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Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts

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Abstract

The anti-glycation activity of four kinds of beans including mung bean, black bean, soybean and cowpea were evaluated. Aqueous alcohol extract of mung bean exhibited the strongest inhibitory activity against the formation of fluorescent advanced glycation endproducts (AGEs) in a bovine serum albumin (BSA)-glucose model, and the inhibitory activities of extracts of the four beans were found to be highly correlated with their total phenolic contents ($R^2 = 0.95$). Subsequent HPLC analysis of mung bean extract revealed two major phenolics which were purified and identified as vitexin and isovitexin by spectral methods. In the anti-glycation assays, both vitexin and isovitexin showed significant inhibitory activities against the formation of AGEs induced by glucose or methylglyoxal with efficacies of over 85% at 100 μM. In another assay, vitexin and isovitexin failed to directly trap reactive carbonyl species, such as methylglyoxal, suggesting that their anti-glycation activities may mainly be due to their free radical scavenging capacity.

Keywords: AGEs; Diabetic complications; Mung bean; Anti-glycation; Vitexin; Isovitexin

1. Introduction

The Maillard reaction (nonenzymatic glycation or browning) in foods has been well studied by the food industry to control food quality. However, it is only forty years ago that a similar glycation process was recognized in human body by the observation of increased formation of glycosylated haemoglobins in diabetic patients (Rahbar, 1968; Rahbar, Blumenfeld, & Ranney, 1969) and this would lead to the formation of detrimental advanced glycation endproducts (AGEs) in humans. AGEs are a group of complex and heterogeneous compounds which are known as brown and fluorescent cross-linking substances such as pentosidine, non-fluorescent cross-linking products such as methylglyoxal-lysine dimers (MOLD), or non-fluorescent, non-crosslinking adducts such as carboxymethyllysine (CML) and pyrraline (a pyrrole aldehyde) (Ikeda et al., 1996; Rahbar & Figarola, 2002). AGE accumulation has been implicated as a major pathogenic process in diabetic complications, including neuropathy, nephropathy, retinopathy and cataract (Ahmed, 2005; Brownlee, 1994) and other health disorders such as atherosclerosis (Kume et al., 1995), Alzheimer's disease (Munch, Thome, Foley, Schinzel, & Riederer, 1997; Vitek et al., 1994) and normal aging (Brownlee, 1995; Munch et al., 1997). Thus, the discovery and investigation of AGE inhibitors would offer a potential therapeutic approach for the prevention of diabetic or other pathogenic complications.

Both synthetic compounds and natural products have been evaluated as AGE inhibitors. Although some synthetic compounds demonstrated strong inhibitory activities

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against the formation of AGEs or in breaking protein crosslinks caused by Maillard reaction *in vivo*, they may also lead to severe side effects. As an example, aminoguanidine, the first AGE inhibitor engaging in clinical study, was terminated in phase III clinical trial in diabetic patients due to safety concerns (Freedman et al., 1999; Thornalley, 2003). Natural products have been proven relatively safer for human consumption when compared with synthetic compounds. In this regard, some plant extracts have been evaluated for their effects on the formation of AGEs in recent years (Gugliucci & Menini, 2002; Imai et al., 1994; Lee, Jang, Lee, Kim, & Kim, 2006; Yamaguchi, Ariga, Yoshimura, & Nakazawa, 2000). It is noted that the inhibitory effects of most of these plant extracts on the formation of AGEs are mainly contributed by the large amount of phenolic antioxidants they contain. As free radicals are involved in the formation of AGEs, it is reasonable to expect that phenolic antioxidants can inhibit the formation of AGEs.

Beans are recommended as suitable foods for diabetic patients in the past mainly for their high fibre and protein contents. Recently, they have also been reported to contain considerable quantities of bioactive phytochemicals, including phenolic compounds (Dabrowski & Sosulski, 1984; Drumm, 1990; Lin & Lai, 2006), which may offer extra benefits for amelioration of diabetes. However, little attention has been paid to the potential applications of leguminous phytochemicals in alleviating or preventing diabetic complications. In this study, we investigated the *in vitro* anti-glycation activity of four kinds of beans including mung bean (*Vigna radiata*), black bean (*Phaseolus vulgaris L.*), soybean (*Glycine max*) and cowpea (*Vigna unguiculata*). Primary focus was on the activity of their aqueous alcohol extracts. Detailed phytochemical analysis, including extraction and separation of phenolic components was also conducted for mung bean. The phenolic compounds obtained were further evaluated for their effects on the formation of AGEs induced by glucose and methylglyoxal. Two major phenolic compounds from mung bean, vitexin and isovitexin were also tested for their activities in direct trapping of methylglyoxal, a key intermediate compound for the formation of AGEs.

2. Experimental

2.1. Chemicals and general procedures

All beans (mung bean, black bean, soybean and cowpea) were obtained from a local supermarket in Hong Kong. Folin-Ciocalteu's phenol reagent (FCR), gallic acid (GA), aminoguanidine (AG), sodium azide, bovine serum albumin (BSA), D-glucose, methylglyoxal (MGO) (40% aqueous solution), 1,2-phenylenediamine (PD), and 2,3-dimethylquinoxaline (DQ) were purchased from Sigma–Aldrich Company (St. Louis, MO, USA). All analytical and HPLC grade solvents used were obtained from BDH Laboratory Supplies (Poole, UK). Silica gel (200–300 mesh) for column chromatography was purchased from Qingdao Marine Chemical Company (Qingdao, PR China). ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker 500 DRX NMR Spectrometer (Billerica, MA, USA). MS spectra were obtained on an Agilent LC-MSD system (Santa Clara, CA, USA) equipped with an electrospray ionization source, Bruker Daltonics 4.0 and Data analysis 4.0 software. Total phenolic content of bean extract was determined by the Folin-Ciocalteu colorimetric method on a Shimadzu UV-1206 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Fluorescent intensity was measured by a Hitachi F-2500 fluorescent spectrometer (Hitachi Corporation, Tokyo, Japan).

2.2. Preparation of bean extracts

All beans were ground into fine powders. Ten grams of each kind of bean powder were extracted with 70% ethanol aqueous solution (250 mL × 3) through sonication for 1 h each time. After vacuum filtration, the 70% ethanol extracts were concentrated under vacuum at 50 °C using a rotary evaporator and then stored in a desiccator before being used for anti-glyation tests.

2.3. BSA-glucose assay

This assay was adopted from the literature (Rahbar, Yerneni, Scott, Gonzales, & Lalezari, 2000; Wu & Yen, 2005) and used as an in vitro model for comparing the anti-glycation activities of various bean extracts. In brief, 5 g BSA and 14.4 g D-glucose were dissolved in phosphate buffer (1.5 M, pH 7.4) to obtain the control solution with 50 mg/mL BSA and 0.8 M D-glucose. 2 mL of the control solution was incubated at 37 °C for 7 days in the presence or absence of 1 mL of bean extracts in phosphate buffer (1.5 M, pH 7.4) (the final concentration of bean extract in the 3 mL test solution was 500 ppm). The test solution also contained 0.2 g/L NaN_3 to assure an aseptic condition. AG (1 mM) and rutin (100 μM) were used as positive controls. For testing of purified compounds, 100 μM vitexin and isovitexin were used. After 7 days of incubation, fluorescent intensity (excitation, 330 nm; emission, 410 nm) was measured for the test solutions. Percent inhibition of AGE formation by each extract or compound was calculated using the following equation, % inhibition = [1 − (fluorescence of the solution with inhibitors/fluorescence of the solution without inhibitors)] × 100%.

2.4. BSA-MGO assay

This assay was adopted from the literature (Lunceford & Gugliucci, 2005) and used to evaluate inhibitory effects of vitexin and isovitexin on protein glycation induced by methylglyoxal, a key reactive intermediate compound formed in Maillard reaction. Briefly, 40 mg BSA was mixed with 31 μL MGO in phosphate buffer (0.1 M, pH 7.4) to obtain the control solution with 1 mg/mL BSA and

5 mM MGO. 2 mL of the control solution was incubated at 37 °C for 6 days with/without 1 mL of flavonoids in phosphate buffer (the final concentration of vitexin or isovitexin in the 3 mL test solution was 100 μM). The test solution also contained 0.2 g/L NaN_3 to assure an aseptic condition. AG (1 mM) and rutin (100 μM) were used as positive controls. Likewise, % inhibition was calculated based on the equation applied in the BSA-glucose assay as described above (excitation, 340 nm; emission, 420 nm).

2.5. Colorimetric analysis of total phenols in bean extracts

Total phenolic contents of the bean extracts were determined by a Folin-Ciocalteu oxidation-reduction colorimetric method (Vinson, Su, Zubik, & Bose, 2001). Briefly, 200 μL of diluted sample (100 ppm bean extracts in 70% ethanol) were added into a cuvette. This was followed with the addition of 1 mL of FCR (10-fold dilution in water) and finally 0.8 mL sodium carbonate solution (75 g/L). After mixing, the reaction mixtures were allowed to incubate at ambient temperature for two hours before measuring their absorbance at 760 nm. Quantification was based on a standard curve generated from different concentrations of gallic acid (GA) solutions. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight of bean extracts. Three measurements were taken for each sample and triplicate experiments were conducted.

2.6. HPLC analysis

Analytical HPLC was carried out using a Shimadzu LC-20AT system equipped with a diode array detector and a LC-Solution software. A pre-packed Alltima C_{18} column (250 × 4.6 mm, 5μm, Metachem Technologies Inc., Torrance, CA, USA) was selected for HPLC analysis of mung bean extract. The absorption spectra were recorded from 200 to 400 nm for all peaks. The flow rate was 0.8 mL/min and the injection volume was 10 μL. The mobile phases were water with 0.2% acetic acid (solvent A) and acetonitrile (solvent B). The elution started with 5% B, linear gradient to 20% B in 5 min. Then it was 20–25% B from 5 to 20 min, 25–90% B from 20 to 30 min and finally kept at 90% B till 35 min. The post running time was 15 minutes. For quantification of MGO derivatives, a Luna Phenyl-hexyl column (150 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA) was chosen and chromatographic analysis was carried out on the same HPLC system as above. The flow rate was 1.0 mL/min and the injection volume was 15 μL. The mobile phase was composed of water and methanol (50:50, *v*/*v*). The total running time was 10 min and the chromatograms were obtained at 315 nm.

2.7. Isolation and identification of two main flavonoids from mung bean

Dried mung bean (2.94 kg) was crushed and extracted with 50% ethanol (3 × 10 L) by sonication. The extracts were combined and concentrated under vacuum at 45 °C. The concentrated extract was suspended in water and then partitioned with *n*-hexane (3 × 1.0 L) to afford *n*-hexane-soluble syrup (29.0 g after drying). The aqueous layer was then partitioned with ethyl acetate (3 × 1.0 L) to give an ethyl acetate-soluble extract (34.8 g after drying). The aqueous layer was finally partitioned with *n*-butanol (3 × 500 mL) to afford 1.8 g of *n*-butanol extract. The ethyl acetate-soluble extract was found to contain the highest levels of phenolics and was selected for further purification. Methanol was first used for dissolution of the extract. The insoluble residues were separated from the liquid solution by filtration and re-crystallized in methanol to give compound 1 (697 mg). The methanol-soluble fraction was further separated by column chromatography using normal phase silica gel as the stationary phase and ethyl acetate (2000 mL) as the mobile phase to give compound 2 (430 mg). The two compounds were elucidated as vitexin and isovitexin, respectively (structures are shown in Fig. 1 by comparison of their spectral data with those in the literature (Hosoya, Yun, & Kunugi, 2005; Tanaka et al., 2005).

Vitexin (**1**): yellow powder. ^{1}H NMR (DMSO-d_6) δ: 13.15 (1 H, s, 5-OH), 8.01 (2 H, d, $J = 8.7$ Hz, 2′-H, 6′-H), 6.88 (2 H, d, $J = 8.7$ Hz, 3′-H, 5′-H), 6.76 (1 H, s, 3-H), 6.27 (1 H, s, 6-H), 4.69 (1 H, d, $J = 9.8$ Hz, glucosyl 1″-H), 3.85–3.22 (6 H, m, glucosyl H). ^{13}C NMR (DMSO-d_6) δ: 164.03 (C-2), 102.50 (C-3), 182.16 (C-4), 160.48 (C-5), 98.23 (C-6), 162.71 (C-7), 104.10 (C-8), 156.09 (C-9), 104.67 (C-10), 121.69 (C-1′), 129.03 (C-2′, 6′), 115.89 (C-3′, 5′), 161.22 (C-4′), 78.74 (C-1″), 73.46 (C-2″), 70.93 (C-3″), 70.62 (C-4″), 81.69 (C-5″), 61.37 (C-6″). Positive ESI-MS: m/z 433 $[M+H]^+$.

Isovitexin (**2**): yellow powder. ^{1}H NMR (DMSO-d_6) δ: 13.55 (1 H, s, 5-OH), 7.91 (2 H, d, $J = 8.8$ Hz, 2′-H, 6′-H), 6.91 (2 H, d, $J = 8.8$ Hz, 3′-H, 5′-H), 6.78 (1 H, s, 3-H), 6.51 (1 H, s, 8-H), 4.58 (1 H, d, $J = 9.8$ Hz, glucosyl 1″-H), 4.03–3.11 (6 H, m, glucosyl H). ^{13}C NMR (DMSO-d_6) δ: 163.54 (C-2), 102.81 (C-3), 181.98 (C-4), 156.24 (C-5), 108.89 (C-6), 163.26 (C-7), 93.64 (C-8), 161.19 (C-9), 103.44 (C-10), 121.12 (C-1′), 128.49 (C-2′, 6′), 116.00 (C-3′, 5′), 160.68 (C-4′), 78.95 (C-1″), 73.56 (C-2″), 71.23 (C-3″), 70.62 (C-4″), 81.59 (C-5″), 61.49 (C-6″). Positive ESI-MS: m/z 433 $[M+H]^+$.

2.8. Evaluation of the capacity of vitexin and isovitexin in direct trapping of MGO

MGO (5 mM), PD (derivatization agent, 20 mM), DQ (internal standard, 5 mM), AG (5 mM), vitexin and isovitexin (5 mM) solutions were freshly prepared by dissolving 15.4 μL of MGO, 21.63 mg of PD, 15.82 mg of DQ, 11.06 mg of AG, 21.6 mg of vitexin and 21.6 mg of isovitexin in 20, 10, 20, 20, 10, and 10 mL of phosphate buffer saline (PBS) (pH 7.4), respectively. 3 mL of the prepared MGO solution was mixed with 1 mL of PBS (blank) or compound solution (AG, vitexin and isovitexin). After

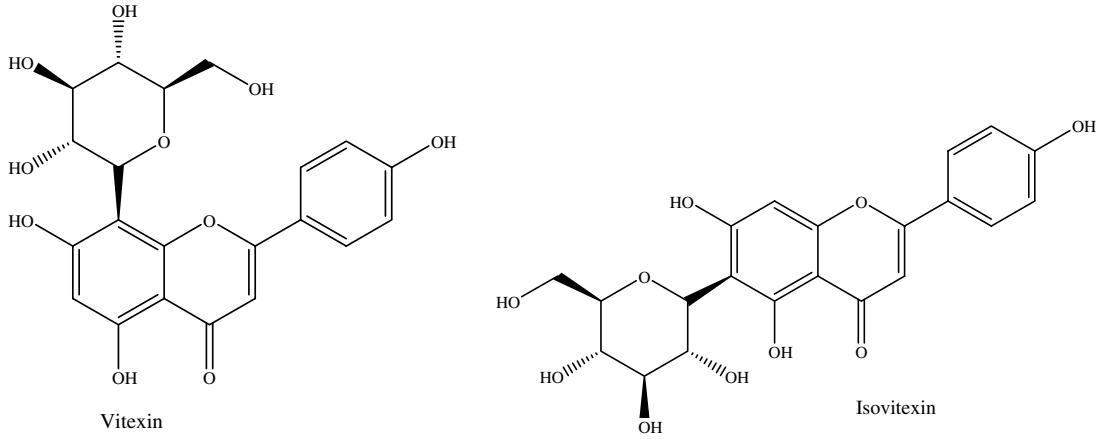

Fig. 1. Structures of vitexin and isovitexin.

mixing, the mixtures were incubated for 50 min in a water bath at 37 °C. Samples were taken at 10-min intervals and 0.5 mL derivatization agent (20 mM PD) and 0.5 mL internal standard (5 mM DQ) were added. After half an hour (when the derivatization reaction was completed), HPLC analysis was performed to quantify the residual MGO based on the amounts of the derivatized product, 1-methylquinoxaline (MQ) in each sample. At each time point, the amounts of unreacted MGO in the four samples [MGO only (control), MGO added with AG, vitexin and isovitexin, respectively] could be worked out based on a calibration curve constructed by plotting the peak area ratios of MQ and DQ versus different concentrations of MGO (0.1, 0.2, 0.5, 1, 2, and 5 mM). Percentage decrease in MGO can be calculated using the following equation, MGO decrease percentage = [(amounts of MGO in control – amounts of MGO in sample with tested compound)/amounts of MGO in control] × 100%. Duplicate samples were taken at each time point.

2.9. Statistical analysis

Statistical analyses were performed using the SPSS statistical package (SPSS, Inc, Chicago, IL). Paired samples *T*-test was applied to determine whether the added bean extracts, vitexin or isovitexin would show significant inhibitory activity against the formation of AGEs compared with the control. $P < 0.01$ was selected as the level decision for significant differences.

3. Results and discussion

The BSA-glucose model adopted in this study provides a useful tool for assessing the effects of various compounds on the non-enzymatic glycation process. Fig. 2 displays the inhibitory effects of four bean extracts on AGE formation in this model. The aqueous alcohol extracts of all beans examined have showed significant inhibitory activities at a concentration of 500 ppm with 80.4% inhibition for mung bean, 72.1% for black bean, 70.1% for soybean, and 67.3% for cowpea extract, respectively. Recently, various phenolic antioxidants from plant extracts have been found to inhibit the formation of AGEs, and their inhibition of free radical generation in the glycation process and subsequent inhibition of modification of proteins have been considered as the major mechanisms for mediating their anti-glycation activities. Thus in the following experiments, total phenolic contents of the four extracts were determined using a colorimetric method. It was found that mung bean extract had the highest phenolic content and anti-glycation activities of these beans were highly correlated with their total phenolic contents ($R^2 = 0.95$) (Table 1 and Fig. 3). Subsequent HPLC analysis was carried out for mung bean extract. Chromatographic spectra were recorded at three different wavelengths, 254, 280 and 360 nm and these spectra clearly showed the presence of various phenolic compounds with two compounds at 16.3 and 17.1 min as the major components (Fig. 4). These two phenolics were thus purified by column chromatography and structurally elucidated using NMR and MS techniques, which confirmed their identity as vitexin and isovitexin, respectively. Vitexin and isovitexin are flavone *C*-glucosides, which also have been identified from other plants, such as bamboo leaves (Zhang et al., 2005), *Xanthosoma violaceum* leaves (Picerno, Mencherini, Lauro, Barbato, & Aquino, 2003) and pigeonpea leaves (Fu et al., 2007). They have been proven to have distinct antioxidant activities (Bramati, Aquilano, & Pietta, 2003; Picerno et al., 2003), anti-hypotensive, anti-inflammatory (Prabhakar, Bano, Kumar, Shamsi, & Khan, 1981), antimicrobial (Agnese, Perez, & Cabrera, 2001) and radioprotective effects (Hien, Huong, Hung, & Duc, 2002). However, there are no previous reports on their anti-glycation activities. In this study, we therefore further evaluated their inhibitory effects on the formation of fluorescent AGEs in the BSA-glucose model. As shown in the Fig. 2, at a concentration of 100 μM, the two compounds showed strong inhibitory effects, comparable to that of rutin, one of the most potent natural AGE inhibitors.

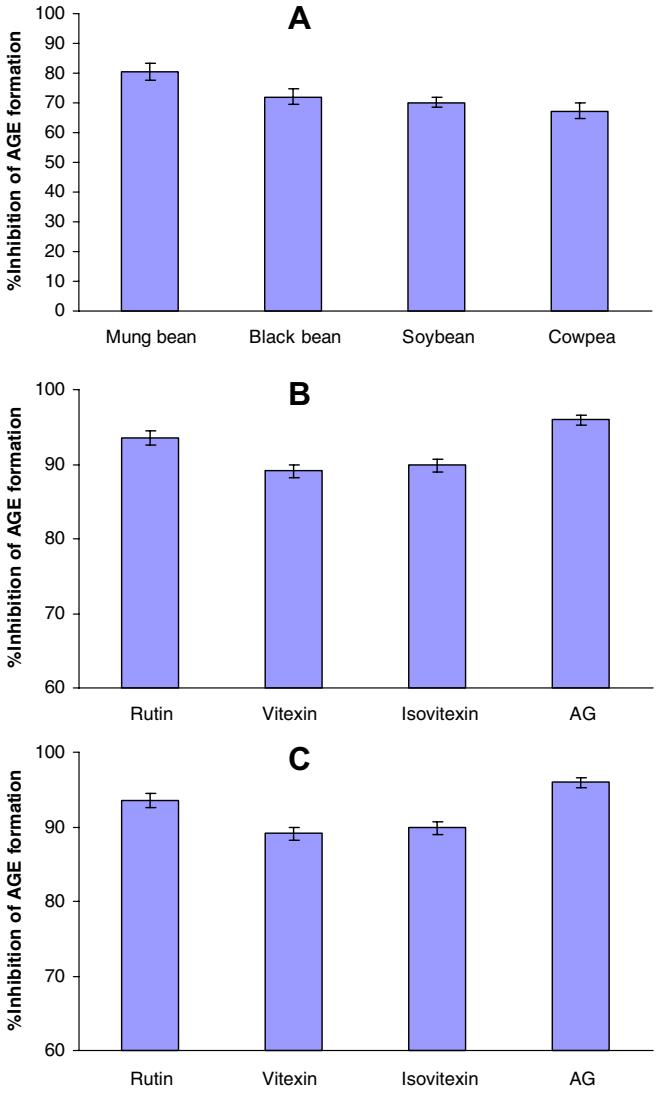

Fig. 2. Panel A: Inhibitory effects of various bean extracts on the formation of AGEs in BSA-Glucose model. The concentration of each bean extract is 500 ppm. Results are means ± SD for $n = 3$. Fluorescent intensities of the solutions with addition of the four bean extracts were all significantly different from that of the control solution ($P < 0.01$). Panel B: Inhibitory effects of vitexin and isovitexin on the formation of fluorescent AGEs in BSA-Glucose model. 100 μM flavonoids (rutin, vitexin or isovitexin) and 1 mM AG were tested in the experiment. Results are means ± SD for $n = 3$. Fluorescent intensities of the solutions with addition of flavonoids and AG were all significantly different from that of the control solution ($P < 0.01$). Panel C: Inhibitory effects of vitexin and isovitexin on formation of fluorescent AGEs in BSA-MGO model. 100 μM flavonoids (rutin, vitexin or isovitexin) and 1 mM AG were examined in this assay. Results are means ±SD for $n = 3$. Fluorescent intensities of the solutions with addition of flavonoids and AG were all significantly different from that of the control solution ($P < 0.01$).

Generally, there are three stages in the nonenzymatic glycation process *in vivo*. First, glycation is initiated by the covalent attachment of reducing sugars to amino groups of proteins, lipids, or nucleic acids to produce reversible and an unstable Schiff base. Then, the Schiff base may undergo Amadori rearrangement and change to a more stable Amadori product. Subsequently, Amadori

Table 1
Total phenolic contents of bean extracts determined by Folin-Ciocalteu method

Bean extracts	Total phenolic content (GAE in mg/g)[a]
Mung bean	9.94 ± 0.184
Black bean	8.04 ± 0.091
Soybean	7.15 ± 0.095
Cowpea	5.70 ± 0.069

[a] Each value is expressed as mean ± standard error of 3 replications.

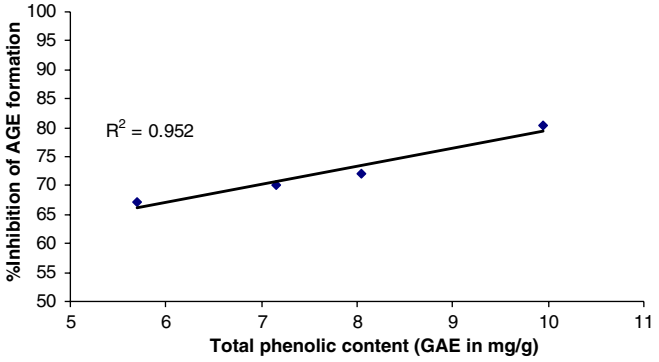

Fig. 3. Linear relationship between % inhibition of AGE formation and total phenolic contents of four bean extracts.

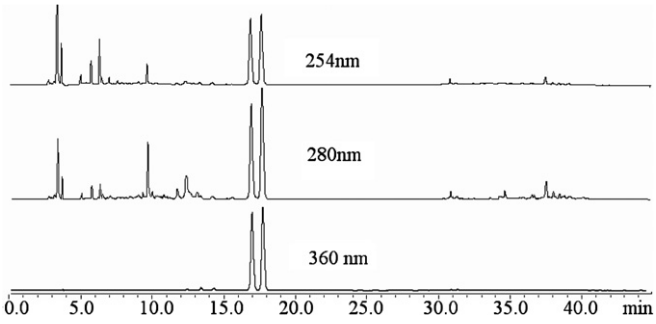

Fig. 4. Chromatograms of mung bean extract at different wavelengths.

products undergo dehydration and rearrangement to form highly reactive carbonyl species (RCS) including 3-deoxyglucosone (3-DG), glyoxal (GO) and methylglyoxal (MGO). Finally, reactions between these reactive carbonyls and amino, sulphydryl and guanidine functional groups of intracellular and extracellular proteins (Frye, Degenhardt, Thorpe, & Baynes, 1998; Lo, Westwood, McLellan, Selwood, & Thornalley, 1994) would result in the formation of AGEs. On the other hand, reactive carbonyl species produced through sugar autoxidation, lipid peroxidation and UV-photo damage may also contribute to the formation of AGEs. For most of the synthetic AGE inhibitors thus far studied, it is believed that direct trapping of RCS is likely one of their principal mechanisms of inhibition in the formation of AGEs. In addition, it was reported that some tea polyphenols could trap MGO under physiological conditions (Lo et al., 2006). Therefore, it would be meaningful to examine whether vitexin and isovetexin are effective in inhibiting direct RCS-induced AGE formation and/

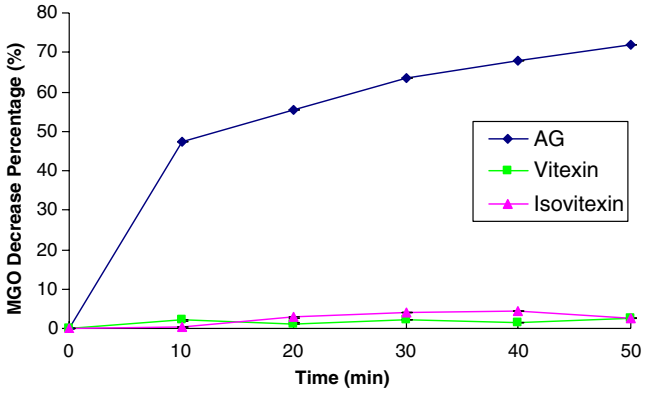

Fig. 5. Kinetic study of direct MGO trapping capacity of AG, vitexin and isovitexin. Results are expressed as mean ± 1/2 range ($n = 2$).

or in the direct trapping of RCS, particularly methylglyoxal. As shown in Fig. 2, both vitexin and isovitexin at a concentration of 100 μM exhibited significant inhibitory activities in the formation of fluorescent AGEs induced by MGO, similar to the activity of AG at 1 mM, but weaker than that of rutin at 100 μM. However, with regard to the capability of direct MGO trapping, both compounds turned out to be much weaker than AG, a typical scavenger for reactive carbonyl compounds (Fig. 5).

Different AGE inhibitors suppress AGE formation at different stages of glycation. For example, aspirin (acetylsalicylic acid) is known to inhibit glycation by acetylating free amino groups of a protein, thereby blocking the attachment of reducing sugars (Caballero, Gerez, Batlle, & Vazquez, 2000; Malik & Meek, 1994) at the early stage of the glycation process. The inhibitory activities against AGE formation of various vitamin B1 and B6 derivatives such as pyridoxamine (Khalifah, Baynes, & Hudson, 1999; Metz, Alderson, Thorpe, & Baynes, 2003; Voziyan, Metz, Baynes, & Hudson, 2002) and thiamine pyrophosphate (Booth, Khalifah, Todd, & Hudson, 1997) have mainly been attributed to their abilities to scavenge reactive carbonyl compounds (Ahmed et al., 2005; Voziyan et al., 2002). In addition, penicillamine could reduce the level of AGEs through decreasing the formation of Amadori products (Jakus, Hrnciarova, Carsky, Krahulec, & Rietbrock, 1999; Keita et al., 1992). Thus, it is hard to decide exactly in what way or at which stage intervention of vitexin and isovitexin is exerted to reduce the formation of AGEs. However, considering their strong antioxidant capacities, it is believed that anti-glycation could be brought about by their free radical scavenging and/or metal ion trapping activities.

4. Conclusions

Legumes have been suggested as dietary foods for diabetic patients for a long time due to their high fibre and protein contents. This study confirmed that legume phytochemicals, especially phenolics, are effective in inhibiting the formation of AGEs *in vitro*. This further supports that legumes are beneficial food choices for diabetics as AGEs have been implicated in the pathogenesis of various diabetic complications and other diseases. Among beans examined in this study, mung bean had the highest anti-glycation activity, largely contributed by its flavone *C*-glucoside constituents, vitexin and isovitexin.

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