Original Article

Comparison of Antioxidant Potential and Rat intestinal α-Glucosidases inhibitory Activities of Quercetin, Rutin, and Isoquercetin

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Summary: Inhibition of α-amylase and α-glucosidases involved in the digestion and absorption of carbohydrates can decrease the postprandial increase of blood glucose level after a mixed carbohydrate diet. Therefore, the inhibitory activity of quercetin and its glycoside derivatives such as rutin and isoquercetin against rat intestinal α-glucosidases (sucrase, maltase, glucoamylase, and isomaltase) and porcine pancreatic α-amylase were compared in vitro. Among the tested three flavonols, quercetin had the highest maltase, glucoamylase, and isomaltase inhibitory activities. The isomaltase and α-amylase inhibitory activities of the above three flavonols were also compared to a known type 2 diabetes drug (Acarbose), strong α-amylase inhibitor. Compared to acarbose, quercetin and its derivatives showed a significant inhibition of isomaltase but did not show high inhibitory activity against porcine pancreatic α-amylase. Furthermore, the oxygen radical absorbance capacity (ORAC) of these flavonols was evaluated. Quercetin had the highest peroxyl radical absorbing activity, followed by rutin and isoquercetin. These results suggest that the selected flavonols which have high ORAC value with α-glucosidase inhibitory activity and low α-amylase activity could be physiologically useful for treatment of diabetes, although in vivo experiments are needed.

Industrial relevance: The α-glucosidase inhibitory activity and antioxidant activity in quercetin would be helpful to manage glucose uptake and the glucose-induced increased levels of mitochondrial reactive oxygen species (ROS) linked to hyperglycemia. This in vitro study therefore provides the biochemical rationale for the benefit of quercetin-based dietary supplement and enzymatic conversion from rutin or isoquercetin to quercetin for enhancing bioactive food components using high rutin-contained grain such as buckwheat.

Keywords: antioxidants, rutin, quercetin, glucosidase inhibitor, oxygen radical absorbance capacity (ORAC).

Introduction

Non-insulin dependent diabetes mellitus (NIDDM), a common disorder of glucose and fat metabolism is strongly associated with diets high in calories and linked to changes in dietary pattern towards high calorie sweetened foods with disaccharides such as maltose and sucrose (Garg et al., 1994). One of the therapeutic approaches for decreasing postprandial hyperglycemia is to retard absorption of glucose by the inhibition of carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase, in the digestive organs (Deshpande et al., 2009). Hyperglycemia is a condition characterized by a rapid rise in blood glucose levels and is due to hydrolysis of starch by pancreatic α–amylase and absorption of glucose in the small intestine by α–glucosidases. The intestinal absorption of dietary disaccharides such as maltose and sucrose is carried out by a group of α-glucosidases which include intestinal maltase, sucrase,
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glucoamylase and isomaltase. Therefore, inhibition of these enzymes can significantly decrease the postprandial hyperglycemia after a mixed carbohydrate diet and can be a key strategy in the control of diabetes mellitus (Hirsh et al., 1997).

However, a main negative aspect of currently used therapeutic α-glucosidase inhibitors such as the drug acarbose which has strong α-amylase inhibitory activity is digestive tract-disorder such as abdominal distention, flatulence, meteorism and possibly diarrhea (Vichayanrat et al., 2002). Therefore, natural inhibitors from dietary plants are useful as they have lower inhibitory activity against α-amylase and a stronger inhibitory activity against α-glucosidase and can be used as effective therapy for postprandial hyperglycemia with minimal side effects (Bischoff, 1994).

Postprandial hyperglycemia has been linked to the onset of the diabetic complications in NIDDM patients and triggers the generation of free radicals and oxidation-related damage in the retina, renal glomerulus and peripheral nerves (Brownlee, 2005; Kwon et al., 2005). Studies have shown that the glucose-induced increased levels of mitochondrial reactive oxygen species (ROS) produced by the mitochondrial electron transport chain seems to be the causal link between elevated levels of glucose and the pathways responsible for hyperglycemia-induced vascular complications (Kaiser et al., 1993; Brownlee, 2005). Therefore it is also important to control both blood glucose level and cellular redox status for managing these diabetic complications.

Important plant foods in traditional diet such as herbs, onion and beans have high phenol phytochemicals. Among these plant foods Allium species, onions and garlic have been traditionally used for a large range of purposes including medicine, nutrition, flavorings, condiment, foodstuff, and the treatment of common aliments as folk medicine (El-Demerdash et al., 2005; Kim and Kim, 2006). Recent research has now reported that phenolic phytochemicals from onion have high antioxidant activity and blood glucose lowering effect in alloxan-induced diabetic rat (El-Demerdash et al., 2005; Kim and Kim, 2006; Azuma et al., 2007). Furthermore it has been reported that onions are rich in flavonoids such as quercetin and its derivatives (rutin and isoquercetin) which have perceived benefits to human health (Lee et al., 2008). Epidemiological studies have also shown that the intake of certain types of flavonoids, including quercetin and myricetin are inversely associated with the risk of incident type 2 diabetes (Griffiths et al., 2002). Additionally quercetin derivatives isoquercetin and isorhamnetin-3-O-rutinoside have shown to exhibit inhibitory activity on rat intestinal α-glucosidase (Rigelsky and Sweet, 2002).

However, the significance of plant intake as a dietary source of quercetin for preventing diabetes-related oxidative stress and hyperglycemia with reduced side-effect caused by excessive α-amylase inhibition is still unclear. Therefore, we (i) evaluated the antioxidant activity of quercetin, its glycoside derivatives (rutin and isoquercetin) and acarbose using ORAC assay system; (ii) investigated the inhibitory activity of these flavonoids and acarbose against α-amylase and α-glucosidase (anti-hyperglycemia potential with reduced side-effect); (iii) compared the enzyme inhibitory specificity of tested compounds using α-glucosidases such as sucrase, maltase, isomaltase, and glucoamylase.

Materials and Methods

Materials: Quercetin (3,3′,4′,5,7-Pentahydroxylavone dihydrate), rutin (Quercetin-3-rutinoside hydrate) and isoquercetin (Quercetin 3-β-D-glucoside) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Porcine pancreatic α-Amylease (EC 3.2.1.1) and rat intestinal acetone powders of α-glucosidase (EC 3.2.1.20) were also purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Unless noted, all chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Oxygen radical absorbance capacity (ORAC) assay: Antioxidant activities of flavonols in different concentrations (between 0.5 μM and 2.0 μM) were investigated for their peroxyl and hydroxyl radical scavenging capacities using ORAC assay system. The ORAC assay was carried out using a Tecan GENios multi-functional plate reader (GENios; Tecan Trading AG, Salzburg, Austria) with fluorescent filters (excitation wavelength: 485 nm, emission filter: 535 nm). In the final assay mixture, fluorescein (40 nM) was used as a target of free radical attack with either 2, 2′-azobis (2-aminodipropene) dihydrochloride (AAPH, 20 mM) as a peroxyl radical generator in peroxy radical scavenging capacity (ORAC_R00) assay (Kurihara et al., 2004) or with H2O2 - CuSO4 (H2O2, 0.75%; CuSO4 5 μM) as a hydroxyl radical generator in hydroxyl radical scavenging capacity (ORAC100) assay (Cao et al., 1997). Trolox (1 μM) was used as a control standard and prepared fresh on a daily basis. The analyzer was programmed to record the fluorescence of fluorescein every 2 min after AAPH or H2O2 - CuSO4 was added. All fluorescence measurements were expressed relative to the initial reading. Final results were calculated based on the difference in the area under the fluorescence decay curve between the blank and each sample. All data were
expressed as micromoles of Trolox equivalents (TE). One ORAC unit is equivalent to the net protection area provided by 1 µM of Trolox.

**α−Amylase inhibition assay:** To evaluate the potency of quercetin and its glycoside derivatives the dose dependency of quercetin, isoquercetin, rutin and acarbose on α-amylase was measured using different concentrations (between 0.05 mM and 1.0 mM). Porcine pancreatic α-amylase inhibition referred to the method of Kwon et al. (2005). Sample solution (200 µL) and 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride, 500 µL) containing α-amylase solution (0.5 mg/mL, 5.0 MU/mL) were incubated at 25°C for 10 min. After pre-incubation, 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer was added. The reaction mixture was then incubated at 25°C for 10 min. The reaction was stopped with 1.0 mL of dinitrosalicylic acid (DNS). The reaction mixture was then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding distilled water, and absorbance was measured at 540 nm with ELISA microplate reader (SUNRISE; Tecan Trading AG, Saltzburg, Austria).

\[ \text{% inhibition} = \left( \frac{\Delta A_{540}^{\text{Control}} - \Delta A_{540}^{\text{Extract}}}{\Delta A_{540}^{\text{Control}}} \right) \times 100 \]

**α−Glucosidase inhibition assay:** To evaluate the potency of quercetin and its glycoside derivatives the dose dependency of quercetin, isoquercetin, rutin and acarbose on rat intestinal α-glucosidase was measured using different concentrations (between 0.05 mM and 1.0 mM). Rat intestinal α-glucosidase assay referred to the method of Kwon et al. (2007) with slight modification. A total of 1 g of rat-intestinal acetone powder was suspended in 3 mL of 0.9% saline, and the suspension was sonicated twelve times for 30 sec at 4°C. After centrifugation (10000 × g, 30 min, 4°C), the resulting supernatant was used for the assay. Sample solution (50 µL) and 0.1 M phosphate buffer (pH 6.9, 100 µL) containing α−glucosidase solution (1.0 U/mL) was incubated at 25°C for 10 min. After pre-incubation, 5 mM p-nitrophenyl-α-D-glucopyranoside solution (50 µL) in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25°C for 5 min. Before and after incubation, absorbance was read at 405 nm and compared to a control which had 50 µL of buffer solution in place of the extract by micro-plate reader (SUNRISE; Tecan Trading AG, Saltzburg, Austria). The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

\[ \text{% inhibition} = \left( \frac{\Delta A_{405}^{\text{Control}} - \Delta A_{405}^{\text{Extract}}}{\Delta A_{405}^{\text{Control}}} \right) \times 100 \]

**Maltase, sucrase, isomaltase, and glucoamylase inhibition assay:** The crude enzyme solution prepared from rat intestinal acetone powder Sigma-Aldrich Co. (St. Louis, MO, USA) was used as the small intestinal maltase, sucrase, isomaltase and glucoamylase, showing specific activities of 0.70, 0.34, 0.20 and 0.45 units/mL, respectively. Rat-intestinal acetone powder (1.0 g) was suspended in 3 mL of 0.9% saline, and the suspension was sonicated twelve times for 30 sec at 4°C. After centrifugation (10000 × g, 30 min, 4°C), the resulting supernatant was used for the assay. Maltase, sucrase, isomaltase and glucoamylase inhibitory activity were assayed by modifying a method developed by Dahlqvist (1964). The inhibitory activity was determined by incubating a solution of an enzyme (50 µL), 0.1 M phosphate buffer (pH 7.0, 100 µL) containing 0.4 mg/ml sucrose or maltose or isomaltose, or 1% soluble starch, and a solution (50 µL) with various concentrations of sample solution (between 0.05 mM and 1.0 mM) at 37°C for 30 min. The reaction mixture was heated in a boiling water bath to stop the reaction for 10 min, and then the amount of liberated glucose was measured by the glucose oxidase method (Bergmeyer and Bernt, 1974). The inhibitory activity was calculated from the formula as follows. Inhibition (%) = (C-T)/C × 100, where C is the enzyme activity without inhibitor and T is the enzyme activity with inhibitor.

**Statistical Analysis:** All data are presented as means ± SD. Statistical analyses were carried out using statistical package SPSS (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA) program and significance of each group was verified with the analysis of One-way ANOVA followed by the Duncan's test of p< 0.05.
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Figure 1. The chemical structures of flavonoid compounds used in this study.

Results and Discussion

Antioxidant activity by ORAC system: The ORAC assay developed by Cao et al. (1997) has been used successfully to determine the reaction capacity with peroxyl radical, one of harmful and reactive oxygen species in biological system. Antioxidant activity of flavonoids (Figure 1) was investigated for their peroxyl radical-scavenging capacity using ORAC assay system, where AAPH was used as a generator of peroxyl radicals. Figure 2 demonstrates that the scavenging activities of flavonols and acarbose on peroxyl radicals generated from AAPH were found to be dose-dependent between 0.5 µM and 2.0 µM. The bars in Figure 2A represents the ORAC activity of 1 µM of the tested compounds equivalent to 1 µM Trolox, a water-soluble α-tocopherol analogue. The ORAC values for the sample extracts ranged from 3.1 µM of Trolox equivalents (TE) to 18.1 µM of TE. The flavonoids (2.0 µM) with high ORAC values were quercetin (18.1 TE), rutin (14.7 TE), isoquercetin (13.1 TE) and acarbose (below 1.0 TE). The hydroxyl radical absorbing activity (ORAC HO·) of quercetin, its glycoside derivatives and acarbose was also measured using ORAC assay in which Cu²⁺ and H₂O₂ were used as hydroxyl radical generator. Excepted for acarbose, quercetin, rutin and isoquercetin showed same ORAC HO· value (Fig. 2B; 13.1 TE).

The most widespread and diverse phenolics are the flavonoids which have the same C15 (C6-C3-C6) skeleton and possess antioxidant capacity toward a variety of easily oxidizable compounds (Robards et al., 1999). For example, the main flavonoid constituents are flavonol aglycones such as quercetin in onion (Kakhonen et al., 1999). Generally, flavonoids containing multiple hydroxyl groups have higher antioxidant activities against peroxyl radicals (Robards et al., 1999). In this study the reduction capacity of quercetin appears to increase with increasing the concentration from 0.5 µM to 2.0 µM (Figure 2). Recent studies also have reported that the water extract of onion showed a dose-dependent free radical scavenging activity using DPPH (1,1-diphenyl-2-picryl hydrazyl radical) assay (Kim and Kim, 2006). Quercetin which has hydroxyl group at position C-3 had higher peroxyl radical scavenging activity than its glycoside derivatives in this study. These data clearly demonstrate that the presence of rutinoside at position C-3 in rutin (glucose in isoquercetin at position C-3) may block of its C-3 hydroxyl group which plays an important role in antioxidant activity such as radical scavenging and (or) transition metal chelating, and result in a reducing of antioxidant activity, which is in agreement with previous study by Heim et al (2002). Therefore, these results could provide the biochemical rationale for the benefit of enzymatic conversion from rutin or isoquercetin to quercetin and...
the basis for further bioconversion studies for producing and enhancing bioactive food components using high rutin-containing grain such as buckwheat.

\[ \text{ORAC} = \frac{\text{area under the curve}}{\text{area under the Trolox curve}} \]

**Figure 2.** Dose dependent changes in peroxyl (A) and hydroxyl (B) radical scavenging activity (Trolox equivalent, µM) of quercetin, rutin, isoquercetin, and acarbose in vitro system. ORAC value is calculated by dividing the area under the sample curve by the area under the Trolox curve, with both areas being corrected by subtracting the area under the blank curve. One ORAC unit is assigned as the net area of protection provided by Trolox at a final concentration of 1 µM. The area under the curve for the sample is compared to the area under the curve for Trolox, and the anti-oxidative value is expressed in micromoles of Trolox equivalent per liter. The results represent the mean±S.D. of values obtained from three measurements. Bar with different letters indicate statistically significance of differences among groups at p < 0.05 by Duncan’s test. First letter is among different samples and second one is among different concentrations within same samples.

**α-Amylase/α-Glucosidase Inhibition of Quercetin and Its Derivatives:** C-glycosylflavones such as vitexin have been reported to have antioxidant, anti-virus, liver protection, and other activities (Rigelsky and Sweet, 2002), however, the inhibitory activities of C-glycosylflavonols such as rutin and isoquercetin on α-glucosidase have rarely been reported. On the other hand, rutin, isoquercetin and quercetin are the three main flavonols in buckwheat, and the only difference between them was the glycosylation at the C-3 position (Figure 1). We therefore tested these C-glycosylflavonols (rutin and isoquercetin) and their aglycone quercetin for their α-glucosidase inhibitory activities.

The α-glucosidase inhibitors, which interfere with enzymatic action in the brush-border of the small intestine, could slow the liberation of D-glucose from oligosaccharides and disaccharides, resulting in delaying glucose absorption and decreasing postprandial plasma glucose levels (Deshpande et al., 2009). Inhibition of these enzymes such as α-amylase and α-glucosidases can significantly decrease the postprandial increase of blood glucose level after a mixed high calorie carbohydrate diet and can be a key strategy in the control of diabetes mellitus (Vichayaranrat et al., 2002; Kwon et al., 2005). However, previous reports have revealed that excessive inhibition of α-amylase could result in the abnormal bacterial fermentation of undigested starch in the colon and therefore low α-amylase inhibitory activity is useful (Kwon et al., 2005). A main negative aspect of currently used therapeutic α-glucosidase inhibitors such as the drug acarbose which has strong α-amylase inhibitory activity is digestive tract disorder such as abdominal distention, flatulence, meteorism and possibly diarrhea (Bischoff, 1994). Our previous study using rat intestinal α-glucosidase with standard phenolic phytochemicals showed that quercetin on a constant weight basis had the highest α-glucosidase inhibitory activity (Kim et al., 2009). Therefore, the inhibitory activity of flavonols and acarbose against α-amylase from porcine pancreas and α-glucosidase prepared from rat small intestine acetone powder were compared.

To evaluate the potency of quercetin and its glycoside derivatives the dose dependency and half-maximal inhibitory concentration (IC\textsubscript{50}) of quercetin, isoquercetin, rutin and acarbose on rat intestinal α-glucosidase was measured using different concentrations (between 0.05 and 1.0 mM) (Figure 3A). Acarbose had been launched in the United States as a medicine, with recommended daily doses of 150 ~ 300 mg. In case of quercetin has been marketed in the United States primarily as a dietary supplement, with recommended daily doses of supplemental...
quercetin of 200–1200 mg. Therefore, we used same dose for evaluating anti-hyperglycemic potential with quercetin and acarbose in this study. All the samples showed a comparable inhibition on rat intestinal α-glucosidase (Figure 3A). Acarbose had the highest α-glucosidase inhibitory activity (0.05 mM of IC₅₀) followed by quercetin (0.48 mM), isoquercetin (0.56 mM) and rutin (0.74 mM) (Figure 3A). Acarbose had also the highest α-amylase inhibitory activity but flavonoids tested did not have any inhibitory activity against porcine pancreatic α-amylase (Figure 3B), indicating that this result was similar to that of Lamiaceae species which has been known to natural α-glucosidase inhibitor with less side-effects due to excessive inhibition of α-amylase (Kwon et al., 2005; Kim et al., 2009).

As shown in Figure 4A, the sucrase inhibitory activity (IC₅₀ > 1 mM) of quercetin was significantly lower than that of rutin and isoquercetin (Figure 4A; 0.353 and 0.435 of IC₅₀, respectively). The difference in the number of sugar in the C ring of quercetin, did significantly affect the inhibitory activity against sucrase (rutin>isoquercetin>quercetin). These results suggest that the rutin and isoquercetin having the glycoside substructure of C-3 in the C rings of the flavonol skeleton showed relatively higher sucrase inhibitory activity than the corresponding their aglycone quercetin (Figure 4A). This result also indicates that the substitution of the sugar moiety to hydroxyl group in flavonol glycosides could decrease their sucrase inhibitory activity.

However, the comparative study on the inhibitory activity of quercetin and its glycoside derivatives against rat intestinal maltase, glucoamylase and isomaltase showed that quercetin was more potent than its glycoside derivatives. Acarbose had the highest inhibitory activity on maltase, glucoamylase, and isomaltase (<0.05 mM of IC₅₀) followed by quercetin, isoquercetin and rutin (Figure 4B, 4C, and 4D).

Among three flavonol compounds, quercetin had the highest inhibitory activity in these three enzymes followed by isoquercetin and rutin (Figure 4B, 4C, and 4D). These results suggest that the quercetin having the hydroxyl substructure of C-3 in the C rings of rutin and isoquercetin skeleton showed relatively higher inhibitory activity on maltase, glucoamylase, and isomaltase than the corresponding their glycoside derivatives.

Glycosylation at C-3 in the C-ring weakens the inhibitory activity of flavonols against maltase, glucoamylase, and isomaltase. These results strongly support the importance of an aglycone structure in the molecule for exerting an effective inhibitory activity on maltase, glucoamylase and isomaltase. The C-3 hydroxylation of the C-ring apparently plays an important part in the maltase, glucoamylase and isomaltase inhibitory activities of flavonols. Similar results have been reported elsewhere by Tadera et al. (2006): hydroxylations at the C-3 positions on the C rings of flavones enhance the inhibitory activity, C-3-OH are favorable to the inhibitory activity (Tadera et al., 2006).
Interestingly, acarbose at a low concentration strongly inhibited the activities of sucrase, maltase and glucoamylase (Figure 4A, 4B, and 4C; <0.05 mM, 0.04 mM, and <0.05 mM of IC$_{50}$, respectively). However, even at a high (1.0 mM) concentration, isomaltase activity was not inhibited (7%) by acarbose (Figure 4D). Quercetin, isoquercetin, and rutin were more potent inhibitors of isomaltase (Figure 4D; 0.50 mM, 0.55 mM, and 0.68 mM of IC$_{50}$, respectively) than acarbose. This result suggests that the main flavonol quercetin and its glycoside derivatives such as rutin and isoquercetin having the glycoside substructure of C-3 in the C rings of the flavonol skeleton showed significantly higher isomaltase inhibitory activity than acarbose (Figure 4C). Similar results have been reported elsewhere by Tadera et al.: 5,6,7-trihydroxyflavone structure is crucial for the potent inhibitory activity (Tadera et al., 2006).

Recent studies have shown that the intake of quercetin was inversely associated with the risk of incident type 2 diabetes. Isoquercetin, isorhamnetine-3-O-rutinoside, and a C-glycosylate apigenin derivative vitexin have also been reported to have strong inhibitory activities against α-glucosidase (Griffiths et al., 2002; Rigelsky and Sweet, 2002). Our results and findings by others, such as the aldose reductase inhibitory activity of quercetin, luteolin, apigenin, and a series of corresponding glycosides (Shin et al., 1995), suggest that grains and herbal medicine containing different types of flavonoids, may contain an important group of candidates as natural anti-type 2 diabetes drugs that warrant further investigation.
In conclusion quercetin and its glycoside derivatives have α-glucosidase inhibitory activity and high peroxyl radical scavenging-linked antioxidant activity. The above benefits (anti-hyperglycemia and antioxidant activity) of quercetin and its glycoside derivatives taken together could support the evidence that diets rich in fruits and vegetables are associated with lower incidences of oxidation-linked diseases such as diabetes (Hertog et al., 1995; Shetty et al., 1995; Paganga et al., 1999; Knekt et al., 2002; Tadera et al., 2006). Compared to acarbose, quercetin and its derivatives showed a significant inhibition of the α–glucosidase but did not show high inhibitory activity against porcine pancreatic α-amylase which reflected potential for reduced side-effects (Bischoff, 1994). This strategy would likely have lower abdominal side effects arising from excessive inhibition of pancreatic α-amylase. Furthermore, our studies show that quercetin, a form of aglycone has higher enzyme inhibitory activity (on maltase, glucoamylase, and isomaltase) than its mono- and diglycoside derivatives, rutin and isoquercetin. These results could be significant evidence for the development of more powerful anti-hyperglycemia supplement and efficacious utilization of rutin enriched-plant like as buckwheat (90% rutin, only a few quercetin and nearly no isoquercetin), which has been proved as an effective food in the diet of diabetic patients.

Therefore, the α-glucosidase inhibitory activity and antioxidant activity in quercetin would be helpful to manage glucose uptake and the glucose-induced increased levels of mitochondrial reactive oxygen species (ROS) linked to hyperglycemia. This in vitro study therefore could provides the biochemical rationale for the benefit of quercetin-based dietary supplement and the basis for further bioconversion studies.

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References