Aldose Reductase and Advanced Glycation Endproducts Inhibitory Effect of Phyllostachys nigra

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To evaluate active principles for diabetic complications from the black bamboo leaves, Phyllostachys nigra, eight compounds were isolated and tested for their effects on rat lens aldose reductase and advanced glycation endproducts. As a result, luteolin 6-C-(6'-O-trans-cafeoylglucoside) was found to show a strong aldose reductase and advanced glycation endproducts inhibition. This compound showed antioxidative activity measured in Photochem® apparatus. It is concluded, therefore, that luteolin 6-C-(6'-O-trans-cafeoylglucoside) (6), a flavone of this plant, has antioxidative as well as aldose reductase and advanced glycation endproducts inhibitory effects. As a result, this compound could be offered as a leading compound for further study as a new natural products drug for diabetic complications.

Key words aldose reductase; advanced glycation endproduct; Phyllostachys nigra; luteolin 6-C-(6'-O-trans-cafeoylglucoside); antioxidant; diabetic complications

Aldose reductase (alditol/NADP+ oxidoreductase, E.C.1.1.1.21, ALR2) is the first enzyme of the polyol pathway which reduces excess d-glucose into d-sorbitol with concomitant conversion of NADPH to NADP+.1,2 which has been demonstrated to play important roles not only in the cataract formation in the lens3 but also in the pathogenesis of diabetic complications such as neuropathy,4 nephropathy5 and retinopathy.6

Prolonged hyperglycemia also results in the formation of advanced glycation endproducts (AGEs) in body tissues. The complex, fluorescent AGE molecules formed during the Maillard reaction can lead to protein cross-linking and contribute to the development and progression of several diabetic complications such as cataract, atherosclerosis, nephropathy and neuropathy.7

Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and their complications.8,9 Diabetes is usually accompanied by increased production of free radicals or impaired antioxidant defenses.10,11

It would therefore seem to be desirable that ALR2 inhibitors have possess AGEs inhibitory and antioxidative potencies.

A number of structurally diverse naturally occurring and synthetic ALR2 and AGEs inhibitors have been studied in vivo to clarify their effectiveness for prevention of diabetic complications in clinical trials as well as experimental animals. Several studies on the inhibition of ALR2 and AGEs and structure–activity relationship of naturally occurring flavonoids have been reported.12–14

Black bamboo, Phyllostachys nigra grows particularly in Southeast Asia and represents a traditionally important commodity used for building materials in traditional medicine, and as a source of food, which are a potential source of natural free radical scavengers.15

In the present study, we investigated the inhibitory effect of the leaves of P. nigra on ALR2 and AGEs to evaluate its potential for treatment of diabetic complications. This investigation led to isolation and identification of the compounds that inhibit the activity of ALR2 and AGEs.

Furthermore, we investigated whether active compound for ALR2 and AGEs was able to scavenge free radical using luminometry (Photochem®).

MATERIALS AND METHODS

Chemicals The first grade solvents were used for extraction, fractionation and column chromatography. Kiesel gel 60 (70—230 mesh, Art, 7734 Merck) was used as the column packing material. Kiesel 60 F254 (precoated plate, Art. 5559, Merck) was used for thin layer chromatography (TLC). Iodine vapor and 10% H2SO4 were used for TLC detection. dL-Glyceraldehyde, d-glucose, bovine serum albumin (BSA) and NADPH were purchased from Sigma Chemical (St. Louis, MO, U.S.A.).

Plant Materials The leaves of P. nigra were collected at the vicinity of Gangneung, Korea and the voucher specimen (No. BBL-0001) was deposited at the Herbarium of Agricultural Technology Center, Korea.

Isolation and Identification The dried leaves of P. nigra (2.5 kg) were extracted three times with hot MeOH for 1 h. This residue was evaporated in vacuo to yield the total extract (26.8 g). This extract was then suspended in distilled water and partitioned sequentially with n-hexane, methylene chloride, and ethyl acetate. The ethyl acetate fraction (10 g) was subjected to ODS column chromatography using H2O–MeOH gradient system (70:30→0:100) to provide 9 fractions (fractions 1—9). From fraction 3, compounds 1(64.2 mg), 2(16) (33.2 mg), and 3(16) (25.3 mg) were isolated using a Sephadex LH-20 column chromatography (MeOH), and then purified by preparative RP-HPLC (YMC J'sphere-H80, 4 μm, 250×20 mm, acetonitrile–H2O=15:85). Compounds 4(17) (17.0 mg) and 5(17) (28.7 mg) were purified from fraction 4 using a Sephadex LH-20 column chromatography (MeOH), and subsequent preparative RP-HPLC (YMC J’sphere-H80, 4 μm, 250×20 mm, acetonitrile–H2O=13:87). Fraction 5 was chromatographed on Sephadex LH-20 (MeOH) to obtain compound 6(18) (46.2 mg). Compound 7(19) (9.74 mg) and 8(19) (49.9 mg) were further purified from fraction 6 using Sephadex LH-20 with MeOH. All compounds

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identified by LC-MS and NMR spectra and direct comparison with authentic compounds. Their chemical structures are shown in Fig. 1.

**Preparation of Rat Lenses ALR2** Crude rat lenses ALR2 was prepared as follows: rat lenses were removed from Sprague-Dawley rats weighing 250—280 g and frozen until use. The supernatant fraction of the rat lenses homogenate was prepared according to Hayman and Kinoshita\(^\text{21}\) and then partially purified according to Inagaki et al.\(^\text{22}\).

**Measurement of Rat Lenses ALR2** Rat lenses ALR2 activities were assayed spectrophotometrically by measuring the decrease in absorption of NADPH at 340 nm over a 5 min period with DL-glyceraldehyde as a substrate.\(^\text{23}\) Each 1.0 ml cuvette contained equal units of enzyme, 0.10 M sodium phosphate buffer (pH 6.2), 0.3 mM NADPH with or without 10 mM substrate and inhibitor. The concentration of inhibitors giving 50% inhibition of enzyme activity (IC\(_{50}\)) was calculated from the least-squares regression line of the logarithmic concentrations plotted against the residual activity.

**Effects on AGEs Formation** The AGEs formation was assessed by characteristic fluorescence reported by Morimitsu et al.\(^\text{24}\) with slight modifications. Briefly, the reaction mixture of 100 mg \(\alpha\)-glucose, 10 mg BSA in 1 ml sodium phosphate buffer (67 mM, pH 7.2) was incubated at 60 °C for 2 d with or without the test compound. The reaction solution (0.2 ml) was diluted with water (2 ml), and the intensity of fluorescence was measured using a fluorophotometer (Luminescence Spectrometer LS50B, Perkin-Elmer Ltd., Buckinghamshire, England) at an excitation wavelength of 370 nm and an emission wavelength of 440 nm. The reaction mixture without \(\alpha\)-glucose was used as a blank solution. Measurements were performed in duplicate, and the concentration required for a 50% inhibition (IC\(_{50}\)) of the intensity of fluorescence was determined graphically.

**Antioxidative Capacity Determination** For the determination of the integral antioxidative capacity of lipid soluble substances in \(P.\ nigra\), the method of photochemiluminescence was used. ACL is the standard kit of Photochem where the luminal plays a double role of photosensitizer as well as the radical detecting agent. A standard plot was plotted and the results were calculated in Trolox equivalents (nmol), according to the manufacturer’s instruction.

**RESULTS AND DISCUSSION**

ALR2 inhibitors have been shown to prevent or delay significantly diabetic complications, and synthetic ALR2 inhibitors are currently available and many have been tested for their clinical use, albeit with limited success\(^\text{25}\) i.e.; Synthetic compounds with diverse structures such as sorbinil,\(^\text{26}\) epalrestat,\(^\text{27}\) other hydantoin derivatives\(^\text{28}\) and flavonoids\(^\text{12}\) from natural origin have been extensively studied and reported to inhibit ALR2.

It has been suggested that free radicals and oxidation reaction are directly involved in glucose-mediated modification of proteins. Several mechanisms appear to be involved in hyperglycemia-mediated oxidative stress, such as glucose autoxidation, protein glycation, and the formation of AGEs.\(^\text{29}\) The present study was carried out in a search for new potential ALR2 and AGEs inhibitors useful for the treatment of diabetic complications from the leaves of \(P.\ nigra\).

In order to identify the active compounds from \(P.\ nigra\), the extract divided into 5 systematic fractions, which were then tested for rat lenses ALR2 inhibitory activity using \(\alpha\)-glyceraldehyde as a substrate and inhibitory effect on BSA glycation by a fluorescence method. From them, ethyl acetate fraction showed significantly higher inhibition in a concentration dependent (Tables 1, 2).

**Table 1. Inhibitory Effects of the Leaves of \(P.\ nigra\) on Rat Lens ALR2**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Concentration ((\mu)g/ml)</th>
<th>Inhibition (%)</th>
<th>IC(_{50}) ((\mu)g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMG(^a)</td>
<td>10</td>
<td>91.5</td>
<td>0.62</td>
</tr>
<tr>
<td>n-Hexane fr.</td>
<td>10</td>
<td>32.7</td>
<td>—</td>
</tr>
<tr>
<td>Methylene chloride fr.</td>
<td>10</td>
<td>68.0</td>
<td>3.97</td>
</tr>
<tr>
<td>Ethyl acetate fr.</td>
<td>10</td>
<td>94.6</td>
<td>0.68</td>
</tr>
<tr>
<td>n-Butanol fr.</td>
<td>10</td>
<td>78.2</td>
<td>0.97</td>
</tr>
<tr>
<td>Water fr.</td>
<td>10</td>
<td>35.5</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) Inhibition rate was calculated as percentage with respect to the control value.

**Table 2. Inhibitory Effects of the Leaves of \(P.\ nigra\) on AGEs**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Concentration ((\mu)g/ml)</th>
<th>Inhibition (%)</th>
<th>IC(_{50}) ((\mu)g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane fr.</td>
<td>200</td>
<td>36.9</td>
<td>—</td>
</tr>
<tr>
<td>Methylene chloride fr.</td>
<td>200</td>
<td>55.2</td>
<td>—</td>
</tr>
<tr>
<td>Ethyl acetate fr.</td>
<td>200</td>
<td>88.4</td>
<td>77.4</td>
</tr>
<tr>
<td>n-Butanol fr.</td>
<td>200</td>
<td>82.8</td>
<td>102.2</td>
</tr>
<tr>
<td>Water fr.</td>
<td>200</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Inhibition rate was calculated as percentage with respect to the control value.
The chemical structures of eight phenolic compounds, two coumaric acids, and six flavone compounds isolated were preparative HPLC and further purification yielded eight compounds by gel chromatography and preparatory HPLC.

The chemical structures of eight phenolic compounds, two coumaric acids, and six flavone compounds isolated were identified, and their inhibitory effects are shown in Tables 3 and 4. Their chemical structures were elucidated by chemical and spectral analysis as isoorientin (1), orientin (2), vitexin (3), cis-coumaric acid (4), p-coumaric acid (5), luteolin 6-C-(6′-O-trans-cafeoylglucoiside) (6), vitexin 3-glucosyl 3-coumaric acid (7), and tricin (8). Their structures were shown in Fig. 1.

Inhibitory activity against ALR2 of isolated compounds was tested using TMG as positive control. Flavones such as isoorientin (1), vitexin (3), luteolin 6-C-(6′-O-trans-cafeoylglucoiside) (6) and tricin (8) were found to exhibit much stronger ALR2 inhibition than other compounds. Of these compounds, luteolin 6-C-(6′-O-trans-cafeoylglucoiside) was found to exhibit most strong rat lens ALR2, and the inhibitory potencies as expressed by IC50 value was 0.0134 μM (IC50 value of TMG is 0.92 μM).

Varma and Kinoshita have indicated some possible relationships of structure to the inhibiting potencies of flavones. The hydroxylation in the 4′-position has beneficial effects, and the abolition of the double bond between C-2 and C-3 leads to a decrease of inhibition. In support of these findings, the 4′-hydroxy group (R2 position of flavone in Fig. 1) of tested compounds was important for showing the inhibition of ALR2 activity.

In our previous study, we postulated the possible relationships of structure to the inhibitory activities of flavonoids: 1) chalcones having 4′-hydroxy at the A ring show stronger activity; 2) chalcones having catechol moiety at the B ring (the 3,4-ortho-dihydroxy moiety) show stronger activity; 3) chalcone shows a stronger activity than dihydrochalcone, in which 3,4,2′,4′-tetrahydroxychalcone was the most potent inhibitor for ALR2; 4) ring-closure form (queretin-like structure) of 3,4,2′,4′-tetrahydroxychalcone exhibit stronger activities than other flavonoids.

In the present study, our results indicated that queretin-like structure of luteolin 6-C-(6′-O-trans-cafeoylglucoiside) (6) also showed stronger inhibition than other flavones.

Matsuda et al. reported that ALR2 inhibitory activities of the compounds having a catechol moiety at the B ring showed stronger activities than those of monohydroxyl and pyrogallol moiety (the 3′,4′,5′-trihydroxy moiety), while opposite examples were found in flavone 8-glucoside [vitexin (3, IC50=2.03 μM)>orientin (2, >10 μM)].

The activity of 8-C-glycosyl 3′,4′,5′,7-tetrahydroxy flavone was weaker than those of 6-C-glycosyl [isoorientin (1, 1.91 μM)>orientin (2, >10 μM)].

In this study, the phenomenon of protein glycation was demonstrated in the reaction mixture of BSA with glucose in vitro. Glucose is used as glycated agent, which is commonly adopted in Maillard reaction studies, and BSA could serve as targets for glycated agent.

Inhibitory activity against AGES formation of isolated compounds was tested using aminoguanidine as positive control. Orientin (2) and luteolin 6-C-(6′-O-trans-cafeoylglucoiside) (6) were found to exhibit much stronger inhibitory activity against AGES formation with IC50 values of 87.3 and 87.7 μM, respectively, compared to a well known positive control, aminoguanidine (IC50 value of 960.0 μM).

It is reported that the hydroxyl groups of flavones at the 3′-, 4′-, 5-, and 7-positions increased the AGES inhibitory activities and the methylation or glycosilation of the 3′- or 4′-hydroxyl group reduced the activity. In agreement with this report, tetrahydroxylation of the 3′-, 4′-, 5-, and 7-positions of luteolin 6-C-(6′-O-trans-cafeoylglucoiside) (6) increased the activity.

It has been suggested that, in diabetes, oxidative stress plays a key role in the pathogenesis of vascular complications, both microvascular and macrovascular, and an early marker of such damage is the development of an endothelial dysfunction. A large body of evidence suggests a possible role of oxidative stress in the pathogenesis of diabetic complications, and this raises the concept that antioxidation therapy may be of great interest in these patients. Recently, a hyperglycemia-induced process of overproduction of the superoxide anion radical (·O2−) by the mitochondrial electron transport chain was reported to partially inhibit the glycoytic enzyme glyceraldehyde phosphate dehydrogenase, thereby diverting upstream metabolites from glycolysis into the polyol pathway and AGES formation, etc.

Table 3. Inhibitory Effects of the Compounds Isolated from the Leaves of *P. nigra* on Rat Lens ALR2

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (μM)</th>
<th>Inhibition (%)</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMG*</td>
<td>10</td>
<td>83.1</td>
<td>0.92</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>61.4</td>
<td>1.91</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>9.1</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>68.2</td>
<td>2.03</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>34.1</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>85.1</td>
<td>0.14</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>75.8</td>
<td>0.0134</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>36.4</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>93.2</td>
<td>2.03</td>
</tr>
</tbody>
</table>

Inhibition rate was calculated as percentage with respect to the control value. a) TMG: tetramethylene glutaric acid.

Table 4. Inhibitory Effects of the Compounds Isolated from the Leaves of *P. nigra* on AGES

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (μM)</th>
<th>Inhibition (%)</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG*</td>
<td>1250</td>
<td>56.9</td>
<td>960.0</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>46.5</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>55.8</td>
<td>87.3</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>42.3</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>61.0</td>
<td>87.7</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>23.6</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>34.1</td>
<td>—</td>
</tr>
</tbody>
</table>

Inhibition rate was calculated as percentage with respect to the control value. *AG*: aminoguanidine.
substance. Luminol acts both as photosensitizer as well as the detecting reagent.36)

If antioxidants are added to this measuring system, the intensity of the photochemiluminescence signal will be attenuated dependent on concentration. In this way, the total antioxidative capacity of the luteolin 6-C-(6'-O-trans-cafeoylglucoside) (6) under investigation can be quantified. The antioxidative capacity is given in equivalent concentration units of Trolox for lipid-soluble substances.

The antioxidative capacity of luteolin 6-C-(6'-O-trans-cafeoylglucoside) (6) was measured as 1.9937, 1.6601 and 0.7095 nmol equivalents of Trolox at 4, 0.4 and 0.04 μM, respectively (Fig. 2).

It is concluded, therefore, that luteolin 6-C-(6'-O-trans-cafeoylglucoside) (6), a flavone of this plant, have antioxidative activity as well as ALR2 and AGEs inhibitory effects. As a result, this compound could be offered as a leading compound for further study as a new natural products drug for diabetic complications.

Acknowledgements This work was supported by grant No. RTI05-01-02 from the Regional Technology Innovation Program of the Ministry of Commerce, Industry and Energy (MOCIE).

REFERENCES