragment size can be directly measured in this method. Therefore, gel electrophoresis is suitable for screening purposes and detailed analysis and confirmation can be achieved using the molecular combing method.

However, there were some potential pitfalls in our procedures. The molecular combing method may have some difficulties for reproducibility without careful sample handling. It is possible that different sizes of Lambda DNA do not attach onto cover glass equally. We found that the length of the Lambda DNA decreased sharply with vigorous pipetting, as previously reported (30). We used a 60x24 mm coverslip as previously reported (24) after we tested several different sizes of coverslips. Interestingly, 55x24 and 45x24 mm coverslips from the same company did not produce a better quality of Lambda DNA stretch. It is not known why Lambda DNA stretched well at 60x24 mm, while other size slides did not, but it may be associated with the different surface tension.

In conclusion, antioxidant and scavenging capacity are well related to DNA DSB formation reduction. Glucosylation affected antioxidant and free radical scavenging abilities to some degree. Although some flavonoids, especially glucosylated ones such as MG-Rutin and MO-Rutin, have good protection for *in vitro* double-stranded DNA, it may be limited to *in vitro* radioprotective effects. Further *in vitro* cell culture studies and most importantly *in vivo* studies are required to prove glucosyl flavonoids are good radioprotectors.

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