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Eucommia ulmoides extracts prevent the formation of advanced glycation end products

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Running head: *Eucommia ulmoides* prevents AGEs formation.

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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^{ε}-(carboxymethyl)lysine (CML) and N^{ω}-(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 *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 [9, 10]. 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 [11, 12] and in humans [13]. 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 N^{ε}-(carboxymethyl)lysine (CML) and N^{ω}-(carboxymethyl)arginine (CMA). CML is known to be a major AGE antigenic determinant [14] that accumulates in lens crystallins in an age-dependent manner [15]. 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 [16]. Furthermore, CML was generated by the oxidative cleavage of Amadori products by hydroxyl radical [17], peroxynitrite [18], and hypochlorous acid [19], 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 [20], and the accumulation of CML is observed in patients who have severe complications, including nephropathy and atherosclerosis [21]. We previously demonstrated that CML was generated by the

oxidative cleavage of Amadori products by hydroxyl radicals [17] and peroxynitrite [18], thus suggesting that CML is an important biological marker of oxidative stress *in vivo*. CMA has been identified in glycated collagen [22], suggesting that it may function as a marker of collagen glycation. *Eucommia ulmoides* Oliv. is a member of the plant family Eucommiaceae. *E. ulmoides* (Cortex Eucommiae) bark crude extract (EBE) and *E. ulmoides* leaves are widely used as herbal medicine, such as analeptic, sedative, antihypertensive, diuretic and antidiabetic agents in China and Korea[23-28], and *E. ulmoides* leaf extract (ELE) is used in beverages (referred to as Tochu-cha (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. *E. ulmoides* leaf extract (ELE) containing leaf glycoside is used as a food for specified health use [29]. Previous studies have reported that ELE also exhibited anti-hypercholesterolemic and anti-hypertriglyceridemic effects [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 [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 [37]. 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 [38] 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.*

114 *ulmoides* leaves were studied to identify its activity components when used as
115 Tochu-cha. Dried *E. ulmoides* leaves (577.6 g) were extracted with hot water for 10 h at
116 60°C to produce *E. ulmoides* leaf extracts (ELE). Commercially available dried bark
117 and root of *E. ulmoides* were also collected at People's Republic of China and these
118 samples were extracted in the same manner as ELE to give *E. ulmoides* bark extracts
119 (EBE) and *E. ulmoides* root extracts (ERE). EBE and ERE were prepared for activity
120 comparison with ELE.
121
122 **Purification of flavonoid glycosides from ELE**
123 As shown in Figure 1A, ELE were subjected to Diaion HP-20P (Mitsubishi Chemical
124 Co., Tokyo, Japan) column chromatography with a gradient of H_2O, 30% MeOH, 50%
125 MeOH, 80% MeOH, and MeOH to yield fractions (Fr.) 1-5, in the order of elution. A
126 part (2.0 g) of Fr. 3 eluted by 50% MeOH on Diaion HP-20 (9.0 g) was subjected to
127 Sephadex LH-20 (GE Healthcare Bioscience Co., Uppsala, Sweden) column
128 chromatography eluted with MeOH to yield isoquercetin (4, 134 mg) and Fr. 3a. Fr. 3a
129 (441 mg) was chromatographed over Chromatorex ODS (Fuji Silysia, Kasugai, Japan)
130 with a gradient of H_2O-MeOH (3:7 to 1:1) to yield quercetin 3-*O*-sambubioside (5, 155
131 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 H_2O-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 ^{1}H- and ^{13}C-NMR data compared with reference literature (Fig. 1B).[39-41]

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 7days 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

150 200 mM of phosphate buffer (pH 7.2) at 37°C for seven days in the presence of natural
151 crude *E. ulmoides* extracts or purified compounds (100 μM), followed by the
152 determination of CML and CMA formation using a noncompetitive ELISA, as
153 previously described [42]. Briefly, each well of a 96-well microtiter plate was coated with
154 100 μL of gelatin in phosphate-buffered saline (PBS) at the indicated protein coating
155 concentration and incubated for 2 h. The wells were washed three times with PBS
156 containing 0.05% Tween 20 (washing buffer). The wells were then blocked with 0.5%
157 gelatin in PBS for 1 h. After three washes, the wells were incubated for 1 h with 100 μL
158 of monoclonal anti-CMA antibody (1 μg/mL) or anti-CML antibody (0.1 μg/mL). The
159 wells were washed three times and incubated with HRP-conjugated anti-mouse IgG
160 followed by 1,2-phenylenediamine dihydrochloride. The reaction was terminated with
161 100 μL of 1.0 M sulfuric acid, and the absorbance was measured at 492 nm using a
162 micro-ELISA plate reader.

163 ***Statistical analysis-***

164 Differences between the groups were examined for statistical significance using
165 either Student's *t*-test or the one-way analysis of variance (ANOVA) with
166 Newman-Keuls *post-hoc* test. P values of <0.05 were considered to indicate
167 statistical significance.

168

169 **The measurement of CMA and CML by LC-MS/MS**

170 Carbohydrate-gelatin samples were digested by enzymes as described previously [43].
171 Standard [$^{13}C_6$] CMA, [$^{2}H_2$] CML (PolyPeptide Laboratories, Strasbourg, France) and
172 [$^{13}C_6$] Lysine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) was added
173 to the pellets, which were digested with enzymes. The dried sample was resuspended in
174 1 ml of distilled water and passed over a Strata-X-C column (Phenomenex, Torrance,
175 CA, USA) which had been pre-washed with 1 ml of methanol and equilibrated with 1
176 ml of distilled water. The column was then washed with 3 ml of 2% formic acid and
177 eluted with 3 ml of 7% ammonia. The pooled elution fractions were dried and
178 resuspended in 1 ml of 20% acetonitrile containing 0.1% formic acid. The samples were
179 subjected to an LC-MS/MS assay using a TSQ Vantage triple stage quadrupole mass
180 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). LC was conducted on a
181 ZIC®-HILIC column (150 x 2.1 mm, 5 μm) (Merck Millipore, Billerica, MA, USA).
182 The mobile phase was performed using solvent A (distilled water containing 0.1%
183 formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The flow rate was
184 0.2 ml/min and the column was kept at 40°C. The retention time for CMA, CML and
185 lysine were approximately 12-14 min. CMA, CML, lysine, and the standard were

186 detected by electrospray positive ionization-mass spectrometric multiple reaction
187 monitoring. The parent ions of CMA and [$^{13}C_6$] CMA were 233 (m/z) and 239 (m/z),
188 respectively. Fragment ions of 116 (m/z) and 121 (m/z) from each parent ion were
189 measured for the analysis of CMA and [$^{13}C_6$] CMA in the samples. The parent ions of
190 CML and [$^{2}H_2$] CML were 205 (m/z) and 207 (m/z), respectively. Fragment ions of 130
191 (m/z) from each parent ion were measured for the analysis of CML and [$^{2}H_2$] CML in
192 the samples.
193

Results

The present study measured the effects of a range of carbohydrates on the formation of CMA and CML. The carbohydrates (glucose, mannose, galactose, fructose, and ribose) were incubated with gelatin for up to one week, followed by ELISA determination of the CMA and CML levels. As shown in Figure 2A, the CMA level increased in a time-dependent manner following exposure of gelatin to ribose, whereas it was only slightly increased following exposure to hexose. A similar tendency was also observed in the formation of CML. Thus, ribose produced the highest CML formation, whereas CML increased gradually from hexoses (Fig. 2B). As shown in Figure 2B, less CML formation was detected following exposure to glucose in a time-dependent manner. These results demonstrated that CMA and CML were rapidly generated in gelatin incubated with ribose. CMA and CML contents in glycated gelatins were also measured by LC-MS/MS. The typical fragment ion chromatograms of CMA and [$^{13}C_6$] CMA (10 pmol) by LC-MS/MS in the samples are shown in Figure 3A. Typical fragment ion chromatograms of CML and [$^{2}H_2$] CML (10 pmol) are also shown in Figure 3B. Using this method, the levels of CML and CMA in glycated gelatins were measured. As shown in Figure 3C, ribose showed the highest contents of CML and CMA. These data demonstrated that the detection of AGEs by the ELISA system using anti-AGE

212 antibodies correlated with the results of LC-MS/MS, thereby validating this assay
213 system.
214 Because CMA and CML were generated more rapidly by ribose than by the hexoses,
215 ribose was used to screen potential inhibitors of CMA and CML formation. As shown in
216 Figure 4A, ELE completely inhibited CMA formation, whereas EBE or ERE partially
217 inhibited CMA formation. Furthermore, ELE partially inhibited CML formation,
218 whereas EBE or EBR did not show any inhibitory effects on CML formation (Fig. 4B).
219 This result strongly demonstrates that ELE contained compound(s) capable of inhibiting
220 the formation of CMA and CML. Therefore, the ELE components were isolated as
221 described in the Materials and Methods section. In the same manner, reported structures
222 such as quercetin and kaempferol were also tested. As shown in Figure 5, several
223 isolated compounds, including isoquercetin, 6"-*O*-acetyl-astragalin, rutin, and astragalin,
224 showed stronger inhibitory effects on CMA and CML formation than aminoguanidine,
225 whereas kaempferol 3-O-rutinoside showed enhancing effects rather than weak
226 inhibitory effects. Furthermore, ELE compounds inhibited the formation of CMA more
227 effectively than that of CML. Acid hydrolyzates of isoquercetin, astragalin, quercetin
228 and kaempferol also showed stronger inhibitory effects on CMA and CML formation
229 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-astragalin, 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

248 formation of CML, one of the major antigenic AGE structures produced by the Maillard
249 reaction and lipid peroxidation. CML is implicated in the pathogenesis of age-related
250 disorders such as diabetic complications and is recognized by the receptor for AGE
251 (RAGE). CML-RAGE interactions activate the NF-κB signaling pathway, while also
252 enhancing the expression of vascular cell adhesion molecule-1 (VCAM-1) in human
253 umbilical vein endothelial cells [45]. Alikhani *et al.* [46] demonstrated that CML-collagen
254 was recognized by RAGE and induced fibroblast apoptosis by activating cytoplasmic
255 and mitochondrial caspase pathways. Thus, the demonstration that the *in vitro* formation
256 of CML was inhibited by ELE points to a potentially novel therapeutic strategy for
257 preventing the development of diabetic complications, such as diabetic nephropathy,
258 retinopathy, and neuropathy, by inhibiting AGE formation.

259 It was reported that ELE controlled the plasma glucose levels in a rat model of
260 type 1 diabetes and a mouse model of type 2 diabetes (C57BL/KsJ-db/db mice) [27,28].
261 Experiments with fructose-drinking rats fed ELE suggested that long-term ELE
262 treatment effectively prevented the development of insulin resistance and ameliorated
263 abnormal perivascular innervation in these animals [47]. Fujikawa *et al*. reported that the
264 chronic administration of ELE or *E. ulmoides* green leaf powder improved insulin
265 resistance in a rat model of high-fat diet (HFD)-induced obesity [48], with concomitant

266 increases in the plasma adiponectin levels and the suppression of both the plasma
267 resistin and TNF-α levels. These findings raised the possibility that ELE may improve
268 atherosclerosis and diabetic nephropathy. This anti-obesity effect was dependent on the
269 asperuloside content of the *E. ulmoides* leaf [49]. Furthermore, the chronic administration
270 of asperuloside decreased body weight, white adipose tissue (WAT) weight, and plasma
271 lipid parameters in mice fed a HFD [50]. Because ELE inhibits the formation of AGEs, we
272 believe that the daily intake of ELE is expected to become an effective means of
273 preventing lifestyle-related diseases. Although the inhibitory effect of ELE on AGEs
274 formation *in vivo* has not yet been clarified, we hope that our findings will encourage
275 further physiological studies. Taken together, there is a possibility that the daily intake
276 of ELE ameliorates AGEs formation, resulting in the prevention of diabetic
277 complications.

278

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283

284

285 **Figure legends**

286 Figure 1. Isolation and purification process and identified structures by ^{1}H- and
287 ^{13}C-NMR data. The isolation and purification process of *E. ulmoides* Oliv. leaves are
288 shown in the flow chart, as described in the Materials and Methods (A). The chemical
289 structures of isolated compounds from ELE are demonstrated (B).

290

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

295

296 Figure 3. The measurement of CMA and CML levels by LC-MS/MS. The parent ions of
297 CMA and [$^{13}C_6$] CMA were 233 (m/z) and 239 (m/z), respectively. Peaks of the
298 fragment ions of CMA and [$^{13}C_6$] CMA (10 pmol) were detected in the samples (A).
299 The parent ions of CML and [$^{2}H_2$] CML were 205 (m/z) and 207 (m/z), respectively.
300 Peaks of the fragment ions of CML and [$^{2}H_2$] CML (10 pmol) were detected in the
301 samples (B). The CMA (open bar) and CML (closed bar) levels in the incubated
302 carbohydrates with gelatin were measured by LC-MS/MS (C), as described in the

303 Materials and Methods.

304

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

312

313 Figure 5. Inhibitory effects of compounds purified from ELE on CMA and CML
314 formation. Gelatin and ribose were incubated in the presence of 0.1 mM of the indicated
315 compounds for 7 days, followed by the determination of CMA and CML by a
316 noncompetitive ELISA. Coated antigen concentration was 10 μg/mL. Data was
317 expressed as inhibition (%) compared with control (without samples). For the control,
318 only gelatin and ribose were incubated. Abbreviations: IQ, isoquercetin; AAs, 6"-
319 *O*-acetyl-astragalin; K, kaempferol; Q, quercetin; R, rutin; KR, kaempferol
320 3-O-rutinoside; As, astragalin; AG, aminoguanidine. For the control, only gelatin and

321 ribose were incubated for 7 days. The data are presented as the means ± SD (n=3). **, P
322 < 0.01, *, P < 0.05 vs. control.
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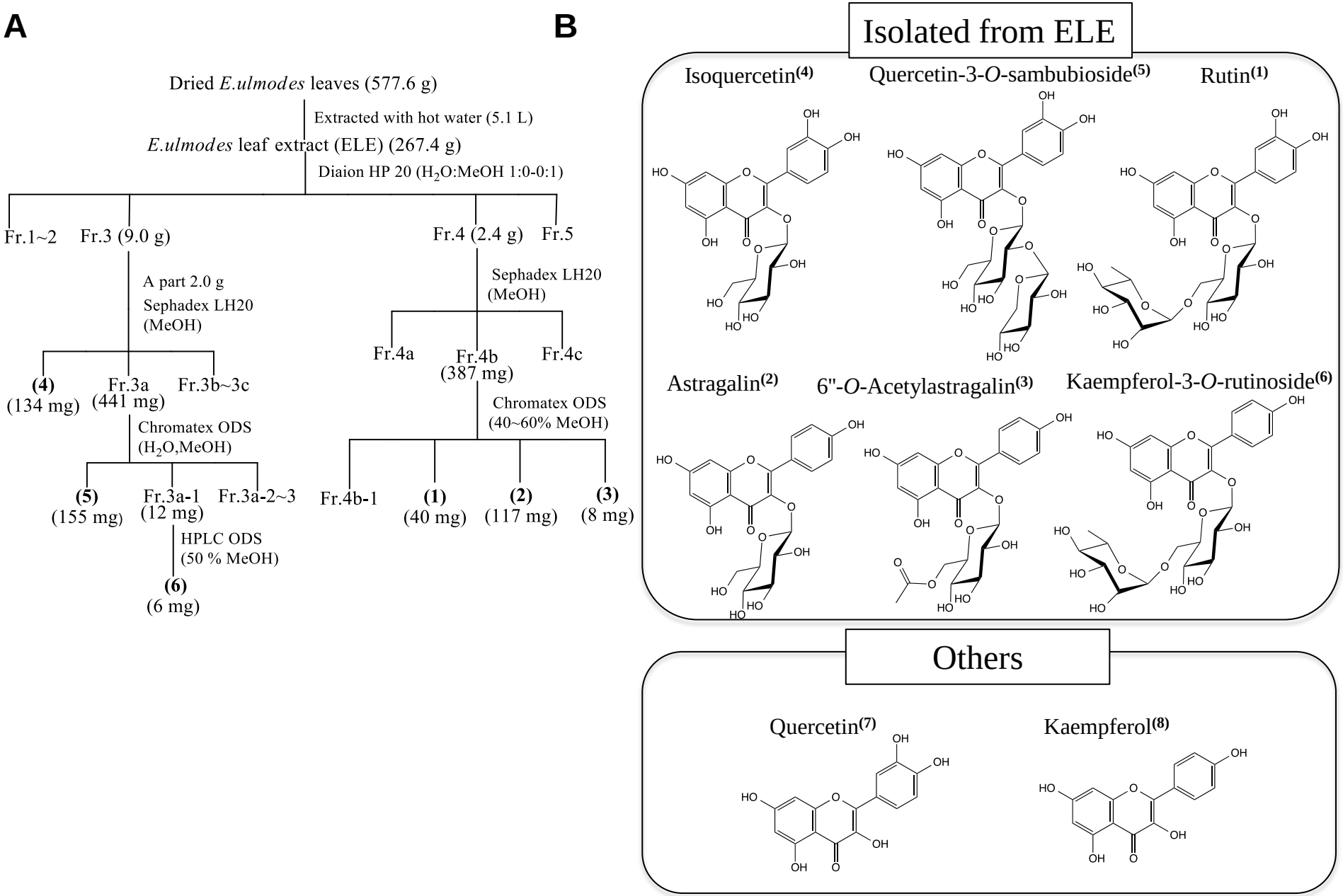
Figure 1. Sugawa et al.,



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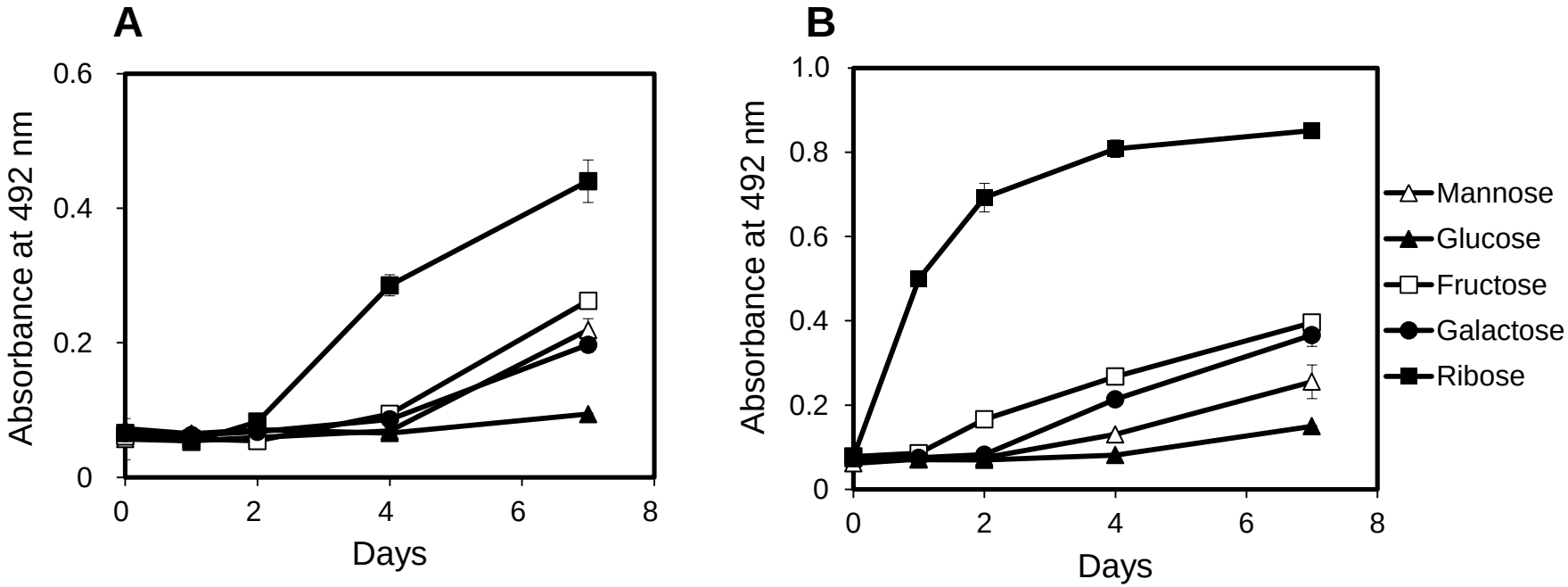

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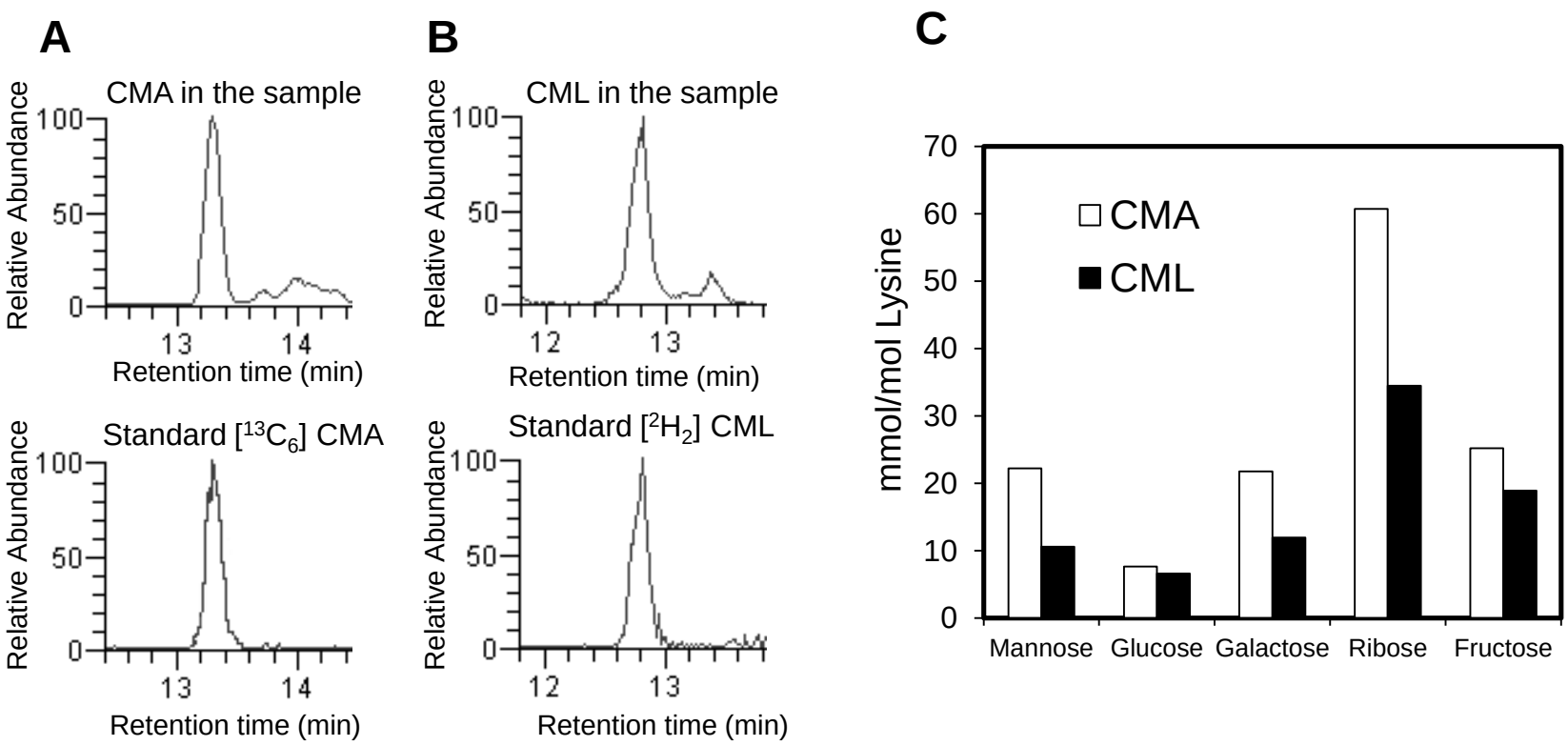

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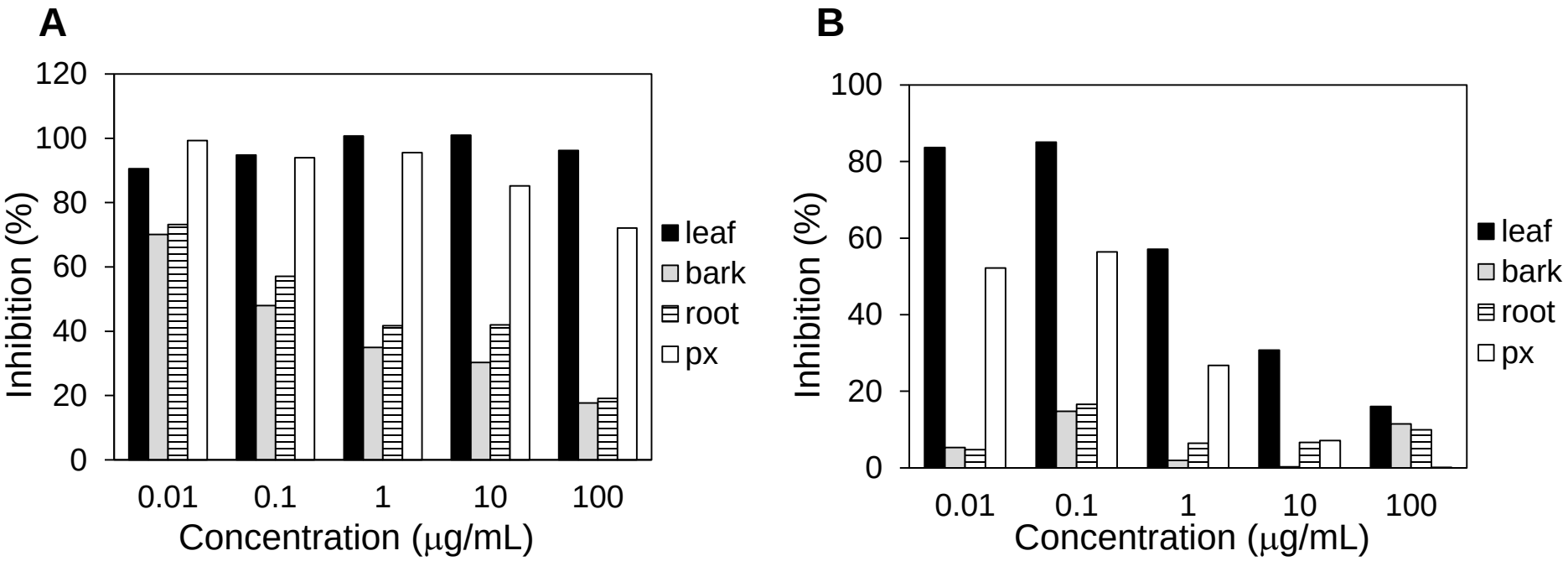

Figure 5. Sugawa et al.,