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Cinnamon Bark Proanthocyanidins as Reactive Carbonyl Scavengers To Prevent the Formation of Advanced Glycation Endproducts

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Cinnamon bark has been reported to be effective in the alleviation of diabetes through its antioxidant and insulin-potentiating activities. In this study, the inhibitory effect of cinnamon bark on the formation of advanced glycation endproducts (AGEs) was investigated in a bovine serum albumin (BSA)−glucose model. Several phenolic compounds, such as catechin, epicatechin, and procyanidin B2, and phenol polymers were identified from the subfractions of aqueous cinnamon extract. These compounds showed significant inhibitory effects on the formation of AGEs. Their antiglycation activities were not only brought about by their antioxidant activities but also related to their trapping abilities of reactive carbonyl species such as methylglyoxal (MGO), an intermediate reactive carbonyl of AGE formation. Preliminary study on the reaction between MGO and procyanidin B2 revealed that MGO−procyanidin B2 adducts are primary products which are supposed to be stereoisomers. This is the first report that proanthocyanidins can effectively scavenge reactive carbonyl species and thus inhibit the formation of AGEs. As proanthocyanidins behave in a similar fashion as aminoguanidine (AG), the first AGE inhibitor explored in clinical trials, they show great potential to be developed as agents to alleviate diabetic complications.

INTRODUCTION

Advanced glycation endproducts (AGEs) are the final products of the nonenzymatic reaction between reducing sugars and amino groups in proteins, lipids, and nucleic acids (*1*). They are a group of complex and heterogeneous compounds that are known as brown and fluorescent cross-linking substances such as pentosidine, nonfluorescent cross-linking products such as methylglyoxal−lysine dimers (MOLD), or nonfluorescent, non-cross-linking adducts such as carboxymethyllysine (CML) and pyrraline (a pyrrole aldehyde) (*2, 3*). Recently, AGE accumulation in vivo has been implicated as a major pathogenic process in diabetic complications, including neuropathy, nephropathy, retinopathy, and cataract (*4, 5*) and other health disorders such as atherosclerosis (*6*), Alzheimer's disease (*7, 8*), and normal aging (*8, 9*). 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 inhibitors against the formation of AGEs. Synthetic AGE inhibitors so far discovered can be divided into three classes: (a) carbonyl trapping agents that attenuate carbonyl stress; (b) metal ion chelators, which suppress glycoxidations; and (c) cross-link breakers that reverse AGE cross-links (*10*). Among them, those which possess reactive carbonyl scavenging capacities remain the dominant candidates for clinical trials, and recent clinical studies have demonstrated the effectiveness of some of these synthetic AGE inhibitors in the amelioration of diabetes complications. Historically, aminoguanidine (AG) was the first AGE inhibitor explored in clinical trials. Aminoguanidine, a hydrazine-like small molecule, is a nucleophilic agent that traps reactive carbonyl intermediates such as methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG) to form relatively nontoxic adducts. However, the drug was not ultimately approved for commercial production because side effects were observed in phase III clinical trials in patients with diabetes, perhaps related to some extent to the sequestration of pyridoxal, resulting in vitamin B6 deficiency (*1*). Despite AG's limitations, the proof-of-concept studies provided strong evidence that inhibition of AGE formation by trapping reactive carbonyl

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species could be a reasonable therapeutic approach for the treatment of diabetes complications.

Natural plant extracts and purified constituents have been evaluated for their role in preventing the formation of AGEs. So far, phenolic antioxidants have been found to be the most promising agents, and their activities against AGE formation in vitro have been shown, with a few exceptions, to correlate highly with their free radical scavenging activities. However, numerous clinical trials have failed to provide conclusive evidence for the efficacy of natural antioxidant therapy in diabetic patients. Therefore, it would be of great interest to discover natural AGE inhibitors that can suppress the formation of AGEs, both through preventing glycoxidation (scavenging free radical and/or chelating metal ions) and by sequestering reactive carbonyl species (especially 1,2-dicarbonyls, the key intermediates in the glycation of proteins). So far, very few natural products have been found to have reactive carbonyl species (RCS) scavenging activities.

Cinnamon (*Cinnamomum zeylanicum*) is a kind of traditional spice, the bark and leaves of which are often added to food preparations to improve taste and aroma. In addition, this herb is also found to possess potent antioxidant (*11, 12*), antimicrobial (*12–14*), and antipyretic (*15*) properties. In recent years, much attention has been paid to the influence of cinnamon on insulin action, which may provide benefits for diabetic patients. Some researchers indicated that cinnamon bark extract could increase cellular glucose uptake and improve glucose metabolism in vivo (*16, 17*). Moreover, its ability to decrease serum glucose, triglyceride, LDL cholesterol, and total cholesterol in people with type 2 diabetes (*18*) proved the insulin-like or insulin-potentiating action of cinnamon extract as well. Anderson et al. found that the insulin-like biological activity of cinnamon is largely due to its insulin-enhancing components, which are water-soluble polyphenols and further identified as type-A proanthocyanidin polymers (*19*). In the present work, we found the aqueous extract of cinnamon bark showed significant inhibitory activity on the formation of AGEs. Subsequently, we identified procyandin B2, epicatechin, catechin, proanthocyanidin oligomers and polymers as AGE formation inhibitors in cinnamon bark extract, which can directly trap reactive carbonyl species. Our research work provides additional evidence to support the health benefits of cinnamon bark for diabetic patients.

MATERIALS AND METHODS

Chemicals and Instruments. Cinnamon barks were obtained from a local pharmacy in Hong Kong. Aminoguanidine (AG), sodium azide, phosphate buffer saline (PBS, pH 7.4), bovine serum albumin (BSA), D-glucose, methylglyoxal (MGO) (40% aqueous solution), 1,2-phenylenediamine (PD), 2,3-dimethylquinoxaline (DQ), catechin, and epicatechin were purchased from Sigma-Aldrich Co. (St. Louis, MO). Procyanidin B2 (reference standard) was purchased from Extrasynthese (Genay, France). Sephadex LH-20 resin was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All analytical and HPLC grade solvents used were obtained from BDH Laboratory Supplies (Poole, U.K.). HPLC analysis was performed on a Shimadzu LC-20AT system equipped with a diode array detector and LC-Solution software. Semipreparative HPLC was carried out on a Waters Delta 600 system equipped with a 2487 dual-wavelength detector, a Masslynx V4.0 software, and a Phenomenex Luna C18 (2) column (250 × 21.2 mm, 5 μm). Fluorescence intensity was measured with a Hitachi F-2500 fluorescent spectrometer (Hitachi Corp., Tokyo, Japan). LC-MS analysis was performed on a Waters Alliance 2695 (Milford, MA) HPLC system coupled to a Micromass ZQ 2000 mass spectrometer equipped with an electrospray ionization source and Masslynx V4.0 software.

Preparation of Different Fractions of Cinnamon Bark Extract. Two hundred grams of cinnamon bark was ground into fine powder and extracted with water (2 × 600 mL) through sonication for 1 h each time. After vacuum filtration, absolute ethanol (twice the volume of the filtrate) was added to the filtrate, which was then stirred for 2 h. The ethanol-diluted extract was centrifuged and filtered through Whatman no. 1 filter paper, then concentrated under vacuum at 50 °C using a rotary evaporator, and then stored in a desiccator. The dried cinnamon extract (10.85 g) was suspended in water, and the resultant suspension was partitioned with ethyl acetate (5 × 0.5 L) to give an ethyl acetate-soluble fraction (0.78 g after drying). The aqueous layer was then partitioned with 1-butanol (5 × 0.5 L) to afford the 1-butanol-soluble extract (2.44 g after drying). The ethyl acetate-soluble fraction was dissolved in 95% ethanol and loaded onto a Sephadex LH-20 column (4 × 45.5 cm). The elution stared with 95% ethanol, followed by 50% acetone, and three subfractions (ethyl-F1, ethyl-F2, and ethyl-F3) were collected. Similarly, the dried 1-butanol-soluble layer was dissolved in methanol and fractionated using Sephadex LH-20 column chromatography eluted with 70% acetone to yield three subfractions (butanol-F1, butanol-F2, and butanol-F3).

BSA−Glucose assay. This assay was adopted from the literature (*20, 21*) and used as an in vitro model for comparison of the antiglycation activities. In brief, 5 g of BSA and 14.4 g of D-glucose were dissolved in phosphate buffer saline (pH 7.4) to obtain the control solution with 50 mg/mL BSA and 0.8 M D-glucose. A 2.76 mL amount of the control solution was incubated at 37 °C for 7 days in the presence or absence of 0.24 mL of the fractions and subfractions of cinnamon bark extract dissolved in phosphate buffer (pH 7.4) (the final concentration of fractions and subfractions in the 3 mL test solution was 200 ppm). The test solution also contained 0.2 g/L NaN_3 to ensure an aseptic condition. AG (1 mM) was used as a positive control. For testing of purified compounds, 100 μM concentrations of catechin, epicatechin, and procyanidin B2 were used. After 7 days of incubation, fluorescence 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%.

Evaluation of Direct MGO Trapping Capacity. Direct MGO trapping capacity was tested using the method described by Peng et al. (*22*). Briefly, MGO (5 mM), PD (derivatization agent, 20 mM), and DQ (internal standard, 5 mM) were freshly prepared in phosphate buffer saline (pH 7.4); 0.25 mL of the prepared MGO solution was mixed with 0.25 mL of PBS (blank), subfractions of cinnamon bark extract (10 mg dissolved in 4 mL PBS), or compound solution (5 mM AG, catechin, epicatechin, and procyanidin B2). After mixing, the mixtures were incubated in a water bath at 37 °C. One hour later, samples were taken out, and 0.125 mL of derivatization agent (20 mM PD) and 0.125 mL of internal standard (5 mM DQ) were added and shaken by vortex for 5 s. After 0.5 h (when the derivatization reaction was completed), HPLC analysis was performed to quantify the residual MGO on the basis of the amount of the derivatized product, 1-methylquinoxaline (MQ), formed in each sample. Separation was carried out on a Luna Phenyl-hexyl column (150 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA). The flow rate was 1.0 mL/min, and the injection volume was 15 μL. Isocratic elution was applied using H_2O/MeOH (50:50, v/v) as the mobile phase. The total running time was 10 min, and chromatograms were recorded at 315 nm. The amounts of unreacted MGO in the samples [MGO added with PBS (control), fractions, compounds, respectively] could be worked out on the basis of the ratios of peak area of MQ and DQ. 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 fraction or compound)/amounts of MGO in control] × 100%.

Analysis of the Chemical Substances in Different Fractions and Isolation of Procyanidin B2 from Fraction Ethyl-F2. Crude cinnamon extract and all of the fractions and subfractions obtained through Sephadex LH-20 column chromatography as described previously were analyzed by HPLC using a prepacked Alltima C_{18} column (250 × 4.6 mm, 5 μm, Metachem Technologies Inc., Torrance, CA). The absorption spectra were recorded from 200 to 400 nm for all peaks. The flow rate was 1.0 mL/min, and the injection volume was 15 μL. The mobile phase contained 0.2% acetic acid (v/v; solvent A) and acetonitrile

(solvent B). The initial ratio of solvent A/solvent B was 90:10. The gradient increased to 80:20 during the first 10 min, to 60:40 (10 min), and then to 45:55 (10 min). The gradient continued to increase to 10:90 in 1 min, and this gradient was kept for 7 min. The total running time was 38 min, and the post running time was 17 min. Mass spectra of ethyl-F1, ethyl-F2, ethyl-F3, and butanol-F3 were obtained in negative ion mode over a range of *m*/*z* 100–1500. The desolvation temperature was 300 °C. Capillary and cone voltages were 3400 and 30 V, respectively. The ionization source worked at 103 °C.

Fraction ethyl-F2 was subjected to detailed separation. It was repeatedly separated by semipreparative HPLC with a Zorbax SB-Phenyl column (250 × 9.4 mm) using H_2O/acetonitrile (80:20) as the mobile phase to obtain compounds **1**−**4**. The flow rate was 3.5 mL/min, and the detection wavelength was set at 278 nm.

Preliminary Study on the Reaction Products of Procyanidin B2 and MGO. It was reported that the products of the reaction between (−)-epigallocatechin gallate (EGCG) and MGO are mainly their adducts at the C_8 position of EGCG A-ring (*23*). This study hereby focused on the analysis of adducts of procyanidin B2 and MGO, which are possibly primary reaction products. Similar to the procedure of the experiment above, each of the reaction systems contained 0.1 mL of 5 mM MGO solution and one of the following agents: 0.2 mL of PBS, 0.2 mL of 5 mM procyanidin B2, or 0.1 mL of 5 mM procyanidin B2 plus 0.1 mL of PBS. After mixing, the samples were incubated in a water bath at 37 °C. One hour later, the samples were taken out, and 60 μL of 20 mM PD was added and shaken by vortex for 5 s. Half an hour later, the samples were analyzed by LC-MS using a prepacked Alltima C_{18} column (250 × 4.6 mm, 5 μm, Metachem Technologies Inc.). The absorption spectra were recorded at 278 and 313 nm for all peaks. The flow rate was 1.0 mL/min, and the injection volume was 10 μL. The mobile phase was composed of water with 0.15% acetic acid (v/v; solvent A) and acetonitrile (solvent B). The elution started with 5% B followed by a linear gradient to 20% B in 10 min. Then it was 20–30% B from 10 to 12 min, 30–90% B from 12 to 13 min, and finally kept at 90% B for 2 min. The post running time was 10 min. Mass spectra were obtained in negative ion mode, and selective ion monitoring was performed at *m*/*z* 577 and 649 for procyanidin B2 and adducts of procyanidin B2 and MGO, respectively. The desolvation temperature was 300 °C. Capillary and cone voltages were 3400 and 30 V, respectively. The ionization source worked at 103 °C.

RESULTS AND DISCUSSION

The BSA−glucose model adopted in this study provides a useful tool for assessing the effects of cinnamon fractions on the nonenzymatic glycation process. Panel **A** of **Figure 1** displays the inhibitory effects of three cinnamon bark extract fractions on AGE formation in this model. The ethyl acetate-soluble fraction and 1-butanol-soluble fraction showed significant inhibitory activities at a concentration of 200 ppm with 66.2 and 59.5% inhibition, respectively. In contrast, the aqueous fraction had only 43.6% inhibition. Subsequently, antiglycation activities of the subfractions from the above two effective fractions were examined (**Figure 1B**). Being capable of resulting in more than 40% reduction in the formation of fluorescent AGEs, ethyl-F1, ethyl-F2, ethyl-F3, and butanol-F3 were shown to be more promising antiglycation candidates than the other two subfractions. It was not a surprise to find these factions showed antiglycation activities in BSA−glucose model as they all had high contents of phenolic compounds. On the basis of a literature search, many purified phenolic compounds (including flavones, flavanones, flavonols, isoflavones, proanthocyanidins, and other phenolics) and phenolic-rich plant extracts have been found to have strong inhibitory activity in this bioassay. For most of the phenolic compounds so far examined, a good correlation exists between their free radical scavenging capacity and AGE inhibitory activity in vitro (*24, 25*). This suggests that they exert their inhibitory activity by interrupting the autoxidative pathways. In fact, there is growing evidence that

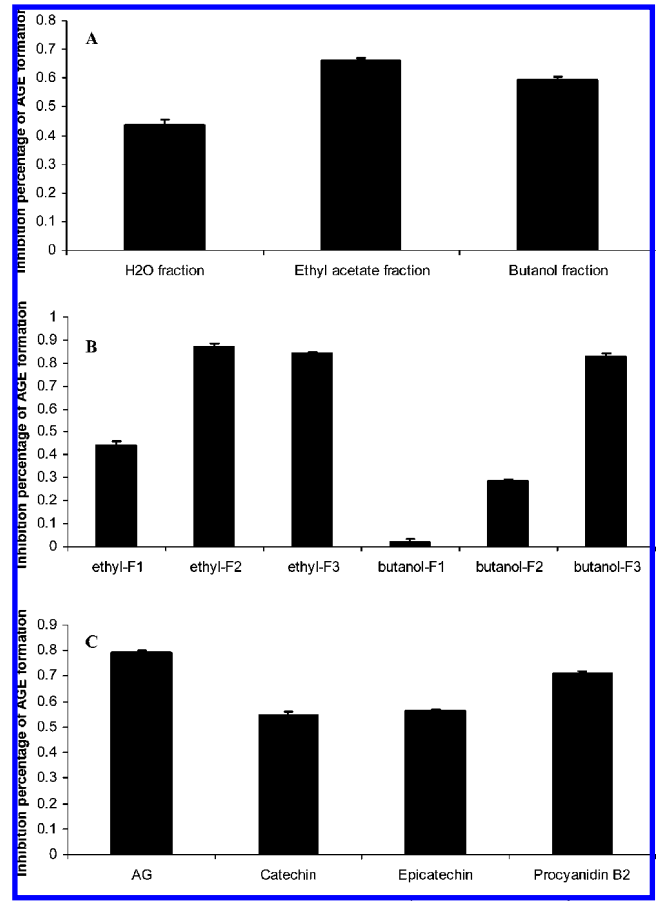

Figure 1. Results for BSA−glucose assay. (**A**) Inhibitory effects of different fractions of cinnamon extract on the formation of AGEs. The concentration of each fraction is 200 ppm. (**B**) Inhibitory effects of subfractions of cinnamon extract on the formation of fluorescent AGEs. The concentration of each subfraction is 200 ppm. (**C**) Inhibitory effects of catechin, epicatechin, and procyanidin B2 on formation of AGEs. Concentrations of 100 μM catechin, epicatechin, procyanidin B2, and 1 mM AG (positive control) were examined in this assay. Results are means ± SD for $n = 3$. Fluorescent intensities of all samples were significantly different from that of the control solution ($P < 0.01$).

production of ROS is increased in diabetes patients and that oxidative stress is associated with diabetic complications. In contrast, numerous clinical trials have failed to provide conclusive evidence for the efficacy of natural antioxidant therapy in diabetic patients (*26*). These findings strongly suggest that free radical scavenging may be effective in suppressing AGE formation only under certain in vitro conditions and that inhibiting autoxidation alone is unlikely an effective way of preventing or treating diabetic complications when more complex physiological environments are involved. It would therefore be of great interest to identify and investigate natural AGE inhibitors that can suppress the formation of AGEs, both through preventing glycoxidations and by sequestering the reactive 1,2-dicarbonyls, the important precursors of AGEs. Therefore, in the following section of this research work, investigation was conducted to see whether these subfractions could directly trap 1,2-dicarbonyls. As in **Figure 2A**, ethyl-F2 and ethyl-F3 showed significant MGO trapping abilities. Butanol-F3 also had an obvious effect on scavenging MGO. Nearly no trapping activity was found for ethyl-F1, butanol-F1, and F2. Subsequently, chromatographic and spectroscopic methods were applied to examine the active components in these fractions. We started with ethyl-F2. As shown in **Figure 3**, the

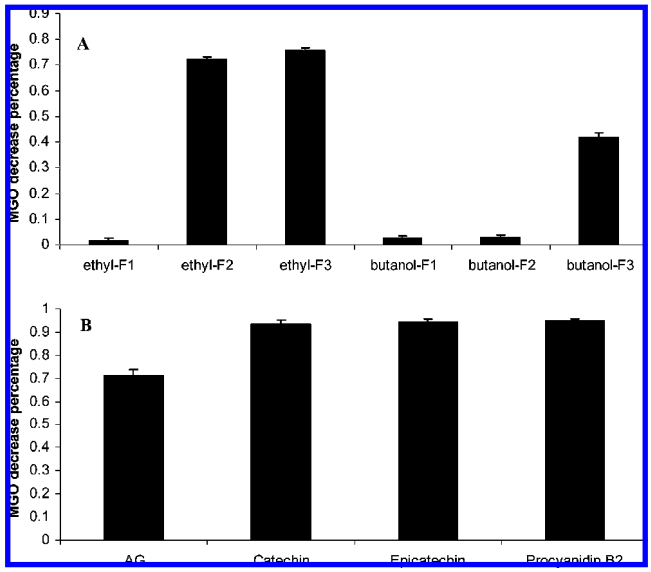

Figure 2. Results for MGO trapping capacity experiment. (**A**) MGO trapping capacities of four subfractions. The concentration of each subfraction is 2.5 mg/mL. (**B**) MGO scavenging abilities of catechin, epicatechin, procyanidin B2, and AG (positive control). All compounds are at the concentration of 5 mM. Results are expressed as mean ± half range ($n = 3$). MGO trapping capacities of all samples were significantly different from that of the control ($P < 0.01$).

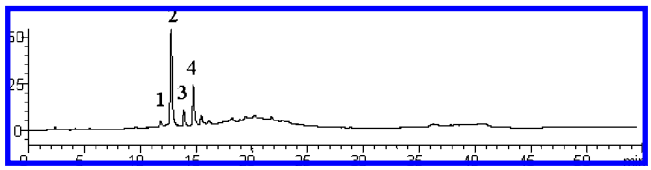

Figure 3. HPLC chromatogram of subfraction ethyl-F2 at 278 nm. Peaks 1, 2, and 3 were identified as catechin, procyanidin B2, and epicatechin, respectively. Peak 4 was unknown.

HPLC chromatogram of ethyl-F2 had five major peaks. Detailed chromatographic separation led to the purification of compounds **1**−**4**. In the MGO trapping assay, all of them, except compound **4**, showed significant trapping capacities. With reference to the retention time and mass spectra obtained from authentic compounds, peaks 1, 2, and 3 were identified as catechin, procyanidin B2, and epicatechin, respectively. As shown in **Figure 1C**, at 100 μM, procyanidin B2 demonstrated the strongest inhibitory effect on AGE formation among the three proanthocyanidins isolated, only slightly weaker than that of 1 mM AG, a typical inhibitor of AGE formation and scavenger for RCS (*27*). It is interesting to observe that catechin, epicatechin, and procyanidin B2 all possessed more potent MGO trapping capacity than AG (**Figure 2B**). This is the first report of procyanidin B2 as a reactive carbonyl (MGO) trapping agent, which behaved in a dose-dependent manner (**Figure 4**). For the subfractions, ethyl-F3 and butanol-F3, LC-MS revealed their main constituents as proanthocyanidin oligomers and polymers. However, attempts to separate them by various chromatographic methods failed. It is not surprising that these compounds can also trap RCS as they all have structures similar to that of procyanidin B2.

Lo et al. recently reported that EGCG could react with MGO (molar ratio of EGCG/MGO = 1:1) to generate isomeric adducts at the C_8-position of the EGCG A-ring (*23*). Thus, it is logical to propose that catechin and epicatechin could react with MGO in a similar way, considering their structural similarity to EGCG. On the basis of the above reasoning, it is probable that procyanidin B2, a condensation dimer of catechin and epicatechin, contains two MGO-reactive sites for MGO as procyanidin B2 is a dimer condensed from catechin and epicatechin. However, the observation that procyanidin B2 had MGO scavenging ability similar to those of catechin and epicatechin (**Figure 2B**) disproved this hypothesis. Therefore, the MGO−procyanidin B2 adducts should be composed of one molecule of MGO and one molecule of procyanidin B2. LC-MS with selective ion monitoring of the reaction products (**Figure 5**) offered concrete evidence. As the molecular weights of procyanidin B2 and MGO are 578 and 72, respectively, selective ion monitoring was set at *m*/*z* 649 under negative ion mode. Four peaks at 8.5, 9.35, 9.85, and 10.35 min were determined in the samples with added procyanidin B2 (**Figure 5b**,**c**). None of these peaks was found in the chromatogram of the control sample (**Figure 5a**). As four peaks were identified at *m*/*z* 649, which corresponds to the molecular ion peak of the MGO−procynaidin B2 adduct, the reaction between procyanidin B2 and MGO likely gave rise to a group of stereoisomers. In addition, peak height was positively correlated with the amount of procyanidin B2 added (panel **c** vs panel **b** of **Figure 5**), suggesting a dose-dependent behavior of procyanidin B2 in trapping MGO.

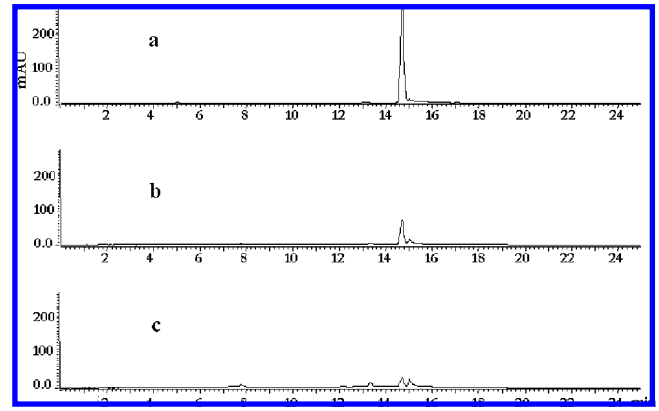

Figure 4. Chromatograms of MGO only (**a**), MGO with an added low concentration of procyanidin B2 (**b**), and MGO with an added high concentration of procyanidin B2 (**c**) after incubation and derivatization at 278 nm.

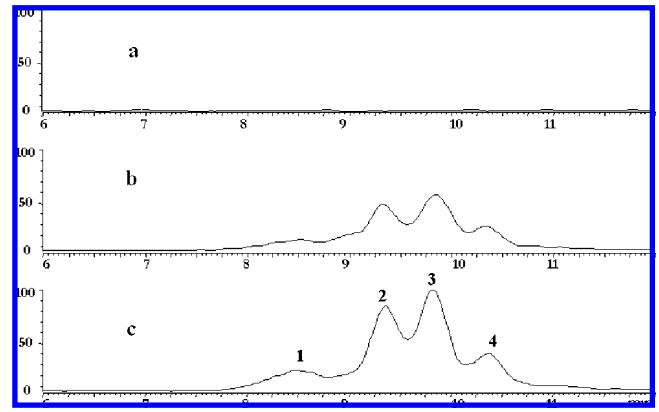

Figure 5. Selective ion monitoring chromatograms of MGO only (**a**), MGO with 0.1 mL of 5 mM procyanidin B2 added (**b**), and MGO with 0.2 mL of 5 mM procyanidin B2 added (**c**) after incubation and derivatization at *m*/*z* 649.

In summary, cinnamon bark has been reported to benefit people with diabetes because of its antioxidant and insulin-enhancing activities. Our study found that cinnamon bark extract could inhibit the formation of AGEs, which have been implicated in the pathogenic process of diabetic complications. The inhibitory effect of cinnamon bark on AGE formation is mainly

attributed to the antiglycation activities of some of its phenolic constituents, such as catechin, epicatechin, and procyanidin B2, likely trapping reactive carbonyl species. Apart from proanthocyanidin dimers, findings from the present study also supported the effectiveness of proanthocyanidin oligomers and polymers as natural antiglycation agents.

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