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Inhibitory Effects of (-) Epigallocatechin Gallate and Quercetin on High Glucose-induced Endothelial Cytotoxicity*

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Functional damage to microvascular endothelial cells by hyperglycemia is thought to be one of the critical risk factors in the impaired wound healing seen with diabetes mellitus. It is also thought that oxidative stress plays a significant role in this endothelial cell dysfunction. The present study examined the differential effects of flavonoids on endothelial cell dysfunction under high glucose conditions. Human endothelial cells exposed to 30 mmol/L glucose for 7 d were pre-treated with various flavonoids and pulse-treated with 0.2 mmol/L H_2O_2 for 30 min. High glucose markedly decreased cell viability with elevated oxidant generation and nuclear condensation. H_2O_2 insult exacerbated endothelial cytotoxicity due to chronic exposure to high glucose. (-)Epigallocatechin gallate and quercetin improved glucose-induced cell damage with the disappearance of apoptotic bodies, whereas apigenin intensified the glucose cytotoxicity. In addition, cell viability data revealed that these flavonoids of (-)epigallocatechin gallate and quercetin substantially attenuated both high glucose- and H_2O_2- induced dual endothelial damage. These results suggest that (-)epigallocatechin gallate and quercetin may be beneficial agents for improving endothelial cell dysfunction induced by high glucose and may prevent or reduce the development of diabetic vascular complications.

Key words: Diabetes, Endothelial cells, Flavonoids, High glucose, Oxidative stress

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INTRODUCTION

Uncontrolled hyperglycemia is the main risk factor in the development of diabetic vascular complications,[1,2] and vascular endothelial cells are the first cells targeted by hyperglycemia.[3] The vascular lesions characteristic of human and experimental diabetes suggest the primary involvement of vascular endothelium.[3] Hyperglycemia induces the production of reactive oxygen species (ROS) and nitric oxide,[2,4] which can subsequently induce endothelial dysfunction and is closely related to diabetic vascular complications.[5] It has been shown that the exposure of endothelial cells to elevated glucose levels causes glucose oxidation, resulting in the generation of excess ROS.[5,6] Accordingly, a reduction in cellular antioxidant reserves might be attributed to endothelial cell dysfunction in diabetes, even in patients with well-controlled glucose levels.[7] However, the mechanism of endothelial injury by hyperglycemia is still poorly understood.

Numerous studies have previously shown that phenolic compounds have considerable antioxidant abilities under various oxidative circumstances.[8-10] There is compelling evidence that the distinct structures of these compounds can partially explain their antioxidant activities.[8,9] Resveratrol blocks the involvement of oxidative stress in high glucose-induced apoptosis of human leukemia cells by virtue of its antioxidant property.[11] In addition, procyanidins have insulin-like effects in insulin-sensitive cells that could help to explain their anti-hyperglycemic effect *in vivo*.[12] These effects must be added to their antioxidant activity to explain why they can improve diabetic situations.

Based evidence in the literature that high glucose-induced oxidative stress is a possible mechanism in the development of diabetic vascular complications,[13] the present study attempted to examine the effects of diverse flavonoids

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including (-)epigallocatechin gallate and quercetin on endothelial cell toxicity and oxidant generation in high glucose-exposed human umbilical vein endothelial cells (HUVEC). The participation of antioxidant features of flavonoids in inhibiting the cytotoxicity was revealed.

MATERIALS AND METHODS

1. Materials

M199 medium chemicals, D-(+)-glucose, polyphenolic flavonoids [flavanol (-)epigallocatechin gallate, flavonol quercetin, flavanones of hesperetin, hesperidin and naringin, flavone apigenin], human epidermal growth factor and hydrocortisone were obtained from Sigma Chemical (St. Louis, MO), as were all other reagents, unless specifically stated elsewhere. Collagenase was purchased from Worthington Biochemicals (Lakewood, NJ). Fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were purchased from Cambrex Corporation (East Rutherford, NJ). 3- (4, 5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT) was purchased from DUCHEFA Biochemie (Haarlem, Netherlands). All test flavonoids were solubilized by dimethyl sulfoxide (DMSO) for culturing with cell;[14] the final culture concentration of DMSO was $\leq 0.5\%$.

2. Preparation of Human Endothelial Cells

HUVEC were isolated from umbilical cords (Department of Obstetrics and Gynecology, Hallym Sacred Hospital, Chuncheon, Korea) using collagenase as described elsewhere.[15] Cultures were maintained in 37 ℃ humidified atmospheres with 5% CO_2 in the air. Cells were cultured in 25 mmol/L HEPES-buffered M199 containing 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin supplemented with 0.075 mg/mL human epidermal growth factor and 0.075 mg/mL hydrocortisone. Cells were passaged at subconfluency with Trypsin-EDTA and used within 10 passages. Endothelial cells were confirmed by their cobblestone morphology and uptake of acetylated LDL (Molecular Probes Inc., Eugene, OR).[16]

3. Cell Culture Experiments and H_2O_2-induced Oxidant Stress

HUVEC were plated at 80~85% confluence in all experiments and treated with 5.5 or 30 mmol/L glucose added to M199 media for 24 h or 7 d. Subsequently, cell viability in the absence and presence of 0.2 mmol/L H_2O_2-induced oxidant stress was measured. In experiments for oxidative stress with H_2O_2, HUVEC exposed to high glucose for 24 h or 7 d were treated with H_2O_2 for 30 min in the absence and presence of 25 μmol/L flavonoids.

4. Cell Viability

At the end of the 24 h incubation period, MTT assay was performed to quantitate cellular viability.[17] HUVEC were incubated in a fresh medium containing 1 mg/mL MTT for 3 h at 37 ℃. After removal of unconverted MTT, the purple formazan product was dissolved in 0.5 mL isopropanol through gentle shaking. Absorbance of formazan dye was measured colorimetrically at λ=570 nm with background subtraction using λ=690 nm.

5. Cellular Oxidant Generation

Oxidant generation of HUVEC was measured as a previously described method with a minor modification.[18] This method was based on ROS conversion of 2′,7′-dichlorodihydrofluorecein diacetate (DCFH, Sigma Chemicals) to 2′,7′-dichlorofluorecein diacetate (DCF), a fluorescent dye. The cellular ability to produce ROS was measured by DCF fluorescence. This reaction is initiated primarily by peroxides, mainly H_2O_2. Cells challenged with high glucose and/or H_2O_2 were washed twice with phosphate buffered saline (PBS) and loaded for 30 min with 10 μmol/L DCFH, freshly prepared in pre-warmed M199 (+2% FBS). Fluorescent images were taken using fluorescence microscopy.

6. Nuclear Morphology

HUVEC grown on a cover glass in a well were cautiously washed with PBS and then fixed with ice-cold 4% formaldehyde for 30 min. The nuclear stain Hoechst 33258 (Molecular Probes inc.) was added to cultures at a final concentration of 5 μg/mL for 15 min at RT to allow uptake and equilibration before microscopic observation. After being rinsed with PBS, the cover slips were mounted while wet in a PermaFluor aqueous mounting solution (Sigma Chemicals). Cells containing fragmented or condensed nuclei were considered apoptotic, while those containing diffuse and irregular nuclei were considered necrotic.[19] The nuclear morphology was observed using an Olympus BX50 fluorescent microscope.

7. Data Analysis

The data are presented as mean±SEM. Statistical analyses were conducted using Statistical Analysis Systems statistical software package version 6.12 (SAS Institute Inc., Cary, NC). Significance was determined by one-way ANOVA, followed by Duncan multiple range test for multiple comparisons. Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

1. High Glucose-induced Cytotoxicity

Diabetes mellitus is characterized by hyperglycemia and vascular complications including microangipathy and macroangiopathy.[1,13] Since endothelial cells are the intimal layer of the vessel wall, it has been proposed as the prominent target of high ambient glucose and other toxic factors under diabetes states. Hyperglycemia induces the production of ROS and hence triggers endothelial dysfunction and diabetic vascular complications.[2,4,5] A reduction in cellular antioxidant reserve might be responsible for endothelial cell dysfunction in diabetes, even in patients with well-controlled glucose levels.[7] However, the mechanisms involved in eliciting endothelial dysfunction by hyperglycemia are still poorly understood.

The present study examined the acute and chronic effects of high glucose on endothelial cells, the prominent target of high ambient glucose in the vasculature. Cell viability data showed that high glucose of 30 mmol/L retarded cell growth and decreased cell viability by ≈10% for 24 h as compared to cells grown in lower glucose medium at 5.5 mmol/L (Fig. 1A). Chronic exposure of high glucose resulted in a≥25% drop in cell viability (Fig. 1B). A 30 min pulse treatment with H_2O_2 caused ≈30% cell death within 24 h under 24 h-acute glucose culture conditions, where H_2O_2 aggravated endothelial cell viability was≥ 50% with marked intracellular oxidant generation under 7 d-chronic culture conditions with high glucose (Figs. 1 and 2). High glucose elicited rapid oxidant generation, which was further enhanced by H_2O_2 insult, manifested by DCF-fluorescence staining (Fig. 2). Both high glucose- and H_2O_2-exposed cells remained DCF with staining more positive and damaged. These results indicate that endothelial cell injury may be partly attributable to an accumulation of intracellular oxidants due to high glucose and that the glucose-induced injury was exacerbated by the dual oxidant effects of H_2O_2.

Oxidative stress contributes to cellular injury and appears to be the common apoptotic mediator.[20] The literature has supported the role of ROS in apoptotic cell death. This study attempted to explore whether high glucose may lead to cell death via apoptotic processes. As shown in Fig. 3, 30 mmol/L high glucose produced cells that exhibited fragmented and/or condensed nuclei and non-nucleated cell fragment apoptotic bodies (Fig. 3A, arrows in microphotographs with Hoechst 33258 nuclear stain). In the H_2O_2-untreated glucose control cells, there were no signs of morphological nuclear damage or chromatin condensation, which are characteristics of apoptosis (Fig. 3A). Therefore, treatment of HUVEC with high glucose

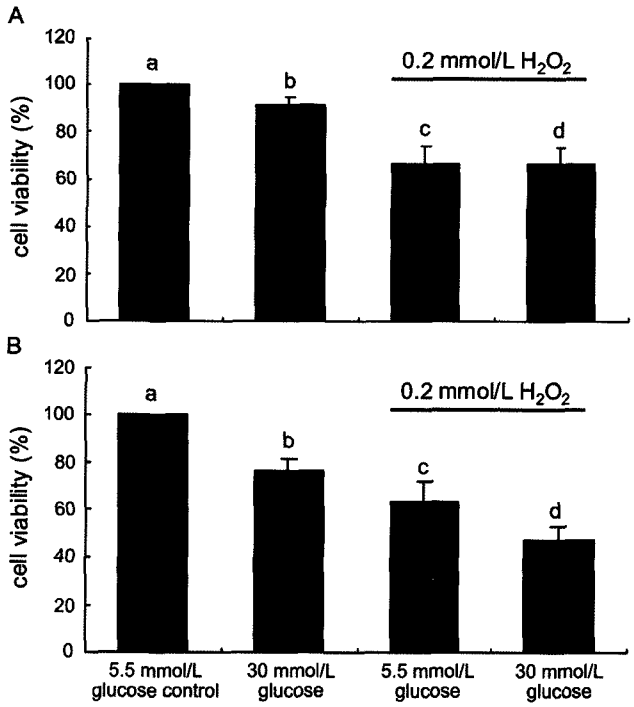

Fig. 1 Cell viability of HUVEC challenged with H_2O_2-induced oxidant stress following treatment with high dose of glucose.

HUVEC were pre-treated with 30 mmol/L glucose for 24 h (A) or 7 d (B). After incubation for 24 h or 7 d, cells were pulse-treated with 0.2 mmol/L H_2O_2 for 30 min and continuously incubated in respective media without H_2O_2 for 18~24 h. Values are means±SEM (n=4) and expressed as percent cell survival relative to control cells solely treated with 5.5 mmol/L glucose (cell viability=100%). Values not sharing a letter are different at P<0.05.

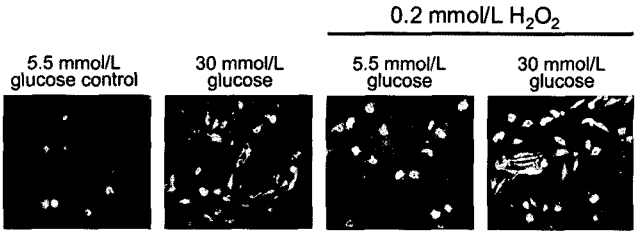

Fig. 2 Glucose activation of H_2O_2-induced intracellular oxidant generation.

Confluent HUVEC were loaded with 2',7'-dichlorodihydrofluorescein diacetate (DCHF) and were left untreated or stimulated with 0.2 mmo/L H_2O_2 for 30 min in the presence of 30 mmol/L glucose for 7 d. Oxidant generation was measured by DCHF-DA fluorescence. Fluorescent images (3 separate experiments) of representative H_2O_2-untreated controls and H_2O_2-treated cells were measured using a fluorescence microscopy.
Magnification : 400-fold.

produced cellular oxidants such as ROS elicited apoptotic cell death. These findings are consistent with previous reports showing high glucose-induced apoptotic death processes in various types of cells.[2,5,21,22]

When exogenous insulin was applied to the glucose culture protocols, there was lack of beneficial effects of insulin in overcoming high glucose-induced cell damage (Fig. 3B). It was assumed that insulin could enhance oxidation of excessive glucose for the metabolic energetics

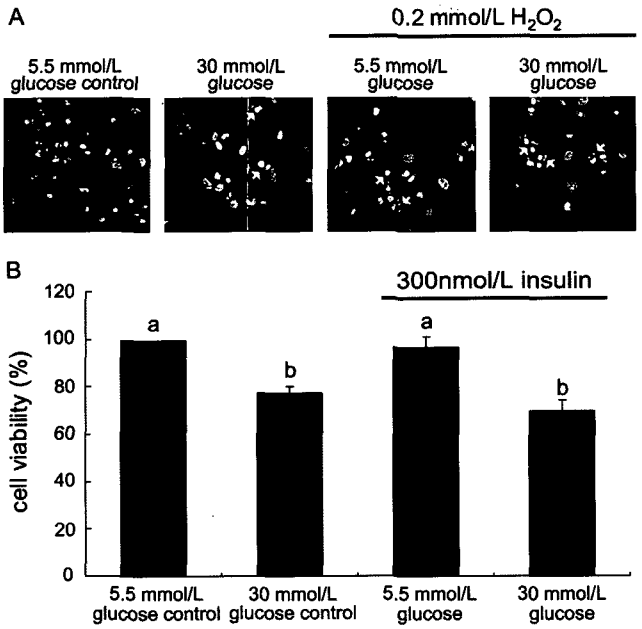

Fig. 3 Typical effects of 0.2 mmol/L H_2O_2 in HUVEC treated with high dose of glucose on nuclear morphology (A) and cell viability in insulin-treated HUVEC challenged with 30 mmol/L glucose (B).

HUVEC were pre-treated with 30 mmol/L glucose for 7 d. Cells were pulse-treated with 0.2 mmol/L H_2O_2 for 30 min and continuously incubated in respective media without H_2O_2 for 18~24 h. High dose of glucose and H_2O_2 caused nuclear condensation and the appearance of apoptosis-like bodies (arrows). This is representative of 3 independent slides.
Magnification : 400-fold.
For the insulin culture protocol, cells exposed to high glucose for 7 d were treated with 300 nmol/L insulin for 24 h. Values are means±SEM, n=5 and are expressed as percent cell survival relative to control cells treated with 5.5 mmol/L glucose alone (cell viability=100%).
Values not sharing a letter are different at $P<0.05$.

and minimize the glucose toxicity. It has been reported that rat insulin-producing cells cultured in high glucose concentrations exhibit increased apoptosis and protein p53 translocation to mitochondria.[22] It has been shown that insulin inhibits the stimulatory effect of high glucose on angiotensinogen expression in non-diabetic rat renal proximal tubular cells, but that it has no effect in diabetic rat renal proximal tubular cells.[23] Hyperglycemia-induced resistance to the insulin-induced inhibition of angiotensinogen gene expression is mediated via the generation of ROS in renal proximal tubular cells, and the blocking of ROS generation improves the inhibitory action of insulin on angiotensinogen expression under conditions of high glucose.[23] Accordingly, in the present study the failure of exogenous insulin to boost cell viability could be inferred from a mediation of cellular oxidants generated due to the incubation of high glucose.

2. Flavonoid Protection Against Cytotoxicity of High Glucose

This study offered insight into ways of finding polyphenolic compounds that are effective in preventing endothelial cells from being damaged by high ambient glucose. As mentioned, high glucose induces the production of ROS and results in endothelial dysfunction. Accordingly, agents that inhibit ROS production or enhance cellular antioxidant defenses can prevent high glucose induced-apoptosis and protect cells from the damaging effects of oxygen radicals due to high glucose. Currently, there is intense interest in polyphenolic compounds such as flavonoids that constitute one of the antioxidant phytochemicals and are found in a large numbers of fruits and vegetables. These flavonoids are natural antioxidants that scavenge various types of radicals in aqueous and organic environments.[8-10] It has been postulated that flavonoids could inhibit glucose toxicity and apoptosis via the blockage of ROS production arising from high glucose and/or H_2O_2.

All the test flavonoids showed little cytotoxicity at≤50 μmol/L when incubated with cells for 24 h.[24] Accordingly, this non-toxic concentration of all the flavonoids used for culture experiments was 25 μmol/L. Each test flavonoid inhibited high glucose-induced cell death differently (Fig. 4A). Among the test flavonoids, (-)epigallocatechin gallate and quercetin at 25 μmol/L reduced the rate of high glucose-i nduced cell death, whereas other flavonoids had no effect on glucose toxicity (Fig. 4A). The nuclear morphology of cells chronically exposed to high glucose with (-)epigallocatechin gallate or quercetin was distinguishable from that of the low glucose (5.5 mmol/L) control cells (Fig 4B). In marked contrast, the morphology of cells treated with other flavonoids in the presence of 30 mmol/L glucose compared poorly with that of the glucose control cells but well with that of the high glucose-alone-treated cells. In cells exposed to high glucose and incubated with these flavonoids including apigenin, cell density was markedly reduced and nuclear condensations and appearance of apoptotic body-like structures became increasingly frequent in response to high glucose (Fig. 4B). During the 24 h incubation, H_2O_2 exacerbated glucose cytotoxicity, with a ≈50% fall in cell viability at 0.2 mmol/L. (Fig. 5), most likely due to the damaging effects of the oxidants generated. Amng the test flavonoids, micromolar (-)epigallocatechin gallate and quercetin reversed the cell death triggered by H_2O_2, whereas equimillimolar apigenin aggravated the high glucose- and H_2O_2-induced dual cytotoxicity significantly ($P<0.05$). On the other hand, the cell viability further reduced by 0.2 mmol/L H_2O_2 was not affected by the flavonones, naringin and hesperidin (Fig. 5).

Resveratrol, a polyphenol in red wine, blocks the involvement of oxidative stress in the high glucose-induced apoptosis of human leukemia cells by virtue of its antioxidant property.[11] Morroniside, a compound in Cornus officinalis

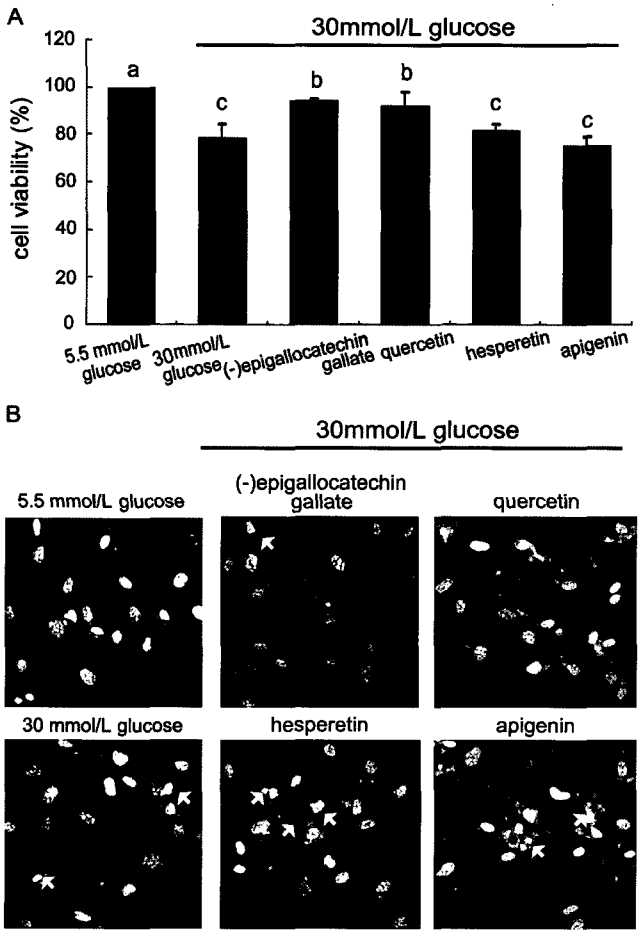

Fig. 4 Cell viability (A) and nuclear morphology (B) in HUVEC treated with flavonoids and exposed to high glucose.

HUVEC were incubated with 5.5 or 30 mmol/L glucose either alone or in the presence of (-)epigallocatechin gallate, quercetin, hesperetin, or apigenin (a final concentration of 25 μmol/L, respectively) for 24 h. Data (means±SEM, 3 separate experiments) are expressed as percent cell survival relative to the viability measured at normal glucose incubation (viability=100%). For the nuclear morphology, HUVEC were stained with Hoechst 33258. The arrows represent apoptosis-like bodies. Observations in five other slide sets were essentially identical. Magnification : 400X.

Sieb et Zucc, protects cultured HUVEC from damage by high ambient glucose.[25] Consistent with these reports, the flavanoids and flavonols, in particular (-)epigallocatechin gallate and quercetin, led to high anti-apoptotic activity in high glucose-treated vascular endothelial cells. In addition, a previous study showed that procyanidins have insulin-like effects on insulin-sensitive cells.[12] It can be postulated that these cytoprotective flavonoids may have such insulin-like effects. The insulin-like effects and antioxidant activity of (-)epigallocatechin gallate and quercetin could explain their anti-hyperglycemic effect *in vivo* and why they can improve diabetic situations.

In summary, this hyperglycemia-mimetic endothelial cell model demonstrates that there are differences in anti-hyperglycemic capacity among flavonoids, in which

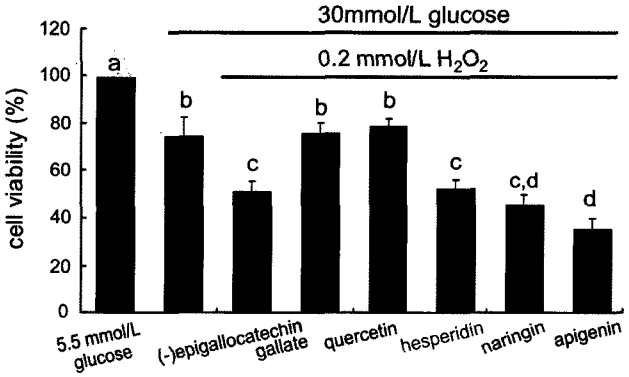

Fig. 5 Survival effects of flavonoids on glucose-induced cytotoxicity of HUVEC.

HUVEC were cultured with 25 μmol/L flavonoids added to M199 culture media with normal (5.5 mmol/L) or high (30 mmol/L) glucose for 7 d and were exposed to H_2O_2-induced oxidant stress. HUVEC viability was assessed by MTT. Data obtained from 5 separate experiments are expressed as percent cell survival relative to H_2O_2- and flavonoid-free controls (viability=100%).

the flavanol (-)epigallocatechin gallate and the flavonol quercetin protected the endothelium from high glucose-induced apoptosis. This anti-apoptotic protection was possibly mediated, at least in part, through blunting ROS-triggered damaging events. (-)Epigallocatechin gallate and quercetin may be beneficial agents for combating endothelial cell dysfunction induced by high glucose and may reduce the development of diabetic vascular complications. It is crucial to elucidate the precise sites of action of anti-hyperglycemic flavonoids in the sequence of events that regulate high glucose-induced cell death and to further evaluate the potential of dietary flavonoids as anti-hyperglycemic agents.

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