Eucommia ulmoides extracts prevent the formation of advanced glycation end products
**Eucommia ulmoides** extracts prevent the formation of  
advanced glycation end products

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Keywords: Advanced glycation end products (AGEs), Glycation, Diabetic complications, *Eucommia ulmoides*
Abstract

Proteins non-enzymatically react with reducing sugars to form advanced glycation end-products (AGEs), resulting in the induction of protein denaturation. Because the levels of AGE increase with age and are elevated in age-related diseases, such as diabetes and atherosclerosis, the intake of compound(s) that inhibit the formation of AGEs by daily meal may represent a potential strategy for preventing age-related disease. In this study, we measured the inhibitory effects of several Eucommia ulmoides extracts on the formation of AGEs, \(N^\epsilon\)-(carboxymethyl)lysine (CML) and \(N^\omega\)-(carboxymethyl)arginine (CMA). Although crude extract obtained from \(E. ulmoides\) bark is widely used as herbal medicine, \(E. ulmoides\) leaf extract (ELE) inhibited CML and CMA formation more effectively during incubation of gelatin with ribose. Therefore, the inhibitory effects of compounds present in ELE on CML and CMA formation were studied. As a result, isoquercetin showed the strongest inhibitory effect of all the tested ELE components. These results indicate that the oral intake of ELE may inhibit the formation of AGEs, thereby ameliorating age-related diseases.

Keywords: Advanced glycation end products (AGEs), Glycation, Diabetic complications, Eucommia ulmoides
Introduction

Lifestyle-related disease such as diabetes and atherosclerosis are serious problems that accounted for 63% of the total deaths worldwide in 2008\(^1\). As the fundamental treatment of lifestyle-related diseases is difficult, the inhibition of disease pathogenesis by daily meal consumption is expected to become an effective means of preventing lifestyle-related diseases. Non-enzymatic reactions between reducing sugars and proteins generate advanced glycation end products (AGEs) via the Maillard reaction. The formation of AGEs proceeds \textit{in vivo} by several reactions, including oxidation and condensation between reducing sugars and proteins, resulting in the induction of protein denaturation\(^2\). In fact, AGEs accumulate in the body along with aging\(^3^4\), with such accumulation being enhanced by aging-related diseases such as diabetic complications. Furthermore, increased levels of AGEs have been reported in several diseases including atherosclerosis, diabetic nephropathy\(^5\), and retinopathy\(^6\), where the accumulation of AGEs in pathological lesions is actively implicated in the development of organ damage. Therefore, AGE inhibitors have been developed to prevent lifestyle-related diseases such as diabetic complications and atherosclerosis. Pyridoxamine was originally proposed to be an inhibitor of the oxidative degradation of fructosamine\(^7\) and has been shown to inhibit the formation of AGEs, as well as products of lipid peroxidation\(^8\).
Pyridoxamine also inhibited the development of retinopathy and neuropathy in rats with streptozotocin (STZ)-induced diabetes. Furthermore, thiamine and its hydrophobic derivative, benfotiamine, decreased the formation of intracellular methylglyoxal-derived AGEs by increasing transketolase activity, thus inhibiting the development of complications in animal models of diabetes and in humans. Therefore, treatment with AGE inhibitors may represent a potential strategy for preventing clinical diabetic complications. A range of AGE structures have been reported and the present study focused on Nε(carboxymethyl)lysine (CML) and Nω(carboxymethyl)arginine (CMA). CML is known to be a major AGE antigenic determinant that accumulates in lens crystallins in an age-dependent manner. The formation of several AGEs, such as CML, and pentosidine are required for oxidation. For instance, in the formation of pentosidine, fluorescent intensity and cross-linking was inhibited under anti-oxidative conditions. Furthermore, CML was generated by the oxidative cleavage of Amadori products by hydroxyl radical, peroxynitrite, and hypochlorous acid, indicating that CML may be an important marker for oxidation in vivo. Reactive oxygen species are reported to be related to aging and age-related diseases, and the accumulation of CML is observed in patients who have severe complications, including nephropathy and atherosclerosis. We previously demonstrated that CML was generated by the
oxidative cleavage of Amadori products by hydroxyl radicals\textsuperscript{17} and peroxynitrite\textsuperscript{18}, thus suggesting that CML is an important biological marker of oxidative stress \textit{in vivo}.

CMA has been identified in glycated collagen\textsuperscript{22}, suggesting that it may function as a marker of collagen glycation. \textit{Eucommia ulmoides} Oliv. is a member of the plant family \textit{Eucommiaceae}. \textit{E. ulmoides} (Cortex \textit{Eucommiae}) bark crude extract (EBE) and \textit{E. ulmoides} leaves are widely used as herbal medicine, such as analeptic, sedative, antihypertensive, diuretic and antidiabetic agents in China and Korea\textsuperscript{23-28}, and \textit{E. ulmoides} leaf extract (ELE) is used in beverages (referred to as Tochu-cha (\textit{E. ulmoides} leaves tea)) in Japan. With the rapidly increasing population of patients with lifestyle-related diseases, disease prevention through daily meal consumption has become more important. \textit{E. ulmoides} leaf extract (ELE) containing leaf glycoside is used as a food for specified health use\textsuperscript{29}. Previous studies have reported that ELE also exhibited anti-hypercholesterolemic and anti-hypertriglyceridemic effects\textsuperscript{30-35}.

In the present study, we measured the inhibitory effects of ELE and its principal compounds on CML and CMA formation as ELE has been reported to have antioxidant activity\textsuperscript{36}. 
Materials and Methods

Chemicals

Gelatin, O-phenylenediamine dihydrochloride (OPD) tablets, and hydrogen peroxide were purchased from Wako (Osaka, Japan). D-Ribose was purchased from the Kanto Chemical Co. (Tokyo, Japan). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies were purchased from Kirkegaard Perry Laboratories (Gaithersburg, MD, USA). Anti-CML and anti-CMA antibodies were prepared as previously described. All other chemicals were of the best grade available from commercial sources.

Preparation of crude extracts.

Fresh *E. ulmoides* Oliv. leaves were collected in August 2005 at Chengdu, Sichuan Province, People’s Republic of China. The leaves were briefly treated with steam at 100–110°C immediately after collection. Then, the extraction and isolation procedures were started successively. A voucher specimen (No. 200) was identified, transferred to a vacuum pack in a well-closed container, and deposited in the herbarium of the Department of Medicinal Plants Garden, Sojo University, Kumamoto, Japan. *E.
ulmoides leaves were studied to identify its activity components when used as Tochu-cha. Dried *E. ulmoides* leaves (577.6 g) were extracted with hot water for 10 h at 60°C to produce *E. ulmoides* leaf extracts (ELE). Commercially available dried bark and root of *E. ulmoides* were also collected at People’s Republic of China and these samples were extracted in the same manner as ELE to give *E. ulmoides* bark extracts (EBE) and *E. ulmoides* root extracts (ERE). EBE and ERE were prepared for activity comparison with ELE.

**Purification of flavonoid glycosides from ELE**

As shown in Figure 1A, ELE were subjected to Diaion HP-20P (Mitsubishi Chemical Co., Tokyo, Japan) column chromatography with a gradient of H$_2$O, 30% MeOH, 50% MeOH, 80% MeOH, and MeOH to yield fractions (Fr.) 1-5, in the order of elution. A part (2.0 g) of Fr. 3 eluted by 50% MeOH on Diaion HP-20 (9.0 g) was subjected to Sephadex LH-20 (GE Healthcare Bioscience Co., Uppsala, Sweden) column chromatography eluted with MeOH to yield isoquercetin (4, 134 mg) and Fr. 3a. Fr. 3a (441 mg) was chromatographed over Chromatorex ODS (Fuji Silysia, Kasugai, Japan) with a gradient of H$_2$O-MeOH (3:7 to 1:1) to yield quercetin 3-O-sambubioside (5, 155 mg) and Fr. 3a-1. Fr. 3a-1 (12 mg) was subjected to preparative HPLC by using a
Cosmosil AR-II column (5 µm, φ10.0 × 250 mm, Nacalai Tesque Inc., Kyoto, Japan) with 50% MeOH to yield kaempferol 3-O-rutinoside (6, 6 mg). Fr. 4 eluted by 80% MeOH on Diaion HP-20 (2.4 g) was chromatographed over Sephadex LH-20 with MeOH to yield Fr.4b. Fr.4b (387 mg) was purified by Chromatorex ODS with a gradient of H₂O-MeOH (6:4 to 4:6) to yield rutin (1,40 mg), astragalin (2, 117 mg), and 6"'-O-acetyl-astragalin (3, 8 mg). Quercetin and kaempferol were prepared by acid hydrolysis of isoquercetin and astragalin, respectively. The chemical structures and purities of all compounds obtained from ELE were confirmed by ¹H- and ¹³C-NMR data compared with reference literature (Fig. 1B).

Carbohydrate-induced AGE formation of gelatin

Each carbohydrate (33 mM glucose, mannose, galactose, fructose, or ribose) was incubated with 2 mg/mL of soluble collagen, gelatin in 200 mM of phosphate buffer (pH 7.2) at 37°C for 7 days prior to the measurement of CMA and CML formation by a noncompetitive enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described below.

Effects of *E. ulmoides* extracts on CML and CMA formation

Ribose-gelatin was prepared by incubating 2 mg/mL of gelatin with 33 mM ribose in
200 mM of phosphate buffer (pH 7.2) at 37°C for seven days in the presence of natural crude *E. ulmoides* extracts or purified compounds (100 µM), followed by the determination of CML and CMA formation using a noncompetitive ELISA, as previously described. Briefly, each well of a 96-well microtiter plate was coated with 100 µL of gelatin in phosphate-buffered saline (PBS) at the indicated protein coating concentration and incubated for 2 h. The wells were washed three times with PBS containing 0.05% Tween 20 (washing buffer). The wells were then blocked with 0.5% gelatin in PBS for 1 h. After three washes, the wells were incubated for 1 h with 100 µL of monoclonal anti-CMA antibody (1 µg/mL) or anti-CML antibody (0.1 µg/mL). The wells were washed three times and incubated with HRP-conjugated anti-mouse IgG followed by 1,2-phenylenediamine dihydrochloride. The reaction was terminated with 100 µL of 1.0 M sulfuric acid, and the absorbance was measured at 492 nm using a micro-ELISA plate reader.

**Statistical analysis**

Differences between the groups were examined for statistical significance using either Student’s *t*-test or the one-way analysis of variance (ANOVA) with Newman-Keuls *post-hoc* test. P values of <0.05 were considered to indicate statistical significance.
The measurement of CMA and CML by LC-MS/MS

Carbohydrate-gelatin samples were digested by enzymes as described previously. Standard $[^{13}C_6]$ CMA, $[^2H_2]$ CML (PolyPeptide Laboratories, Strasbourg, France) and $[^{13}C_6]$ Lysine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) was added to the pellets, which were digested with enzymes. The dried sample was resuspended in 1 ml of distilled water and passed over a Strata-X-C column (Phenomenex, Torrance, CA, USA) which had been pre-washed with 1 ml of methanol and equilibrated with 1 ml of distilled water. The column was then washed with 3 ml of 2% formic acid and eluted with 3 ml of 7% ammonia. The pooled elution fractions were dried and resuspended in 1 ml of 20% acetonitrile containing 0.1% formic acid. The samples were subjected to an LC-MS/MS assay using a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). LC was conducted on a ZIC®-HILIC column (150 x 2.1 mm, 5 µm) (Merck Millipore, Billerica, MA, USA). The mobile phase was performed using solvent A (distilled water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The flow rate was 0.2 ml/min and the column was kept at 40°C. The retention time for CMA, CML and lysine were approximately 12-14 min. CMA, CML, lysine, and the standard were
detected by electrospray positive ionization-mass spectrometric multiple reaction monitoring. The parent ions of CMA and $^{13}\text{C}_6$ CMA were 233 (m/z) and 239 (m/z), respectively. Fragment ions of 116 (m/z) and 121 (m/z) from each parent ion were measured for the analysis of CMA and $^{13}\text{C}_6$ CMA in the samples. The parent ions of CML and $^{2}\text{H}_2$ CML were 205 (m/z) and 207 (m/z), respectively. Fragment ions of 130 (m/z) from each parent ion were measured for the analysis of CML and $^{2}\text{H}_2$ CML in the samples.
194 **Results**

195 The present study measured the effects of a range of carbohydrates on the formation of
196 CMA and CML. The carbohydrates (glucose, mannose, galactose, fructose, and ribose)
197 were incubated with gelatin for up to one week, followed by ELISA determination of
198 the CMA and CML levels. As shown in Figure 2A, the CMA level increased in a
199 time-dependent manner following exposure of gelatin to ribose, whereas it was only
200 slightly increased following exposure to hexose. A similar tendency was also observed
201 in the formation of CML. Thus, ribose produced the highest CML formation, whereas
202 CML increased gradually from hexoses (Fig. 2B). As shown in Figure 2B, less CML
203 formation was detected following exposure to glucose in a time-dependent manner.
204 These results demonstrated that CMA and CML were rapidly generated in gelatin
205 incubated with ribose. CMA and CML contents in glycated gelatins were also measured
206 by LC-MS/MS. The typical fragment ion chromatograms of CMA and [\(^{13}\)C\(_6\)] CMA (10
207 pmol) by LC-MS/MS in the samples are shown in Figure 3A. Typical fragment ion
208 chromatograms of CML and [\(^{3}\)H\(_2\)] CML (10 pmol) are also shown in Figure 3B. Using
209 this method, the levels of CML and CMA in glycated gelatins were measured. As shown
210 in Figure 3C, ribose showed the highest contents of CML and CMA. These data
211 demonstrated that the detection of AGEs by the ELISA system using anti-AGE
antibodies correlated with the results of LC-MS/MS, thereby validating this assay system.

Because CMA and CML were generated more rapidly by ribose than by the hexoses, ribose was used to screen potential inhibitors of CMA and CML formation. As shown in Figure 4A, ELE completely inhibited CMA formation, whereas EBE or ERE partially inhibited CMA formation. Furthermore, ELE partially inhibited CML formation, whereas EBE or EBR did not show any inhibitory effects on CML formation (Fig. 4B). This result strongly demonstrates that ELE contained compound(s) capable of inhibiting the formation of CMA and CML. Therefore, the ELE components were isolated as described in the Materials and Methods section. In the same manner, reported structures such as quercetin and kaempferol were also tested. As shown in Figure 5, several isolated compounds, including isoquercetin, 6''-O-acetyl-astragal, rutin, and astragal, showed stronger inhibitory effects on CMA and CML formation than aminoguanidine, whereas kaempferol 3-O-rutinoside showed enhancing effects rather than weak inhibitory effects. Furthermore, ELE compounds inhibited the formation of CMA more effectively than that of CML. Acid hydrolyzates of isoquercetin, astragal, quercetin and kaempferol also showed stronger inhibitory effects on CMA and CML formation than aminoguanidine.
Discussion

CMA, an acid-labile AGE, was originally identified in glycated collagen using enzymatic digestion \(^{44}\). Our previous study employing monoclonal anti-CMA antibody demonstrated that CMA was generated specifically on glycated collagen, thereby altering dermal fibroblast functions such as collagen secretion \(^{37}\). Therefore, ingestion of natural compounds that inhibit CMA formation on a daily basis may help to prevent pathologies associated with glycation \(^{5,6}\). The detection of CMA is difficult due to its susceptibility to acid hydrolysis. Furthermore, because there were no reports of a specific CMA antibody until 2011 \(^{37}\), the biological significance of CMA remains poorly understood. Nevertheless, natural compounds such as isoquercetin, \(^{6''}\)-O-acetyl-astragal, rutin, and astragalin, which inhibited the formation of both CMA and CML (Fig. 5), would be helpful to evaluate the role of glycation in age-related diseases. Although *E. ulmoides* bark contains lignans, which possess antioxidant activity, the lignan content of the leaves is very low \(^{39,40}\), indicating that lignans in the leaves may not work as inhibitors of AGEs formation. Furthermore, as our preliminary experiments using iridoids such as geniposidic acid and asperuloside did not show a significant inhibitory effect on AGEs formation (data not shown), we focused on flavonoids in ELE. We also measured the inhibitory effects of natural compounds on the
formation of CML, one of the major antigenic AGE structures produced by the Maillard
reaction and lipid peroxidation. CML is implicated in the pathogenesis of age-related
disorders such as diabetic complications and is recognized by the receptor for AGE
(RAGE). CML-RAGE interactions activate the NF-κB signaling pathway, while also
enhancing the expression of vascular cell adhesion molecule-1 (VCAM-1) in human
umbilical vein endothelial cells. Alikhani et al. demonstrated that CML-collagen
was recognized by RAGE and induced fibroblast apoptosis by activating cytoplasmic
and mitochondrial caspase pathways. Thus, the demonstration that the in vitro formation
of CML was inhibited by ELE points to a potentially novel therapeutic strategy for
preventing the development of diabetic complications, such as diabetic nephropathy,
retinopathy, and neuropathy, by inhibiting AGE formation.

It was reported that ELE controlled the plasma glucose levels in a rat model of
type 1 diabetes and a mouse model of type 2 diabetes (C57BL/KsJ-db/db mice). Experiments with fructose-drinking rats fed ELE suggested that long-term ELE
treatment effectively prevented the development of insulin resistance and ameliorated
abnormal perivascular innervation in these animals. Fujikawa et al. reported that the
chronic administration of ELE or E. ulmoides green leaf powder improved insulin
resistance in a rat model of high-fat diet (HFD)-induced obesity, with concomitant
increases in the plasma adiponectin levels and the suppression of both the plasma resistin and TNF-α levels. These findings raised the possibility that ELE may improve atherosclerosis and diabetic nephropathy. This anti-obesity effect was dependent on the asperuloside content of the *E. ulmoides* leaf. Furthermore, the chronic administration of asperuloside decreased body weight, white adipose tissue (WAT) weight, and plasma lipid parameters in mice fed a HFD. Because ELE inhibits the formation of AGEs, we believe that the daily intake of ELE is expected to become an effective means of preventing lifestyle-related diseases. Although the inhibitory effect of ELE on AGES formation *in vivo* has not yet been clarified, we hope that our findings will encourage further physiological studies. Taken together, there is a possibility that the daily intake of ELE ameliorates AGES formation, resulting in the prevention of diabetic complications.

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**Figure legends**

**Figure 1.** Isolation and purification process and identified structures by $^1$H- and $^{13}$C-NMR data. The isolation and purification process of *E. ulmoides* Oliv. leaves are shown in the flow chart, as described in the Materials and Methods (A). The chemical structures of isolated compounds from ELE are demonstrated (B).

**Figure 2.** Comparison of AGE formation by carbohydrates. The indicated carbohydrates were incubated with gelatin and the formation of CMA (A) and CML (B) was measured by a noncompetitive ELISA, as described in the Materials and Methods. Coated antigen concentration was 10 µg/mL. The data are presented as the means ± SD (n=3).

**Figure 3.** The measurement of CMA and CML levels by LC-MS/MS. The parent ions of CMA and $[^{13}\text{C}_6]$ CMA were 233 (m/z) and 239 (m/z), respectively. Peaks of the fragment ions of CMA and $[^{13}\text{C}_6]$ CMA (10 pmol) were detected in the samples (A). The parent ions of CML and $[^{2}\text{H}_2]$ CML were 205 (m/z) and 207 (m/z), respectively. Peaks of the fragment ions of CML and $[^{2}\text{H}_2]$ CML (10 pmol) were detected in the samples (B). The CMA (open bar) and CML (closed bar) levels in the incubated carbohydrates with gelatin were measured by LC-MS/MS (C), as described in the
Materials and Methods.

Figure 4. Inhibitory effects of *E. ulmoides* extracts from bark, root, and leaves (ELE) on CMA and CML formation. Gelatin and ribose were incubated in the presence of 0.1 mg/mL ELE or pyridoxamine for 7 days, followed by the determination of CMA (A) and CML (B) by a noncompetitive ELISA, as described in the Materials and Methods. Data was expressed as inhibition (%) compared with control (without samples). For the control, only gelatin and ribose were incubated. Abbreviation: px, pyridoxamine. X axis shows the coated antigen concentration.

Figure 5. Inhibitory effects of compounds purified from ELE on CMA and CML formation. Gelatin and ribose were incubated in the presence of 0.1 mM of the indicated compounds for 7 days, followed by the determination of CMA and CML by a noncompetitive ELISA. Coated antigen concentration was 10 µg/mL. Data was expressed as inhibition (%) compared with control (without samples). For the control, only gelatin and ribose were incubated. Abbreviations: IQ, isoquercetin; AAs, 6"-O-acetyl-astragal; K, kaempferol; Q, quercetin; R, rutin; KR, kaempferol 3-O-rutinoside; As, astragal; AG, aminoguanidine. For the control, only gelatin and
ribose were incubated for 7 days. The data are presented as the means ± SD (n=3). **, P < 0.01, *, P < 0.05 vs. control.
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Figure 1. Sugawa et al.,

A

Dried *E. ulmodes* leaves (577.6 g)

*E. ulmodes* leaf extract (ELE) (267.4 g)

Diaion HP 20 (H₂O:MeOH 1:0-0:1)

Extracted with hot water (5.1 L)

Fr.1~2 Fr.3 (9.0 g)

A part 2.0 g

Sephadex LH20 (MeOH)

Fr.3a Fr.3b~3c

(134 mg) (441 mg)

Chromatex ODS (H₂O,MeOH)

Fr.3a-1 Fr.3a-2~3

(155 mg) (12 mg)

HPLC ODS (50 % MeOH)

Fr.4a Fr.4b-1

(40 mg) (40 mg)

Fr.4b Fr.4c

(117 mg) (8 mg)

Fr.4 (2.4 g)

Sephadex LH20 (MeOH)

Fr.4a Fr.4b Fr.4c

B

Isolated from ELE

Isoquercetin(4) Quercetin-3-O-sambubioside(5) Rutin(1)

Astragalin(2) 6''-O-Acetylastragalin(3) Kaempferol-3-O-rutinoside(6)

Others

Quercetin(7) Kaempferol(8)
Figure 2. Sugawa et al.,
Figure 3. Sugawa et al.,

A

CMA in the sample

Relative Abundance

0 20 40 60 80 100

Retention time (min)

13 14

Standard $^{[13]}C_6$ CMA

Relative Abundance

0 20 40 60 80 100

Retention time (min)

13 14

B

CML in the sample

Relative Abundance

0 20 40 60 80 100

Retention time (min)

12 13

Standard $^{[2]}H_2$ CML

Relative Abundance

0 20 40 60 80 100

Retention time (min)

12 13

C

CMA

mmol/mol Lysine

0 10 20 30 40 50 60 70

CML

Mannose Glucose Galactose Ribose Fructose

Relative Abundance

0 10 20 30 40 50 60 70

CMA

CML
Figure 4. Sugawa et al.,
Figure 5. Sugawa et al.,