Potent α-Glucosidase Inhibitors Isolated from *Ginkgo biloba* Leaves

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**Abstract:** *In vitro* α-glucosidase inhibitory activity of *Ginkgo biloba* leaves was investigated. The inhibitory activity of methanol extracts from yellow and green leaves was 13.8 and 40.1 μg mL⁻¹, respectively. Each methanol extract was separated into its respective fraction by solvent-solvent extraction with n-hexane, chloroform, ethyl acetate and n-butanol. The n-hexane fractions (in both methanol extracts from green and yellow leaves) exhibited high α-glucosidase inhibitory activity with IC₅₀ values of 13.6 and 13.4 μg mL⁻¹, respectively. Further fractionation of the n-hexane fractions by silica gel column chromatography gave the most active fraction which was identified as ginkgolic acid (C13:0) and a mixture (C13:0, C15:0, C15:1, C17:1 and C17:2). Ginkgolic acid (C13:0) exhibited the highest α-glucosidase inhibitory activity. This is the first study to successfully isolate ginkgolic acids as α-glucosidase inhibitors.

**Key words:** *Ginkgo biloba*, α-glucosidase inhibitor, ginkgolic acids

**INTRODUCTION**

Diabetes Mellitus (DM) is a common metabolic disease characterized by high blood glucose levels due to the lack of pancreatic insulin secretion, with or without an interruption in the action of insulin. Diabetes has been estimated to currently affect 177 million people worldwide and this figure is expected to increase to 300 million by 2025 (Porter and Barret, 2005). Epidemiological studies and clinical trials have demonstrated that hyperglycemia is the main cause of complications associated with coronary artery disease, cerebrovascular disease, kidney failure, blindness, limb amputation, neurological complications and premature death (Lopez-Candales, 2001). Recent studies reported that postprandial hyperglycemia may induce non-enzymatic glycosylation of proteins, resulting in the development of chronic complications (Gholamhoseinian et al., 2008). Hence, control of postprandial plasma glucose levels is critical in the initial treatment of DM as well as reducing chronic vascular complications (Shim et al., 2003).

One therapeutic approach used to reduce postprandial hyperglycemia in patients with DM is preventing the absorption of carbohydrates following the consumption of food (Gholamhoseinian et al., 2008). Only monosaccharides, such as glucose and fructose, can be transported from the intestinal lumen into the bloodstream. Complex starches, oligosaccharides and disaccharides must be broken down into individual monosaccharides before being absorbed in the duodenum and upper jejunum. Enteric enzymes including pancreatic α-amylase and α-glucosidase which are attached to the brush border of the intestinal cells, facilitate this digestion. An increasing number of studies are screening α-glucosidase inhibitors from plants and synthetic sources (Christudas et al., 2009). Inhibitors of these enzymes have recently been developed from natural sources (Shim et al., 2003).

*Ginkgo biloba* is among the most valued medicinal plants and has been since it was described in medical treatises in the ancient world; people have used its fruit for thousands of years (Hobbs, 1991). A vast number of chemical and pharmacological studies have enhanced its traditional reputation, making ginkgo one of the most widely used phytopharmaceuticals in the world. The active compounds in ginkgo extract have been shown to improve blood circulation, discourage clot formation, reinforce the walls of capillaries and protect nerve cells from harm when deprived of oxygen and its leaf extract is also used to treat cognitive disorders, such as concentration difficulties and memory impairment (Singh et al., 2008). This extract has also been shown to

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exhibit antioxidant (Bridi et al., 2001), antiasthmatic (Mahmoud et al., 2000), radical-scavenging (Louajri et al., 2001), wound healing (Bairy and Rao, 2001) and neuroprotective properties as well as improving the mental capacities of Alzheimer’s patients (Kennedy et al., 2000). The usefulness of a standardized extract of Ginkgo biloba (EGB 761) has also been demonstrated in the treatment of retinal impairment in diabetes sufferers (Doly et al., 1986), decreasing fasting blood glucose levels (Shankar et al., 2005) and reducing blood glucose values to normal levels following its administration (Cheng et al., 2013). However, no studies have yet examined the α-glucosidase inhibitor active compounds from G. biloba. In this study, we describe the isolation and identification of α-glucosidase inhibitors from G. biloba leaves.

MATERIALS AND METHODS

Plant materials: Green and yellow leaves of Ginkgo biloba were collected from the Faculty of Agriculture, Ehime University, Japan. Green leaves were collected in August 2011 while, yellow leaves were collected in December 2011. The green and yellow leaves were kept dry and away from direct sunlight during storage. All specimens were deposited in the Laboratory of Plant Chemistry, Faculty of Agriculture, Ehime University, Japan, for further use.

Chemicals: Quercetin dehydrate, dimethylsulfoxide, p-nitrophenyl-D-glucopyranoside, α-glucosidase type I, from yeast of Saccharomyces cerevisiae, bovine serum albumin and quercetin as a control were purchased from Wako Chemical Co. Ltd. (Osaka, Japan). The silica gel used for column chromatography (Wakogel C-200) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). TLC (thin layer chromatography) aluminum sheet (0.25 mm) silica gel 60 F254, 20×20 cm was obtained from Merck (Darmstadt, Germany). All solvents were purchased from Wako Chemical Co. Ltd. (Osaka, Japan) and distilled before use.

Apparatus: Melting points were measured on a Yanaco micro melting point apparatus (Yanaco Co., Ltd., Kyoto, Japan) and were uncorrected. Ultraviolet (UV) spectra were measured with a Shimadzu UV-Vis 1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Preparative HPLC was conducted with a Waters system consisting of a 600 Delta pump controller equipped with a Charater Sample Loader SL964, Waters module column oven, Cappell pak C18 UG column and UV-Visible Detector 2489 (Waters, Japan). The mass spectra of compounds were obtained using TOF-MS (Waters, Japan) and high resolution FAB-MS. Nuclear Magnetic Resonance (NMR) spectra were recorded on a JEOL JNM-EX 400 (JEOL Ltd., Tokyo, Japan) at 500 and 125 MHz. Chemical shifts were expressed as δ in ppm and TMS was used as the internal standard. The coupling constant (J) was recorded in Hz.

Isolation of active compounds from the methanol extract:

The leaves were cut into small pieces and air-dried before extraction. The dried leaves (1,225 g) were extracted for 48 h at room temperature with methanol solvent. The extraction was repeated twice. The methanol extract was concentrated with a rotary evaporator under reduced pressure. The methanol extract was a dark yellow semisolid. The methanol extract of G. biloba leaves (300.16 g) was suspended in methanol-water (1:4) and then partitioned with n-hexane, chloroform, ethyl acetate and n-butanol solvents to give n-hexane, chloroform, ethyl acetate and n-butanol fractions. Each fraction was subjected to bioassay-guided fractionation using an in vitro α-glucosidase inhibitory activity assay.

As the most active fraction, a portion of the n-hexane fraction was chromatographed over silica gel (C-200) and eluted with the n-hexane-ethyl acetate solvent system which increased in polarity (1:0-0:1), to afford 37 fractions (F1-F37). Fractions 6 and 7 were the most active. The recrystallization of fraction 6 using n-hexane resulted in compound 1. Fraction 7 contained five compounds that were analyzed by HPLC and GLC. Spots were detected on TLC plates under a UV lamp (254 nm) by spraying with the DSA reagent, bromocresol green or by heating after spraying with concentrated sulfuric acid.

The isolated active fraction was analyzed by gas chromatography with a mass spectrometer (GC-MS 2010), equipped with a TC-5 column (30 m, id: 0.24 mm). The carrier gas was helium delivered at a constant rate of 1.5 mL min⁻¹, with a column pressure of 100 kPa and interface temperature of 280°C. The temperature program was 60°C which was increased at 10°C min⁻¹ to 300°C and 300°C for 18 min to allow the late eluting peak to exit the column. The injection volume was 1 μL and the injector temperature was maintained at 280°C. The GC-MS conditions consisted of 40-700 mass ranges.

Trimethylsilylation (TMS) derivative of compound 1:

Compound 1 was trimethylsilylated and analyzed by gas chromatography with a mass spectrometer (GC-MS 2010), equipped with a TC-5 column (30 m, id: 0.24 mm). The helium carrier gas was delivered at a constant rate of 1.5 mL min⁻¹, with a column pressure of 100 kPa and interface temperature of 280°C. The temperature program was 60°C which was increased at 10°C min⁻¹ to 300°C and 300°C for 18 min to allow the late eluting peak to exit the column. The injection volume was 1 μL and the injector temperature was maintained at 280°C. The GC-MS conditions consisted of 40-700 mass ranges.
**Spectrometric analysis:** $^1$H NMR and $^{13}$C NMR spectra were recorded on a JEOL INX-EX400 (JEOL Ltd., Tokyo, Japan) at 400 MHz and 100 MHz, respectively. Ultraviolet (UV) spectra were measured with a UV-Vis spectrophotometer (Shimadzu UV-1600). Mass spectra were recorded at an electron energy of 70 eV with a direct inlet and on a Shimadzu GC-MS QP-2010. Preparative HPLC was conducted with a Waters system consisting of a 600 Delta pump controller equipped with a Characte Sample Loader SL064, Waters module column oven, UV-visible Detector 2489 and Capcell pak C18 UG column.

**Structural analysis of compound 1:** White crystal (n-hexane), m.p. 65-67°C, UV $A_{max}$ nm (e): 310 (3100), 243 (5000). EI-MS [M]+ $m/z$ 320; FAB-MS [M+H]$^+$ $m/z$ 321; HRFAB-MS [M+H]$^+$ $m/z$ 321.2416 [Caled. C$_3$H$_7$O$_2$, as 321.2421]; $^1$H-NMR $\delta$ (CDCl$_3$): 0.87 (3H, t, $J = 7.0$ Hz), 1.34 (20H, m), 1.59 (2H, q, $J = 4.0$ Hz), 2.97 (2H, t, $J = 8.0$ Hz), 6.77 (1H, d, $J = 7.5$ Hz), 6.87 (1H, d, $J = 8.0$ Hz), 7.36 (1H, dd, $J = 8.0, 7.5$ Hz), 11.07 (1H, s, COOH), $^{13}$C-NMR $\delta$ (CDCl$_3$): 14.11 (C1'), 22.69 (C2'), 31.93 (C11'), 29.36 (C10'), 29.49 (C9'), 29.63 (C8'), 29.66 (C7', C6'), 29.69 (C5'), 29.69 (C4'), 29.82 (C3'), 32.05 (C2'), 36.50 (C1'), 111.85 (C7), 110.30 (C8), 122.74 (C5), 135.42 (C4), 147.74 (C6), 163.67 (C2), 175.36 (COOH).

**Assay for $\alpha$-glucosidase inhibitory activity:** Inhibition of methanol extract was monitored by $\alpha$-glucosidase activity. The reaction mixture consisted of 250 $\mu$L of 5 mM p-nitrophenyl-1-glucopyranoside and 1 mL of 100 mM phosphate buffer (pH 7.0) and was added to a flask containing 250 $\mu$L of the sample dissolved in DMSO at various concentrations. The reaction mixture was pre-incubated for 5 min at 37°C and the reaction was then initiated by adding 250 $\mu$L $\alpha$-glucosidase and the incubation was continued for 15 min. The reaction was stopped by adding 1 mL of 200 mM Na$_2$CO$_3$. The activity of $\alpha$-glucosidase was determined by measuring the release of p-nitrophenol at a UV$A_{max}$ of 400 nm. The percentage inhibitory activity was calculated as:

\[
\frac{A_0 - A}{A_0} \times 100
\]

where $A_0$ is the absorbance of the control and $A$ is the absorbance of the extract or standard. The 50% inhibition concentration (IC$_{50}$) of each compound was determined from the graph of percentage $\alpha$-glucosidase inhibitory activity against sample concentration. Assays were performed in triplicate and the results were expressed as Mean Values ± Standard deviations. Quercetin was used as the positive control.

**RESULTS AND DISCUSSION**

**$\alpha$-Glucosidase inhibitory activity of solvent fraction:** The methanol extract from both the green and yellow leaves of *G. biloba* exhibited $\alpha$-glucosidase inhibitory activities with IC$_{50}$ values of 40.1±3.43 and 13.8±2.64 $\mu$g mL$^{-1}$, respectively while that of quercetin as the positive control was 3.8±0.12 $\mu$g mL$^{-1}$. Since, *G. biloba* yellow leaves showed potent inhibitory activity, the active principles in this plant extract were isolated and purified further. The isolation procedures for active compounds are shown in Fig. 1. Figure 1 also shows the yield of the n-hexane fraction to the methanol extract was higher (45%) than those of the chloroform, ethyl acetate and n-butanol fractions (8, 12 and 13%, respectively). However, the yield of the n-hexane fraction from green leaves was lower than that of the n-butanol fraction (32 and 34%, respectively), but higher than those of the chloroform and ethyl acetate fractions (6 and 3%, respectively).

The n-hexane fraction exhibited activity on the $\alpha$-glucosidase inhibition assay with an IC$_{50}$ value of 13.6±0.53 $\mu$g mL$^{-1}$ while the chloroform, ethyl acetate and n-butanol fractions had IC$_{50}$ values of 61.0±12.8, 81.0±2.89 and 95.0±1.92 $\mu$g mL$^{-1}$, respectively as shown in Table 1. The $\alpha$-glucosidase inhibitory activity exhibited by the n-hexane fraction obtained from the green leaves of *G. biloba* was investigated and an IC$_{50}$ value of 13.6±0.65 $\mu$g mL$^{-1}$ was recorded while those of the chloroform, ethyl acetate and n-butanol fractions had IC$_{50}$ values of 30.7±3.21, 287.5±6.17 and 151.1±5.51 $\mu$g mL$^{-1}$, respectively. The n-hexane fraction of the yellow leaves showing the highest $\alpha$-glucosidase inhibitory activity was then fractionated further to isolate the active compounds.

**Isolation of active compounds from the n-hexane fraction:** A portion of the n-hexane fraction was separated by chromatography with a silica gel column (gradient of n-hexane-ethyl acetate) into 37 fractions. The $\alpha$-glucosidase inhibition assay was conducted and fractions 6 and 7 were found to be the most active with IC$_{50}$ values of 3.9±0.12 and 5.1±0.97 $\mu$g mL$^{-1}$, respectively. Fraction 6 was separated by HPLC using the Capcell pak C18 UG column which resulted in two fractions (Fr. 6.1 and Fr. 6.2), as shown in Fig. 2. The $\alpha$-glucosidase inhibition

| Table 1: $\alpha$-glucosidase inhibitory activities of each fraction in methanol extract from yellow and green leaves of *Ginkgo biloba* |
|---------------------------------|-----------------|-----------------|
| Fraction                        | Yellow leaves   | Green leaves    |
| n-hexane                        | 13.4±0.53       | 13.6±0.65       |
| Chloroform                      | 61.0±12.8       | 30.7±3.21       |
| Ethyl acetate                   | 81.0±2.89       | 287.5±6.17      |
| n-butanol                       | 95.0±1.92       | 151.1±5.51      |
Fig. 1: Procedure for isolating α-glucosidase inhibitor active compounds from the methanol extract of *G. biloba.* *Active fraction or compound, †percentage yield of each fraction to methanol extract.

![Diagram showing the procedure for isolating active compounds from the methanol extract of *G. biloba.*]

An assay of each fraction revealed that Fr. 6.1 was the most active with an IC₅₀ value of 3.7±0.06 µg mL⁻¹. Compound 1 was obtained as a white crystal with a melting point of 65-67°C by recrystallizing fraction 6.1 using n-hexane. The structure of the isolated compound was elucidated by the spectroscopic data of EI-MS, the TMS derivative, FAB-MS, †H-NMR and ‡C-NMR.

Structure determination of the isolated compound 1: The yellow colorization obtained by spraying the TLC plate with bromoresol green and DSA reagent after heating indicated the presence of carboxylic acid (R-COOH) and phenolic hydroxyl groups (R-OH), respectively. The electron ion mass spectrum (EI-MS) analyzed by GC-MS showed a molecular ion peak at m/z 320 (M⁺), as shown in Fig. 3, which corresponded to the molecular formula C_{33}H_{32}O_{2}. The fragment ion at m/z 302 was formed by the loss of H₂O (M-18) (Silverstein et al., 2005). The base fragment ion at m/z 161 observed in the spectrum was the stable bicyclic aromatic phenol cation form. Various fragment ions at m/z 147 and 121 mainly formed through eliminating a CH₂O and C₃H₂O₂ group or both. The rearrangement peak at m/z 108 and its molecular formula revealed the presence of a hydroxyl group. Analyzing trimethylsilylated (TMS) compound 1 using GC-MS showed a molecular ion and fragment ion peak at m/z 464 (M⁺), 449 (M⁺-15) (100%) which corresponded to the molecular formula C₉₈H_{36}O₈Si₃. The UV spectrum of compound 1 had bands at λ_{max} 240 and 310 nm.
Fig. 3: Electron ion mass spectra of compound 1

Fig. 4: Chemical structure of ginkgolic acid (C13:0) isolated as compound 1 from yellow leaves of *Ginkgo biloba*

The signals of a 1,2,6-tri-substituted aromatic ring at 6.77, 6.87 and 7.36 ppm were observed in the ¹H-NMR spectrum of the compound. Furthermore, three protons of the methyl groups (triplet, J = 7.0 Hz) attached at the C1 3’ position at 0.87 ppm, twenty-four protons of methylene attached at the C1’-C12’ positions at 1.34-2.97 ppm and one hydroxyl group in carboxylic acid at the C1 position at 11.07 ppm were observed in the spectrum. The signal at 175.36 ppm on their ¹³C-NMR and the fragmentation peak at m/z 276 (M⁻CO₂) on their MS exhibited the presence of a carboxyl group. The carbon signal of one methyl group (C13’) at 14.11 ppm; methanediyl groups at 36.50 (C1’), 32.05 (C2’), 31.93 (C11’), 29.82 (C3’), 29.69 (C4’), 29.69 (C5’), 29.66 (C6’, C7’), 29.63 (C8’), 29.49 (C9’), 29.36 (C10’) and 22.69 ppm (C12’); three hydrocarbons (C3, C4 and C5) at 110.30, 122.74 and 135.42 ppm and one carbon of the hydroxyl group at 147.74 ppm, respectively, were observed. ¹H-NMR and ¹³C-NMR data coincided with the data reported by Irie *et al.* (1996) and Van Beek and Wintemans (2001). Based on its melting point, UV spectrum, EI-MS, MS of the TMS derivative, FAB-MS, and ¹H-NMR and ¹³C-NMR spectra strongly indicated that compound 1 was ginkgolic acid (C13:0), with the molecular formula C₂₀H₂₂O₅ as shown in Fig. 4.

The amounts of ginkgolic acid (C13:0) in the n-hexane fraction and methanol extract of *Ginkgo biloba* yellow leaves were higher (8.26 and 3.747%, respectively) than those in the green leaves (5.77 and 2.249%, respectively) and seed extracts (0.04 and 0.0002%, respectively), as shown in Table 2. The different α-glucosidase inhibitory activities observed in yellow and green leaves may be associated with their ginkgolic acid content.

Fraction 7 was examined as the active fraction using GC-MS and revealed a five compound mixture, as shown in Fig. 5. The EI mass spectrum of peaks 1, 2, 3, 4 and 5 of fraction 7 after the TMS derivative is shown in Fig. 6. The EI mass spectrum of peak 1 in fraction 7 showed a molecular ion peak at m/z 464. Peak 2 was analyzed by GC-MS which showed a molecular ion peak at m/z 490. Peaks 3 and 4 were analyzed by GC-MS after the TMS derivative and had molecular ion peaks at m/z 492 and 516, respectively. Peak 5 was analyzed using GC-MS after the TMS derivative and had a molecular ion peak at m/z 518.

The MS of the compound was consistent with the structure proposed by Cartayrade *et al.* (1990). According to the EI-MS spectrum analysis, peak 1, 2, 3, 4 and peak 5
contained in fraction 7 had molecular ion peaks at m/z (M⁺) 320, 346, 348, 372 and 374, respectively and were strongly indicated as ginkgolic acids C13:0 (C₂₀H₃₂O₃), C15:1 (C₂₀H₃₄O₃), C15:0 (C₂₀H₃₂O₃), C17:2 (C₂₀H₃₆O₃) and C17:1 (C₂₀H₃₄O₃), respectively. The five compounds of ginkgolic acids (C13:0, C15:1, C15:0, C17:2 and C17:1) contained in G. biloba leaves were also consistent with the findings of Fuzzati et al. (2003).

Fig. 5: GC-MS profile of several compounds in fraction 7

Fig. 6: Continue
Alpha-glucosidase inhibitory activity of the isolated compounds: The α-glucosidase inhibition assay on ginkgolic acid (C13:0) isolated from *G. biloba* yellow leaves and the mixture of ginkgolic acids revealed IC₅₀ values of 3.5±0.13 and 5.1±0.87 µg mL⁻¹ while that of quercetin as the positive control was 3.8±0.12 µg mL⁻¹, as shown in Table 3. Ginkgolic acid consists of both aromatic and aliphatic structures. The structure responsible for inhibiting α-glucosidase has not yet been determined. This inhibitory activity may be related to the presence of

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC₅₀ (µg mL⁻¹)</th>
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<tbody>
<tr>
<td>Ginkgolic acid (C13:0) in fraction 6</td>
<td>3.5±0.13</td>
</tr>
<tr>
<td>Ginkgolic acids mixture in fraction 7</td>
<td>5.1±0.87</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.8±0.12</td>
</tr>
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Table 3: α-Glucosidase inhibitory activities of ginkgolic acid (C13:0) in fraction 6 and ginkgolic acids mixture in fraction 7 isolated from *Ginkgo biloba* yellow leaves.

Fig. 6(a-e): El Mass spectra of ginkgolic acid mixture in fraction 7 after TMS derivatization (a) C13:0, (b) C15:1, (c) C15:0, (d) C17:2 and (e) C17:1.
a hydroxyl group and carboxylic acid group. Hydroxyl groups (R = OH) have been reported to play an important role in α-glucosidase inhibitors (Zhang et al., 2009; Kuang et al., 2011) due to hydrogen bonding and electrostatic interactions between hydroxyl moieties and the binding pocket of α-glucosidase (Uddin et al., 2012). Based on the structure-activity relationship of pentacyclic triterpenes, the carboxylic acid group (R = COO⁻) has been shown to play an important role in the optimum activity of α-glucosidase inhibitors and replacing hydrogen with glycoside (R = COO-Glycoside) can decrease this inhibitory activity (Uddin et al., 2012).

Ginkgolic acids have been shown to contain possible hazardous constituents which have immunotoxic properties (Koch et al., 2000; Liu and Zeng, 2009). This experiment revealed that ginkgolic acids were potent α-glucosidase inhibitors. Baron-Ruppert and Lupeke (2001) reported that biflavones or some other constituents in the different fractions may amplify the adverse effects of these substances. However, a limit has yet to be set on the level of these compounds in ginkgo preparations (Lepoittevin et al., 1989; Hauser, 1998). A limit of 5 ppm as a maximum concentration of ginkgolic acids in G. biloba extracts has been set in the draft monographs, of Kramer et al. (1990) and EMA (2014). Although, further detailed investigations are required for more extensive biological evaluations to elucidate plausible mechanisms of these inhibitions before bringing the isolated ginkgolic acid into commercial use, it is hoped that these preliminary observations will provide the basis information of G. biloba as a medicinal supplement that contributes toward the treatment and prevention of diabetes.

CONCLUSION

Ginkgo biloba was determined to have α-glucosidase inhibitory activity. Bioassay-guided fractionation of the yellow leaf extracts yielded the active compounds. The active compounds isolated from Ginkgo biloba were identified as a ginkgolic acid (C13:0) and a mixture (C13:0, C15:0, C15:1, C17:1 and C17:2). However, ginkgolic acid (C13:0) was isolated as a main compound exhibiting the highest α-glucosidase inhibitory activity with an IC₅₀ value of 3.9 μg mL⁻¹.

REFERENCES


