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ARTICLE

# Astragalosides Isolated from the Root of *Astragalus Radix* Inhibit the Formation of Advanced Glycation End Products

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Because advanced glycation end product (AGE) inhibitors such as pyridoxamine significantly inhibit the development of retinopathy and neuropathy in the streptozotocin-induced diabetic rat, treatment with AGE inhibitors is believed to be a potential strategy for the prevention of lifestyle-related diseases such as diabetic complications. A crude extract of *Astragali Radix* (AR; roots of *Astragalus membranaceus*) inhibits the formation of $N^{\varepsilon}$-(carboxymethyl)lysine (CML) and pentosidine during the incubation of bovine serum albumin with ribose. In the present study, compounds were isolated from AR that prevented CML and pentosidine formation. Astragalosides significantly inhibited the formation of both CML and pentosidine, and astragaloside V had the strongest inhibitory effect among all if the isolated compounds. These data suggest that AR and astragalosides may be a potentially useful strategy for the prevention of clinical diabetic complications by inhibiting AGEs.

**KEYWORDS: Advanced glycation end products; $N^{\varepsilon}$-(carboxymethyl)lysine; pentosidine; *Astragali Radix*; astragaloside**

## INTRODUCTION

Long-term incubation of proteins with glucose leads, through the formation of early products such as Schiff base and Amadri products, to the formation of advanced glycation end products (AGEs) (*1*). $N^{\varepsilon}$-(Carboxymethyl)lysine (CML), a major antigenic AGE structure, accumulates in several human and animal tissues during aging (*2*) and various diseases including diabetic nephropathy (*3*). Cellular interactions with CML-modified proteins induce several biological responses, which are involved in the development of diabetic vascular complications (*3*). Pentosidine is an AGE structure that possesses fluorescent and cross-linking properties (*4*) and can be a glycoxidation marker for AGEs in proteins (*5*). Pentosidine has been identified in skin collagen (*1*,*4*), glomerular basement membranes (*4*, *6*), and plasma proteins of diabetic patients (*7*). The pentosidine level in skin is correlated with the severity of diabetic complications (*8*). Furthermore, a dramatic increase in pentosidine levels is observed in the plasma of patients with end-stage renal failure (*7*, *9*), $\beta$2-microglobulin amyloid deposits (*10*), and skin collagens (*11*), irrespective of the presence or absence of diabetes. Pentosidine has thus been implicated in tissue damage in not only diabetic patients but also in hemodialysis patients with end-stage renal failure.

CML is generated by the oxidative cleavage of Amadori products by the hydroxyl radical (*12*), peroxynitrite (*13*), and hypochlorous acid (*14*), thus suggesting that CML is an important biological marker of oxidative stress in vivo. Furthermore, pentosidine is also an oxidation-dependent AGE structure because its formation is inhibited by antioxidative conditions (*5*).

Pyridoxamine, which was originally proposed to be an inhibitor of the oxidative degradation of fructosamine to AGEs (*15*), inhibits the formation of AGEs and lipid peroxidation products (*16*). Pyridoxamine inhibits the development of retinopathy and neuropathy in streptozotocin (STZ)-induced diabetic rats (*17*, *18*). Therefore, treatment with AGE inhibitors and antioxidants may be a potential strategy for the prevention of clinical diabetic complications. However, the screening of new AGE inhibitors by instrumental analyses such as high-performance liquid chromatography (HPLC) is required for several preparation steps, and it is difficult to estimate the inhibitory effects of a great number of candidate compounds. In fact, although the inhibitory effects of several crude extracts from natural products on AGE formation have been documented, the active compound(s) in those crude extracts and AGE structure(s) that are inhibited have not yet been identified (*19*).

In the present study an assay system was developed to estimate the formation of CML and pentosidine using antibodies, and then the inhibitory effect of 50 crude extracts from natural products and Chinese herbs such as *Epimedii Herba*, *Artemisiae Capillari Flos*, *Foeniculi Fructus*, and *Astragali Radix* were measured. Under the assay conditions employed, *Astragali Radix* (AR; roots of *Astragalus membranaceus* Bunge, Leguminosae family) extract significantly inhibited CML and pentosidine

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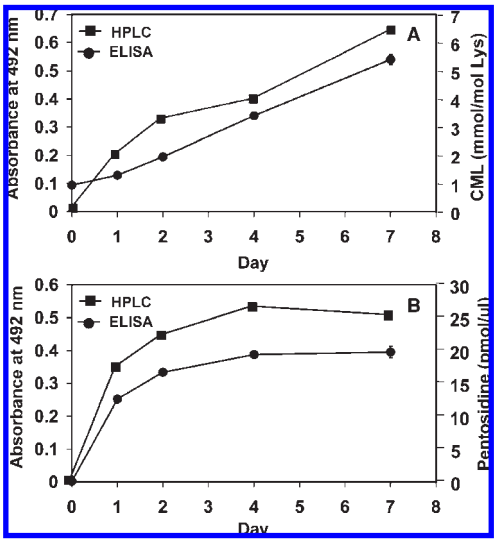


**Figure 1.** Time dependency of CML and pentosidine formation. BSA and ribose were incubated in phosphate buffer for 7 days followed by determination of CML (**A**) and pentosidine (**B**) formation by noncompetitive ELISA and HPLC analysis as described under Materials and Methods.

formation. Furthermore, compounds such as astragalosides and isoastragalosides were isolated from AR, and the inhibitory effect of those compounds on CML and pentosidine formation was also measured. As a result, astragaloside V significantly inhibited CML and pentosidine formation. These results suggest that astragaloside V could be a new therapeutic compound inhibiting the development of diabetic complications such as diabetic nephropathy, retinopathy, and neuropathy by inhibiting AGE formation.

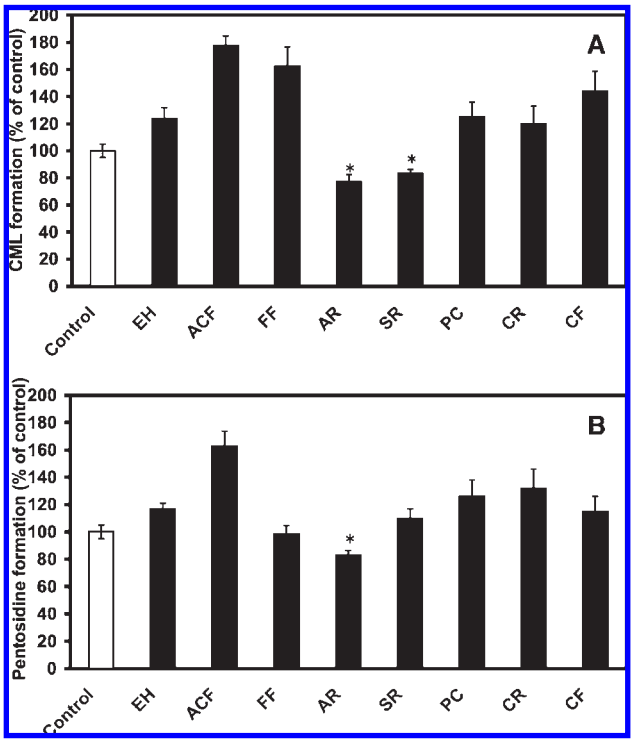


**Figure 2.** Inhibitory effect of AR extract on CML and pentosidine formation. BSA and ribose were incubated with 1 mg/mL natural products (EH, *Epimedii Herba*; ACF, *Artemisiae Capillari Flos*; FF, *Foeniculi Fructus*; SR, *Scutellariae Radix*; PC, *Phellodendori Cortex*; CR, *Coptidis rhizoma*; CF, *Catalpae Fructus*) extract in phosphate buffer for 7 days followed by determination of CML (**A**) and pentosidine (**B**) by noncompetitive ELISA as described under Materials and Methods. Data are presented as the mean ± SD. ∗, $P < 0.01$ versus control.

## MATERIALS AND METHODS

**Chemicals.** *Astragalus Radix* (lot SU262625) inspected and certified by regulation of the Japanese Pharmacopeia was purchased from Uchida Wakan-yaku Co. Ltd. (Tokyo, Japan). Fatty acid-free BSA and methyl hydroquinone were purchased from Wako (Osaka, Japan). Ribose was purchased from Acros. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody was purchased from Kirkegaard Perry Laboratories. Monoclonal anti-pentosidine antibody, 1C12, was prepared by immunizing diacetylpentosidine-conjugated human serum albumin HSA (*20*). The reaction of the antibody with pentosidine−HSA was effectively inhibited by free pentosidine; the concentration for 50% inhibition ($IC_{50}$) was 6 nM. In contrast, L-arginine and L-lysine, starting materials for pentosidine formation, were not recognized by the antibody. Furthermore, other compounds, such as creatine, creatinine, *N*-monomethylarginine, and *N*-propylpyridinium bromide, did not compete with the antibody for binding to pentosidine−HSA. We have confirmed that this monoclonal anti-pentosidine antibody does not cross-react with CML. Microtitration plates (96-well, Nunc Immunoplate II) were purchased from Nippon Iner Med (Tokyo, Japan). CML standard was prepared as described previously (*13*). Briefly, 0.26 M $N^{\alpha}$-acetyllysine was incubated overnight with 0.13 M glyoxylic acid in the presence of 0.65 M $NaCNBH_3$ in 0.5 mL of 0.1 M sodium carbonate buffer (pH 10.0) at room temperature. Pentosidine standard was prepared as described previously (*20*). Briefly, 2.19 g of D-ribose, 2.5 g of *N*-acetyllysine, and 3.69 g of *N*-acetylarginine were dissolved in 58 mL of 100 mM sodium phosphate, the pH was adjusted to 9.0 by the addition of 5 M sodium hydroxide, and the mixture was stirred for 48 h at 65 °C. The reaction mixture was diluted 5-fold with water and passed through 800 mL of Diaion HP-20 (Mitsubishi Chemical, Tokyo) cation exchange resin. The column was eluted with 2 L of water, 5% methanol, 10% methanol, and 20% methanol, followed by elution with 2 L of 40% methanol. Our analytical HPLC system described below detected $N,N^{\phi}$-diacetyl pentosidine in the fraction eluted with 40% methanol. The crude product was then purified according to a modification of the HPLC system reported by Grandhee and Monnier (*21*). All other chemicals were of the best grade available from commercial sources.

**Preparation of Crude Extracts.** *Astragali Radix*, *Epimedii Herba*, *Artemisiae Capillari Flos*, *Foeniculi Fructus*, *Scutellariae Radix*, *Phellodendori Cortex*, *Coptidis rhizoma*, *Catalpae Fructus*, *Gambir*, *Mume Fructus*, *Corydalis Tuber*, *Cuculus canorus*, *Puerariae Radix*, *Zingiberis Rhizoma*, *Aurantii Fructus*, *Immaturus*, *Armeniacae Semen*, *Sophorae Radix*, *Schizonepetae Spica*, *Cinnamomi Cortex*, *Magnoliae Cortex*, *Schisandrae Fructus*, *Asiasari Radix*, *Rehmanniae Radix*, *Paeoniae Radix*, *Cimicifugae Rhizoma*, *Cnidii Rhizoma*, *Atractylodis Lanceae*, *Zizyphi Fructus*, *Alismatis Rhizoma*, *Ophiopogonis Tuber*, *Atractylodis Rhizoma*, *Hoelen*, *Coicis Semen*, *Polygalae Radix*, *Glycyrrhizae Radix*, *Anemarrhenae Rhizoma*, *Zedoariae Rhizoma*, *Cassiae Semen*, *Pharbitidis Semen*, *Cyperi Rhizoma*, *Schisandrae Fructus*, *Bupleuri Radix*, *Lithospermi Radix*, *Plantaginis Semen*, *Houttuyniae Herba*, *Nupharis Rhizoma*, *Polyporus*, *Aurantii Nobilis Pericarpium*, *Angelicae Radix*, and *Angelicae Dahuricae Rhizoma* were extracted with MeOH (three times) by refluxing for 2 h, and the extracts were concentrated in vacuo to afford residues. The residues were loaded onto a Diaion HP-20 column and eluted by $H_2O$ and MeOH. Those MeOH eluates were used as crude extracts in the AGE inhibition assay.

**Extraction and Isolation of Astragalosides and Isoastragalosides from AR.** AR (1.3 kg) was extracted with MeOH (three times) by refluxing for 2 h, and the extract was concentrated in vacuo to afford residues (311.5 g). The residues were loaded onto a Diaion HP-20 column (60 × 300 mm) and eluted by a stepwise gradient of $H_2O$/MeOH (100:0, 20:80, 40:60, 60:40, 80:20, 100:0). The obtained fractions, 20% MeOH (5.4 g), 40% MeOH (5.6 g), 60% MeOH (8.3 g), 80% MeOH (6.1 g), and 100% MeOH (0.65 g), were tested for inhibitory effect on CML and pentosidine formation. The 80% MeOH eluate (0.47% from AR) was the

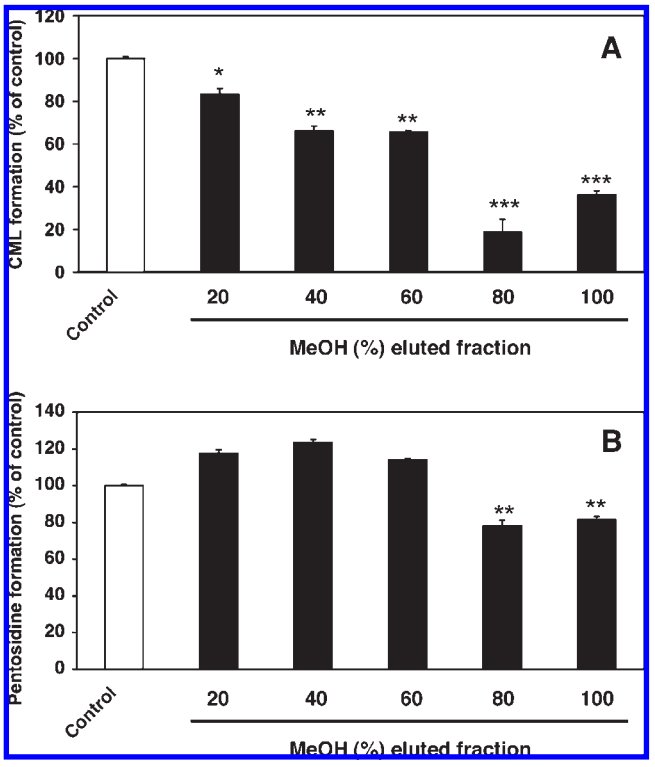


**Figure 3.** Inhibitory effect of the fractions isolated by Diaion HP-20 column chromatography on CML and pentosidine formation. BSA and ribose were incubated with indicated MeOH eluted fractions in phosphate buffer for 7 days followed by determination of CML (**A**) and pentosidine (**B**) by noncompetitive ELISA as described under Materials and Methods. Data are presented as the mean ± SD. ∗, $P < 0.01$; ∗∗, $P < 0.001$; ∗∗∗, $P < 0.0001$ versus control.

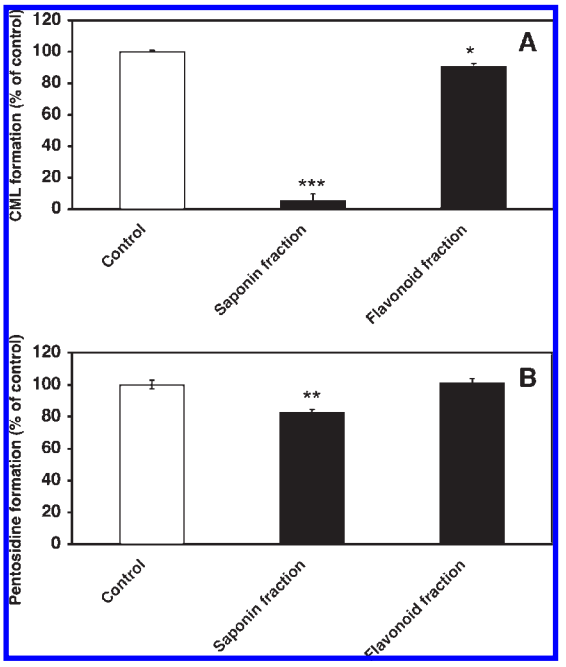


**Figure 4.** Inhibitory effect of the saponin fraction and the flavonoid fraction on CML and pentosidine formation. BSA and ribose were incubated with the saponin fraction and the flavonoid fraction in phosphate buffer for 7 days followed by determination of CML (**A**) and pentosidine (**B**) by noncompetitive ELISA as described under Materials and Methods. Data presented as the mean ± SD. ∗, $P < 0.01$; ∗∗, $P < 0.001$; ∗∗∗, $P < 0.0001$ versus control.

most effective of all fractions and was further separated by Sephadex LH-20 column (∅ 40 × 300 mm, 80% MeOH) to give a saponin fraction (5.2 g, 0.40%) and a flavonoid fraction (0.7 g, 0.054%). By comparison of the saponin and flavonoid fractions, the saponin fraction was found to be significantly effective (see **Figure 4**). As a result, the saponin fraction was further purified with a silica gel column (∅ 45 × 410 mm) and eluted by a stepwise gradient of $CHCl_3$/MeOH/$H_2O$ (C/M/W) (9:1:0.1, 8:2:0.2, 7:3:0.5, 6:4:1). The eluates (20 mL in each test tube) were combined into 22 fractions (1−22) on the basis of silica gel TLC. Fraction 5 (751.3 mg) was chromatographed on a silica gel column (∅ 26 × 290 mm) using C/M/W (gradient elution, from 9:1:0.1 to 8:2:0.2) to give 10 fractions (5-1−5-10). Fraction 5-6 (116.8 mg) was subjected to evaluation by a reversed-phase column (∅ 30 × 150 mm, Chromatorex ODS), using MeOH/$H_2O$ (gradient elution, from 70 to 75% MeOH) to afford three fractions (5-6-1−5-6-3). Fraction 5-6-2 (90.0 mg) was subjected to a silica gel column (∅ 20 × 330 mm) using C/M/W (gradient elution, from 10:1:0 to 8:2:0.2) to furnish **OG-3** (21.8 mg, 0.00168%) and **OG-4** (16.1 mg, 0.00124%). Fraction 8 (183.7 mg) was chromatographed on a reversed-phase column (∅ 38 × 90 mm, Chromatorex ODS), using MeOH/$H_2O$ (gradient elution, from 60 to 70% MeOH) to give seven fractions (8-1−8-7). Fraction 8-5 (123.6 mg) was subjected to a silica gel column (∅ 20 × 310 mm) using C/M/W (gradient elution, from 9:1:0.1 to 8:2:0.2) to afford **OG-6** (17.3 mg, 0.00 133%), **OG-5** (47.4 mg, 0.00365%), and **OG-1** (22.4 mg, 0.00172%). Fraction 11 (200.8 mg) was chromatographed on a reversed-phase column (∅ 30 × 150 mm, Chromatorex ODS), using MeOH/$H_2O$ (gradient elution, from 60 to 70% MeOH) to give six fractions (11-1−11-6). Fraction 11-4 (101.7 mg) was subjected to a silica gel column (∅ 18 × 500 mm) using C/M/W (gradient elution, from 9:1:0.1 to 25:5:0.3) to give **OG-2** (20.9 mg, 0.00161%) and **OG-1** (72.9 mg, 0.00561%). Fraction 16 (142.6 mg) was chromatographed on a reversed-phase column (∅ 20 × 330 mm, Chromatorex ODS), using MeOH/$H_2O$ (gradient elution, from 60 to 65% MeOH) to give **OG-7** (9.6 mg, 0.00074%) and **OG-8** (43.9 mg, 0.00338%). Fraction 18 (372.5 mg) was chromatographed on a reversed-phase column (∅ 30 × 150 mm, Chromatorex ODS), using MeOH/$H_2O$ (gradient elution, from 50 to 60% MeOH) to give six fractions (18-1−18-6). Fraction 18-4 (65.1 mg) was subjected to evaluation by a silica gel column (∅ 18 × 250 mm) using C/M/W (gradient elution, from 8:2:0.2 to 7:3:0.5) to give **OG-9** (27.2 mg, 0.00209%). The structures of the obtained astragalosides **OG-1**−**9** were identified to be astragaloside IV (*22*), astragaloside III (*23*), astragaloside I (*22*), isoastragalside I (*22*), astragaloside II (*22*), isoastragalside II (*22*), astragaloside V (*23*, *24*), astragaloside VI (*23*, *24*), and astragaloside VII (*23*, *24*), respectively, based on the findings of a combined spectroscopic analysis. The NMR data of these compounds closely correlated with those reported previously (*22*−*24*).

***OG-1*** (*astragaloside IV*)*:* white amorphous powder; $[\alpha]_D^{18}$ +28.2° (*c* 0.1, MeOH); negative FAB-MS *m*/*z* 783 $[M - H]^-$; $^1$H NMR (pyridine-$d_5$) $\delta$ 0.21 (1H, d, $J$ = 4.3 Hz, H-19A), 0.56 (1H, d, $J$=4.3 Hz, H-19B), 0.96, 1.31 × 2, 1.37, 1.42, 1.59, 2.03 (each 3H, s, *tert*-$CH_3$), 2.53 (1H, d, $J$=3.7 Hz, H-17), 3.52 (1H, dd, $J$=4.3, 11.6 Hz, H-3), 3.89 (1H, m, H-24), 4.85 (1H, d, $J$ = 7.9 Hz, xyl H-1), 4.90 (1H, d, $J$=7.9 Hz, glc H-1), 5.00 (1H, m, H-16).

***OG-2*** (*astragaloside III*)*:* brown amorphous powder; $[\alpha]_D^{18}$ +13.8° (*c* 0.1, MeOH); negative ESI-MS *m*/*z* 783 $[M - H]^-$; $^1$H NMR (pyridine-$d_5$) $\delta$ 0.29 (1H, m, H-19A), 0.58 (1H, m, H-19B), 1.01, 1.31, 1.32, 1.44 × 2, 1.63, 1.95 (each 3H, s, *tert*-$CH_3$), 3.56 (1H, m, H-3), 4.92 (1H, d, $J$=6.7 Hz, xyl H-1), 5.41 (1H, d, $J$=7.9 Hz, glc H-1).

***OG-3*** (*astragaloside I*)*:* white amorphous powder; $[\alpha]_D^{18}$ +9.6° (*c* 0.1, MeOH); negative FAB-MS *m*/*z* 867 $[M - H]^-$; $^1$H NMR (pyridine-$d_5$) $\delta$ 0.22 (1H, m, H-19A), 0.57 (1H, m, H-19B), 0.94, 1.27, 1.31 × 2, 1.42, 1.59, 1.79 (each 3H, s, *tert*-$CH_3$), 1.97 (3H, s, Ac), 2.03 (3H, s, Ac), 3.40 (1H, m, H-3), 4.82 (1H, d, $J$=7.9 Hz, xyl H-1), 4.93 (1H, d, $J$=7.3 Hz, glc H-1).

***OG-4*** (*isoastragaloside I*)*:* white amorphous powder; $[\alpha]_D^{18}$ +21.1° (*c* 0.1, MeOH); negative FAB-MS *m*/*z* 867 $[M - H]^-$; $^1$H NMR (pyridine-$d_5$) $\delta$ 0.19 (1H, m, H-19A), 0.56 (1H, m, H-19B), 0.93, 1.27, 1.31 × 2, 1.42, 1.59, 1.82 (each 3H, s, *tert*-$CH_3$), 1.96 (3H, s, Ac), 2.03 (3H, s, Ac), 3.41 (1H, m, H-3), 4.81 (1H, d, $J$=7.9 Hz, xyl H-1).

***OG-5*** (*astragaloside II*)*:* white amorphous powder; $[\alpha]_D^{18}$ +30.4° (*c* 0.1, MeOH); negative FAB-MS *m*/*z* 825 $[M - H]^-$; $^1$H NMR (pyridine-$d_5$) $\delta$ 0.20 (1H, d, $J$ = 3.7 Hz, H-19A), 0.57 (1H, d, $J$ = 3.7 Hz, H-19B), 0.95, 1.28, 1.30, 1.31, 1.41, 1.56, 1.81 (each 3H, s, *tert*-$CH_3$), 2.05 (3H, s, Ac),

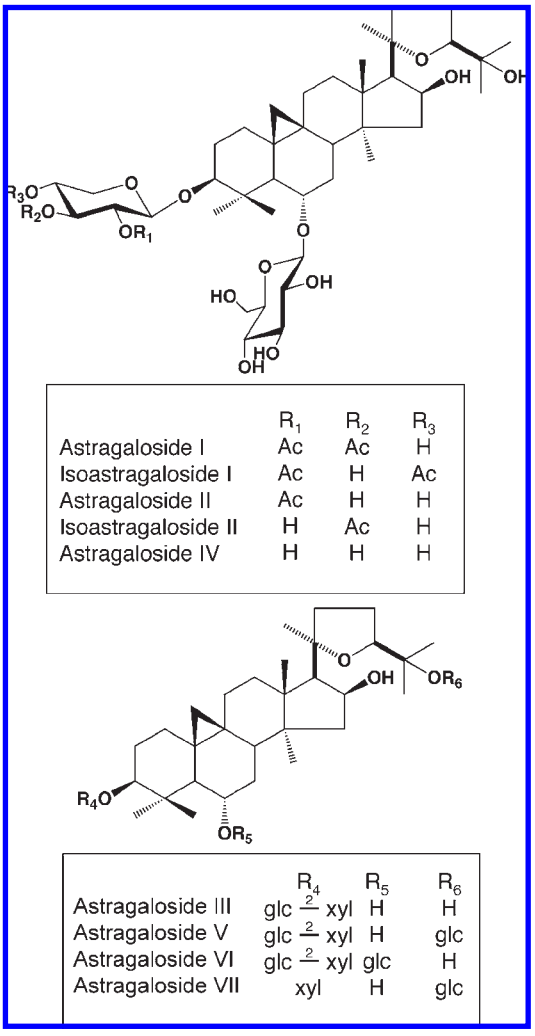


**Figure 5.** Structures of astragalosides and isoastragalosides.

3.41 (1H, m, H-3), 4.79 (1H, d, $J$ = 7.9 Hz, xyl H-1), 4.91 (1H, d, $J$ = 7.3 Hz, glc H-1), 5.54 (1H, br, xyl H-2).

***OG-6*** (*isoastragaloside II*)*:* white amorphous powder; $[\alpha]_D^{18}$ +15.1° (*c* 0.1, MeOH); negative ESI-MS *m*/*z* 825 $[M - H]^-$; $^1$H NMR (pyridine-$d_5$) $\delta$ 0.22 (1H, m, H-19A), 0.59 (1H, m, H-19B), 0.96, 1.31, 1.33, 1.43, 1.59, 1.67, 1.97 (each 3H, s, *tert*-$CH_3$), 2.00 (3H, s, Ac), 3.50 (1H, m, H-3), 4.84 (1H, d, $J$ = 7.3 Hz, xyl H-1), 5.91 (1H, d, $J$ = 7.3 Hz, glc H-1), 5.71 (1H, m, xyl H-3).

***OG-7*** (*astragaloside V*)*:* brown amorphous powder; $[\alpha]_D^{18}$ +9.2° (*c* 0.1, MeOH); negative FAB-MS *m*/*z* 945 $[M - H]^-$; $^1$H NMR (pyridine-$d_5$) $\delta$ 0.27 (1H, d, $J$ = 3.7 Hz, H-19A), 0.56 (1H, d, $J$ = 3.7 Hz, H-19B), 0.94, 1.29, 1.35, 1.42, 1.43, 1.67, 1.94 (each 3H, s, *tertiary*-$CH_3$), 3.55 (1H, dd, $J$ = 4.3, 11.6 Hz, H-3), 4.91 (1H, d, $J$ = 6.1 Hz, xyl H-1), 5.06 (1H, m, glc3 H-1), 5.40 (1H, d, $J$ = 7.3 Hz, glc1 H-1).

***OG-8*** (*astragaloside VI*)*:* brown amorphous powder; $[\alpha]_D^{18}$ +17.2° (*c* 0.1, MeOH); negative FAB-MS *m*/*z* 945 $[M - H]^-$; $^1$H NMR (pyridine-$d_5$) $\delta$ 0.18 (1H, d, $J$ = 4.3 Hz, H-19A), 0.66 (1H, d, $J$ = 4.3 Hz, H-19B), 0.98, 1.26, 1.30, 1.42, 1.43, 1.58, 1.91 (each 3H, s, *tert*-$CH_3$), 3.42 (1H, m, H-3), 3.77 (1H, m, H-6), 4.86 (1H, d, $J$ = 7.9 Hz, xyl H-1), 4.97 (1H, m, glc2 H-1), 5.34 (1H, d, $J$ = 7.9 Hz, glc1 H-1).

***OG-9*** (*astragaloside VII*)*:* brown amorphous powder; $[\alpha]_D^{18}$ +11.2° (*c* 0.1, MeOH); negative FAB-MS *m*/*z* 945 $[M - H]^-$; $^1$H NMR (pyridine-$d_5$) $\delta$ 0.18 (1H, d, $J$ = 4.3 Hz, H-19A), 0.58 (1H, d, $J$ = 4.3 Hz, H-19B), 0.92, 1.26, 1.33, 1.36, 1.42, 1.65, 2.01 (each 3H, s, *tert*-$CH_3$), 3.52 (1H, m, H-3).

**Determination of the Inhibitory Effect of Plant Extracts and Natural Compounds on CML Formation.** BSA (2 mg/mL) and ribose (33 mM) were incubated with either plant extracts (1 mg/mL) or natural compounds (1 mM) in PBS at 37 °C for 7 days, followed by the determination of CML by a noncompetitive enzyme-linked immunosorbent assay (ELISA) and HPLC analysis.

**Determination of the Inhibitory Effect of Plant Extracts and Natural Compounds on Pentosidine Formation.** BSA (2 mg/mL) and ribose (100 mM) were incubated with plant extracts (1 mg/mL) or natural compounds (1 mM) in 200 mM sodium phosphate buffer at 60 °C for 7 days, followed by the determination of pentosidine by both noncompetitive ELISA and HPLC analyses.

**Determination of the Inhibitory Effect of Astragaloside V on CML Formation from Amadori Products.** Glycated HSA (2 mg/mL) was incubated with astragaloside V (1 mM) in PBS at 37 °C for 5 days, followed by the determination of CML by ELISA.

**Determination of the Inhibitory Effect of Astragaloside V on CML Formation from Glyoxal Pathway.** BSA (2 mg/mL) and glyoxal (5 mM) were incubated with astragaloside V (1 mM) in PBS at 37 °C for 2 days, followed by the determination of CML by ELISA.

**ELISA.** ELISA was performed as described previously (*25*). Briefly, each well of a 96-well microtiter plate was coated with 100 $\mu$L of the sample to be tested in PBS, blocked with 0.5% gelatin, and washed three times with PBS containing 0.05% Tween 20 (washing buffer). The wells were incubated with 0.1 mL of anti-CML antibody, 6D12 (1 $\mu$g/mL), or anti-pentosidine antibody (1 $\mu$g/mL) dissolved in washing buffer for 1 h. The wells were then washed with washing buffer three times and reacted with HRP-conjugated anti-mouse IgG antibody, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by the addition of 0.1 mL of 1 M sulfuric acid, and the absorbance at 492 nm was read by a micro-ELISA plate reader.

**Detection of CML by HPLC.** The CML content of the samples were quantified by acid hydrolysis with 6 N HCl for 24 h at 110 °C, followed by amino acid analysis on a Hitachi L-8500A instrument equipped with an ion-exchange HPLC column (2622 SC, 4.6 × 80 mm; Hitachi) and a ninhydrin postcolumn detecztion system, as described previously (*26*).

**Detection of Pentosidine by HPLC.** The pentosidine content of the samples were quantified by acid hydrolysis with 6 N HCl for 24 h at 110 °C, followed by an analysis using HPLC equipped with an ODS column (Capcell pak $C_{18}$ UG-80, 4.6 × 250 mm; Shiseido) and fluorescence detector (ex/em = 335/385 nm) as described previously (*20*).

**Statistical Analysis.** All data are expressed as the mean ± SD. Differences between groups were examined for statistical significance using the Mann−Whitney U test and the nonrepeated measures ANOVA. A $p$ value of $< 0.05$ denoted the presence of a statistically significant difference.

## RESULTS AND DISCUSSION

BSA was incubated with ribose in sodium phosphate buffer, and CML and pentosidine formation was measured. As shown in **Figure 1**, the reactivity of anti-CML antibody increased in a time-dependent manner during incubation of BSA (2 mg/mL) with 33 mM ribose in PBS at 37 °C. A similar tendency was observed when CML content was measured by HPLC (**Figure 1A**). Similarly, the reactivity of anti-pentosidine antibody increased in a time-dependent manner during incubation of BSA (2 mg/mL) with 100 mM ribose in 200 mM sodium phosphate buffer at 60 °C, and those reactivities were correlated with the finding of the HPLC analysis (**Figure 1B**).

We first measured the inhibitory effect of 50 crude extracts from natural products and Chinese herbs on CML and pentosidine formation. Incubation of BSA with ribose increased CML and pentosidine formation. Under the assay conditions employed, AR extract inhibited CML and pentosidine formation (**Figure 2**). These data suggest that AR contains compound(s) that can inhibit AGE formation. Next, the AR extract was applied to Diaion HP-20 column chromatography and eluted with 20−100% MeOH to give five fractions. As shown in **Figure 3**, the 80 and 100% MeOH fractions significantly inhibited both CML and pentosidine formation. Because the extractive content of the 80% MeOH fraction was 10-fold higher than that of the 100% MeOH fraction, the 80% MeOH fraction was further separated into a saponin fraction and a flavonoid fraction. As a

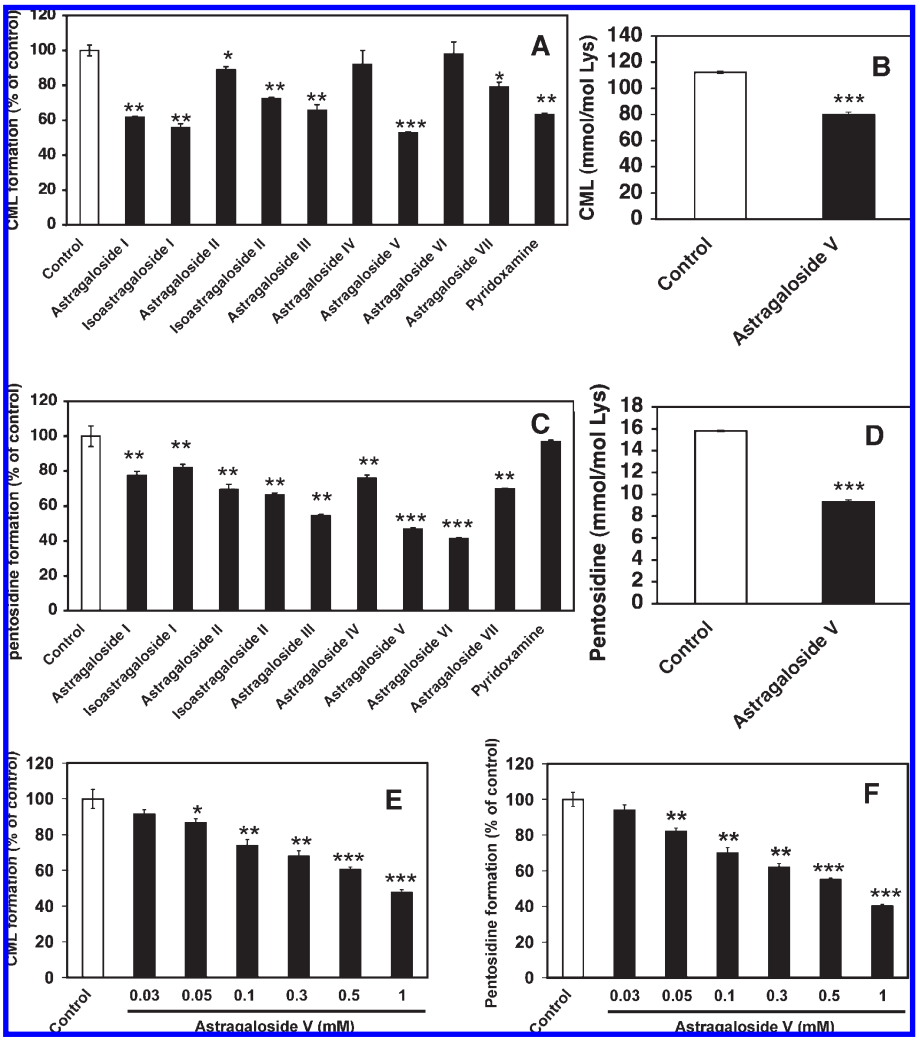


**Figure 6.** Inhibitory effect of astragalosides and isoastragalosides on CML and pentosidine formation. BSA and ribose were incubated with astragalosides and isoastragalosides in phosphate buffer for 7 days followed by determination of CML and pentosidine by noncompetitive ELISA (**A**, **C**, **E**, and **F**) and HPLC (**B** and **D**) as described under Materials and Methods. Data are presented as the mean ± SD. ∗, $P < 0.05$; ∗∗, $P < 0.0005$; ∗∗∗, $P < 0.0001$ versus control.

result, the saponin fraction inhibited CML and pentosidine formation, whereas the flavonoid fraction slightly inhibited CML formation (**Figure 4**). Then, the saponin fraction was further applied to ODS column chromatography and/or silica gel column chromatography to yield astragalosides and isoastragalosides (**Figure 5**). As shown in **Figure 6**, isolated compounds other than astragaloside IV and astragaloside VI inhibited CML formation. Among those compounds, astragaloside I, isoastragaloside I, and astragaloside V have inhibitory effects on CML formation the same as that of pyridoxiamine (**Figure 6A**). Furthermore, all compounds inhibited pentosidine formation (**Figure 6C**), and astragaloside V and astragaloside VI showed the highest inhibitory effect on pentosidine formation, whereas pyridoxiamine did not inhibit pentosidine formation (**Figure 6C**). Moreover, astragaloside V inhibited both CML and pentosidine formation in a dose-dependent manner (**Figure 6E**,**F**). These results suggest that those compounds in the AR extract played a key role in the inhibition of CML and pentosidine formation and that astragalosides are more effective AGE inhibitors than pyridoxamine. We next measured the inhibitory mechanism of astragaloside V on CML formation. It is known that CML is generated from the oxidation of Amadori products and glucose autoxidation-derived glyoxal pathway. Astragaloside V inhibited CML formation from incubation of BSA with glyoxal (**Figure 7B**), whereas it did not inhibit CML formation from Amadori products (**Figure 7A**). These data indicate that astragaloside V inhibits CML formation by inhibiting the glyoxal pathway.

AGEs are a heterogeneous group of reaction products that form between a protein's primary amino group and a carbohydrate-derived aldehyde group. A substantial number of previous papers have indicated that AGEs exacerbate and accelerate the changes associated with aging while also contributing to the early phases of age-related disease, including atherosclerosis, cataracts, neurodegenerative disease, renal failure, arthritis, and age-related macular degeneration (*2*, *3*). CML and pentosidine are known as AGE structures associated with pathogenesis and development of these diseases. For these reasons, it is believed that the use of AGE inhibitors may therefore be a potentially effective strategy to prevent the pathogenesis of age-related diseases. In this study, CML and pentosidine inhibitors were screened from 50 crude extracts of natural products and Chinese herbs. Among those extracts, AR extract significantly inhibited CML and pentosidine formation (**Figure 2**). On the other hand, extracts such as EH, ACF, PC, CR, and CF enhanced CML and pentosidine formation (**Figure 2**). Akagawa et al. (*27*) demonstrated that some polyphenols such as epicatechin gallate and epigallocatechin gallate generate hydrogen peroxide, and the EH, ACF, PC, CR, and CF extracts contain plenty of polyphenols and may have enhanced CML and pentosidine formation by producing

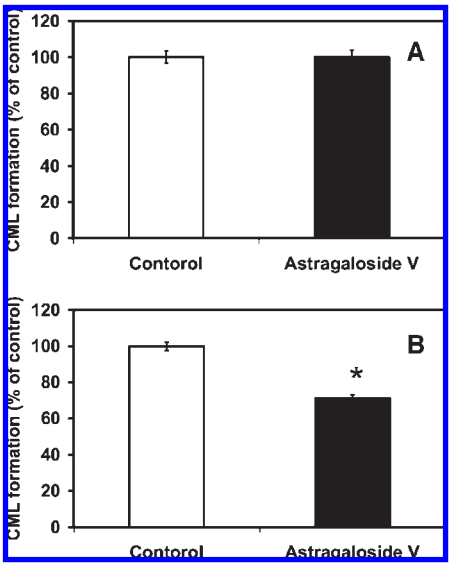


**Figure 7.** Inhibitory effect of astragaloside V on CML formation from Amadori products and glyoxal pathway. Glycated HSA (2 mg/mL) was incubated with astragaloside V (1 mM) in PBS at 37 °C for 5 days (**A**). BSA (2 mg/mL) and glyoxal (5 mM) were incubated with astragaloside V (1 mM) in PBS at 37 °C for 2 days (**B**), followed by the determination of CML by ELISA.

hydrogen peroxide. AR is a traditional Chinese medicinal herb that originated in northern China and has been used for more than 2000 years (*28*). AR contains 2′4′-dihydroxy-5,6-dimethoxyisoflavone, kumatakenin, choline, betaine, polysaccharides, saponins, glucuronic acid, sucrose, amino acids, traces of folic acid, and astraisoflavanin (*29*). AR is a widely used Chinese medicinal herb that is well-known for its vital-energy tonifying, skin reinforcing, diuretic, and tissue generative actions. Furthermore, AR has been extensively used as a tonic to enhance the body's defense system (*30*). Evidence has indicated the importance of AR polysaccharide fractions in the modulation of immune functions in both humans and experimental animals (*31*). The current study demonstrated that astragalosides, isolated from AR by successive purification with Diaion HP-20 and Sephadex LH-20 column chromatography, inhibited AGE formation (**Figure 6**). Among these astragalosides, astragaloside V significantly inhibited both CML and pentosidine formation (**Figure 6**). However, astragaloside VI significantly inhibited only pentosidine formation (**Figure 6**). Both CML and pentosidine are known as oxidation-dependent AGE structures. However, those formation pathways are different from each other. CML is generated from the oxidation of Amadori products and the glucose autoxidation-derived glyoxal pathway. On the other hand, pentosidine is generated from oxidation of glycated protein formed by cyclization reaction of ribose with lysine and arginine. Therefore, these results suggest that astragalosides V and VI showed different inhibitory effects on AGE formation because of differences in inhibitory mechanism and chemical structure between astragalosides V and VI. Astragalosides are known as cycloartane-type triterpenoid compounds. In a previous study, several types of triterpenoid compounds such as oleanane-type, ursane-type, and lupane-type triterpenoid compounds could hardly inhibit AGE formation under our assay conditions (data not shown). However, astragalosides significantly inhibited CML and pentosidine formation in this study, suggesting that the cycloartane skeleton may play an important role in the inhibition of AGE formation. Furthermore, astragaloside V inhibited both CML and pentosidine formation, whereas astragaloside VI, which has the same aglycon as astragaloside V, inhibited only pentosidine formation, thus suggesting that a difference in the binding position of glucose (C-6 and C-25) between astragaloside V and astragaloside VI might affect the inhibition of CML formation. In traditional Chinese medicine, AR is often mixed with other herbs, such as angelica and ginseng, in various complex prescription formulas. Such herbal formulas have been used for centuries in Asia to treat cancers, diabetes, kidney infections, strokes, and many other diseases (*32*, *33*). For this reason, the astragalosides in AR may play an important role in those beneficial effects by inhibiting AGE formation. Therefore, astragalosides could be candidate agents for use as a potential new medicine.

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