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RESEARCH ARTICLE

Anti-hyperglycaemic effects of the Japanese red maple *Acer pycnanthum* and its constituents the ginnalins B and C

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Abstract

The anti-hyperglycaemic effects of the leaves of *Acer pycnanthum* K. Koch, and the purification and identification of the active compounds were investigated. Extracts of the leaves showed a potent inhibitory effect on the α-glucosidase in both *in vivo* and *in vitro* experiments. The fractionation of the crude extract gave two active compounds, ginnalin B (6-*O*-galloyl-1,5-anhydro-D-glucitol) and ginnalin C (2-*O*-galloyl-1,5-anhydro-D-glucitol), by spectroscopic analysis. This is the first report that *A. pycnanthum* and its constituents may be useful for the prevention or treatment of diabetes mellitus.

Keywords: Acer pycnanthum, diabetes mellitus, α-glucosidase inhibitory effect, ginnalin B, ginnalin C

Introduction

As a possible approach to the treatment of diabetes, decreasing postprandial hyperglycaemia has been proposed [1]. Different types of anti-hyperglycaemic drugs are conceivable [2]. By inhibiting carbohydrate hydrolysis enzymes such as α-glucosidase and α-amylase in the digestive tract, we can delay carbohydrate digestion. We could also reduce postprandial hyperglycaemia by impairing glucose production and diminishing glucose absorption from the small intestine. Such an inhibitory action has been shown to be one of the most effective approaches for preventing diabetes in recent clinical studies [3]. Thus, it is believed that an anti-hyperglycaemic effect caused by the inhibition of α-glucosidase may be important for diabetes care. Worldwide, many kinds of herbs are used for diabetic care [4,5] and in medicinal therapy for diabetes. To identify new compounds that may be useful for preventing or treating diabetes, we screened many kinds of natural products that exert an anti-hyperglycaemic effect and identified foods that may be useful for diabetic care, as described previously [6–8]. As part of these ongoing experiments, we found that an extract from the leaves of *Acer pycnanthum* had an anti-hyperglycaemic effect.

A. pycnanthum belongs to *Aceraceae* and is called "Hana-noki" in Japan. The trees grow wild in the Aichi, Gifu, Nagano, and Shiga prefectures in Japan [9] and are planted in gardens and by the roadside. The genus *Acer* includes more than 200 species that are distributed mainly in the temperate zone of the northern hemisphere [10]. In the autumn, the leaves change colour from green to yellow or red.

Acer nikoense Maxim is used in Japan as a folk medicine for the eyes and some of its constituents have been reported together with their actions [11,12]. *A. pycnanthum* is also used as a folk medicine for eye care [13]. Other pharmacological effects of *Acer* genus have also been reported [14,15]. However, the phytochemical constituents of *A. pycnanthum* and their biological activities have not yet been reported.

In this study, we found that the extract of the leaves of *A. pycnanthum* had an anti-hyperglycaemic effect and we have elucidated the mechanism based on an inhibitory effect toward α-glucosidase. We also identified ginnalin B (6-*O*-galloyl-1,5-anhydro-D-glucitol) and ginnalin C (2-*O*-galloyl-1,5-anhydro-D-glucitol) in the extract.

Materials and Methods

Preparation of sample extract

The methanol extract of the leaves of *A. pycnanthum* (APM) was obtained as described below. The leaves were

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collected in July and extracted with a 10-fold volume of methanol at room temperature for 2 days. After filtration, the extract was evaporated by a rotary evaporator. The recovery rate for the APM was 15% of the weight of the fresh leaves.

Animals

Male ddY mice (6–8 weeks of age) were purchased from SLC, Shizuoka, Japan. The room was maintained at 24 ± 1°C and 50 ± 10% humidity under a 12 h light/dark cycle (lights on from 8:00 AM to 8:00 PM), and the animals had free access to water and food. Animal studies were conducted according to the 2006 guidelines entitled Notification No 88 of the Ministry of the Environment in Japan and Guidelines for Animal Experimentation of Tokyo University of Marine Science and Technology with the approval of the Animal Care and Use Committee of Tokyo University of Marine Science and Technology

Assay for inhibitory activity against α-glucosidase

Intestinal α-glucosidase inhibitory activity was determined as described in the literature with some modification [16]. Fasted ddY male mice were sacrificed and the small intestines were immediately removed. The contents of the small intestines which included brush border enzymes were squeezed out and used to prepare samples at 25 mg/mL. Ice-cold phosphate buffer (pH 6.9) containing 0.2% bovine serum albumin was used as a solvent. The samples were centrifuged at 4500 × *g* for 5 min at 4°C. The supernatant was used as a crude enzyme solution in the experiment.

The activity of α-glucosidase was assayed with 27.7 mM maltose or 55.3 mM sucrose (final concentrations) with or without APM at different concentrations. Acarbose was used as a positive control. After the samples and substrate were pre-incubated for 5 min at 36.5°C, the enzyme solution was then added. The samples were then incubated at the same temperature for 20 min for maltose, or 40 min for sucrose. The reaction was terminated by heating with boiling water for 5 min, and the quantity of glucose produced was measured with a commercial GOD-POD kit (Glucose C II-test, WAKO Pure Chem, Osaka, Japan). The reaction rate (%) was calculated and the IC_{50} value of each sample was determined.

Carbohydrate-loading tests in mice

The mice were fasted for 18 h and divided into groups according to body weight ($n = 6$). Each sample was dissolved in water and administered orally. In the control group, mice were given sucrose (2,000 mg/kg body weight) or glucose (1,000 mg/kg body weight). In the APM-treated group, mice were given a mixture of 100 mg/kg body weight of APM or, as a positive control, 3 mg/kg of acarbose, which is known to be a major inhibitor of α-glucosidase and each carbohydrate. Blood from the tail vein was collected sequentially in test tubes and centrifuged at 4,500 × *g* for 2 min. The serum was extracted and the serum glucose level was measured. The serum glucose level was measured before the administration, and at 30, 60, 90, and 120 min after administration. The serum glucose level was measured by GOD-POD kit (Glucose C II-test, WAKO Pure Chem).

Statistical analysis

Data are presented as the mean ± S.E. The significance of differences was calculated using Student's *t*-test, and the results were considered statistically significant when $p < 0.05$.

Spectroscopic analysis

To determine the chemical structures of the purified compounds, NMR spectra were recorded with a 400 MHz NMR (AV-400, Bruker Biospin, Yokohama, Japan) and MS spectral data were obtained on an ESIMS spectrometer (microOTOF-QII, Bruker).

Results

Effect of APM on α-glucosidase activity

To confirm the effects of APM on α-glucosidase activity, *in vitro* experiments were performed with sucrose or maltose as a substrate. The IC_{50} (μg/mL) values of APM and acarbose against sucrase activity were 38.5 ± 5 and 0.5 ± 0.1, respectively. Against maltase these were 180.5 ± 5.1 and 0.8 ± 0.2, respectively.

Effect of APM on the serum glucose level in carbohydrate-loaded mice

Figure 1 shows the anti-hyperglycaemic effects of APM in sucrose (A)- and glucose (B)-loaded mice. The serum

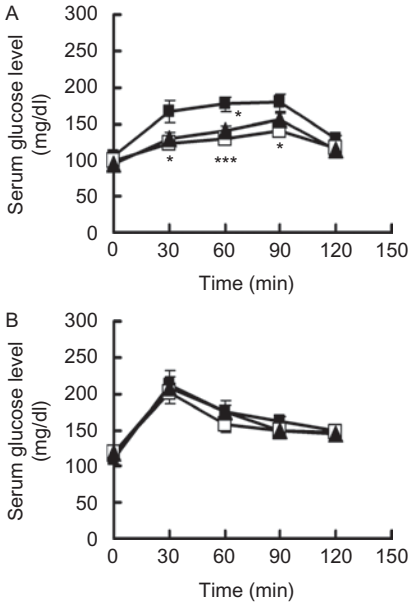

Figure 1. Anti-hyperglycaemic effects of APM in mice. Fasted mice were given 2,000 mg/kg of sucrose (A), 1,000 mg/kg of glucose (B), with or without 100 mg/kg of APM (methanol extract of *Acer pycnanthum*: open squares), 3 mg/kg of acarbose (closed triangles) and vehicle (control: closed squares), Data are presented as the mean ± S.E. ($n = 6$). ***; $p < 0.005$, *; $p < 0.05$ versus the control.

glucose level in the APM group was significantly lower than that of the control group. In the sucrose-loading test, the serum glucose levels at 30, 60 and 90 min after administration in the APM group were 121.9 ± 2.4, 129.1 ± 4.6, and 140.9 ± 4.1 mg/dl, while in the control group these values were 165.6 ± 15.2, 177.1 ± 9.8, and 178.5 ± 12.2 mg/dl, respectively. The administration of 100 mg/kg APM had a suppressive effect on hyperglycaemia that was similar to that found with the administration of 3 mg/ kg acarbose. On the other hand, there was no significant difference in the glucose-loading test.

Purification of the active ingredients from APM

APM was fractionated as described below to give two active compounds. APM (3 g) was partitioned between 90% methanol and hexane. The 90% methanol layer was chromatographed on an octodecyl silane (ODS) column (Wakogel, WAKO Pure Chem) with a water-methanol gradient and an ethyl acetate elution to give three fractions. The active fraction, Fraction 1 (205 mg) eluted with 40% methanol was subjected to HPLC, using an ODS column (Develosil HG-5 20 × 250 mm; Nomura Chemical, Aichi, Japan) with 30% methanol at a flow rate of 4 mL/min, detected at UV 215 nm to give five fractions. The active fraction, Fraction 1-2 was further purified by the same HPLC system except using a more polar mobile phase 10% methanol and then divided into two fractions to give Fraction 1-2-1 (**1**, 2.4 mg, t_R: 34 min) and Fraction 1-2-2 (**2**, 1.2 mg, t_R: 37 min) as the active compounds.

Identification of the structures of 1 and 2

Ginnalin B, 6-*O*-galloyl-1,5-anhydro-D-glucitol (**1**) was isolated as a reddish brown crystal. The molecular formula of **1** was determined to be $C_{13}H_{16}O_9$ [(M+H)$^+$, *m/z*: 317.0868, δ -0.1 mmu] by HR-ESIMS (positive mode). Spectroscopic data for **1**; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.8 (2H, *s*, galloyl-H), 4.51 (1H, *dd*, *J* = 11.9, 1.1 Hz, H-6a), 4.3 (1H, *dd*, *J* = 11.9, 5.3 Hz, H-6b), 3.92 (1H, *dd*, *J* = 10.9, 5.2 Hz, H-1a), 3.43 (1H, *m*, H-5), 3.38 (1H, *m*, H-4), 3.35 (1H, *m*, H-2), 3.32 (1H, *t*, *J* = 10.8 Hz, H-3), 3.2 (1H, *t*, *J* = 10.9 Hz, H-1b); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 168.9 (C-7'), 147 (C-3' and C-5'), 140.5 (C-4'), 121.8 (C-1'), 110.7 (C-2' and C-6').

Ginnalin C, 2-*O*-galloyl-1,5-anhydro-D-glucitol (**2**) was isolated as a reddish brown crystal. The molecular formula of **2** was determined to be $C_{13}H_{16}O_9$ [(M+H)$^+$, *m/z*: 317.0869, δ -0.2 mmu] by HR-ESIMS (positive mode). Spectroscopic data for **2**; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.8 (2H, *s*, galloyl-H), 4.85 (1H, *dd*, *J* = 10.4, 5.4 Hz, H-2), 4.08 (1H, *dd*, *J* = 10.8, 5.4 Hz, H-1a), 3.85 (1H, *dd*, *J* = 11.6, 1.4 Hz, H-6a), 3.65 (1H, *dd*, *J* = 11.6, 6.2 Hz, H-6b), 3.63 (1H, *dd*, *J* = 6.2, 5.4 Hz, H-3), 3.5 (1H, *m*, H-4), 3.28 (1H, *m*, H-1b), 3.22 (1H, *m*, H-5); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 168.3 (C-7'), 146.9 (C-3' and C-5'), 140.4 (C-4'), 121.6 (C-1'), 110.6 (C-2' and C-6').

The similarities of the spectral data for **1** and **2** revealed that the two compounds were isomers of each other. NMR data showed that both compounds possessed a galloyl moiety and a 1-deoxyglucose moiety. The heteronuclear multiple bond coherence (HMBC) correlations to the carbonyl carbon of the galloyl moiety differed between **1** and **2**. As a result, **1** was determined to be 6-*O*-galloyl-1,5-anhydro-D-glucitol (ginnalin B) based on δ_H 4.49 and 4.24 (H-6a and 6b) and **2** was determined to be 2-*O*-galloyl-1-,5-anhydro-D-glucitol (ginnalin C) based on δ_H 4.65 (H-2). Other spectral data correspond to those in a previous report [17]. These structures are shown in Figure 2.

Effects of ginnalins B and C on sucrase activity

Ginnalins B (**1**) and C (**2**) exerted an sucrase-inhibitory effect. The IC_{50} values of **1** and **2** were calculated to be 79.8 ± 5.6 and 42.1 ± 2.9 μg/mL, respectively.

Kinetics analysis of sucrase inhibition by ginnalins B and C

In order to examine the type of inhibition of ginnalins, the sucrase activities were measured with increasing concentrations of sucrose (6.91–110.67 mM) with or without **1** or **2**. The amount of protein was determined by the Bradford protein assay kit (Pierce Biotech, IL, USA). One unit of enzyme activity was taken as the amount of enzyme which liberates one micromole of glucose from sucrose under the experimental conditions. The mode of inhibition by **1** and **2** were assessed by a Lineweaver-Burk plot analysis. The results revealed that **1** and **2** inhibited sucrase activity in a mixed competitive manner (Figure 3).

Effects of ginnalins B and C on serum glucose levels in sucrose-loaded mice

Both compounds (24 mg/kg) isolated from APM were used in an *in vivo* experiment in sucrose-loaded mice (2,000 mg/ kg). As shown in Figure 4, the serum glucose levels of in the treated groups were significantly lower than that in the control group at 30 min. In the ginnalin B and C group, each value was 223.7 ± 10.1, 196.4 ± 8.8 respectively, while in the control group it was 274.2 ± 10.9 for the equivalent time.

Discussion

This report is the first to show that *Acer pycnanthum* contains ginnalins B (**1**) and C (**2**), which exert anti-hyperglycaemic effects through a mechanism that involves the inhibition of α-glucosidase.

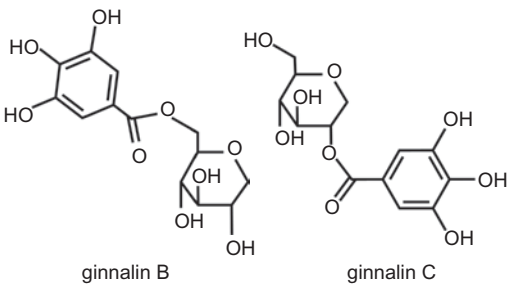

Figure 2. Chemical structures of ginnalins B and C.

As revealed in Figure 1, APM showed an anti-hyperglycaemic effect in a sucrose-loading test with the oral administration of APM (100 mg/kg) to the same degree as with the oral administration of acarbose (3 mg/kg), which is a major α-glucosidase inhibitor. However, APM did not have a similar effect in a glucose-loading test. Thus, the anti-hyperglycaemic effect of APM is due to an inhibitory effect on α-glucosidase, which is a disaccharide hydrolysis enzyme, and an *in vitro* test showed such an effect. In fact, both *in vivo* and *in vitro,* APM showed the same degree of anti-hyperglycaemic effect and α-glucosidase-inhibitory effect as an extract of mulberry (*Morus alba*) leaf, which is a medicinal herb that is used to treat diabetes (data not shown) [18].

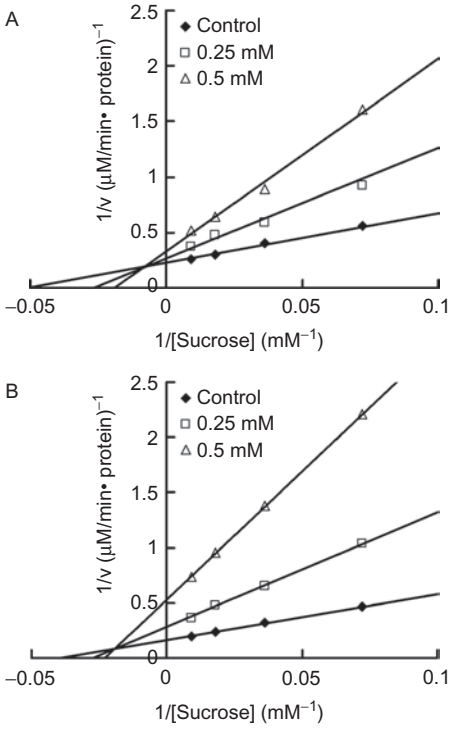

Figure 3. Kinetics analysis of sucrase inhibition by ginnalins B and C. Linewever-Burk plots for inhibitory activity of ginnalin B (A) and C (B) on intestinal sucrase.

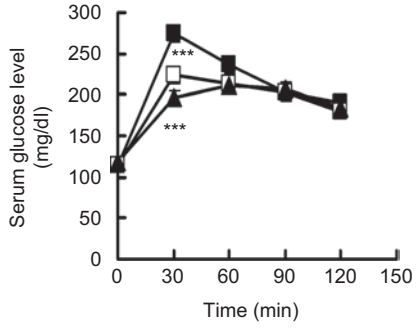

Figure 4. Anti-hyperglycaemic effects of ginnalins B and C in sucrose-loaded mice. Fasted mice were given 2,000 mg/kg of sucrose with 24 mg/ kg of ginnalin B (open squares) or 24 mg/kg of ginnalin C (closed triangles) and vehicle (control: closed squares). Data are presented as the mean ± S.E. (n = 8). ***; p <0.005 versus the control.

We purified two active ingredients from APM and a spectroscopic analysis of their structures revealed that these compounds were **1** and **2**, as shown in Figure 2. And both compounds also showed sucrase-inhibitory effect and had an anti-hyperglycaemic effect in a sucrose-loading test as shown in Figure 4. Compared with the IC_{50} values of APM and **1** or **2**, each compound showed less sucrase-inhibitory action. The recovery rate of **1** and **2** purified from APM were 0.08 and 0.04%, respectively. Therefore **1** or **2** may not be the core of the active compound and the other compounds may also contribute to the inhibitory effect of APM on α-glucosidase.

Conpounds **1** and **2** were previously isolated from the leaves of *Acer ginnala* Maxim by Song et al. [17] and used as antibacterial compounds, *in vitro*, toward Flexner's dysentery bacillus, Sonne dysentery bacillus and *Aurococcus* [19]. *A. ginnala* has also been used as a folk medicine in Korea as a stomachic and for the treatment of diarrhea [20].

With regard to **1** and **2**, it has been reported that some galloyl derivatives have shown α-glucosidase-inhibitory activity, such as epigallocatechingallate from tea (*Camellia sinensis*) [21] and ellagitannins from clove (*Syzgium aromaticum*) [22]. Gallotannins show high affinity for sugars [23], and gallotannin and sugars may form hydrogen bonds through the galloyl part of these compounds in the small intestine, and this may prevent their absorption. In addition, the galloyl part may inhibit enzymes through a high affinity for proteins [24]. And these tannins inhibit α-glucosidase in an uncompetitive manner [21,25]. It is also conceivable that the 1-deoxyglucose part of **1** or **2** may imitate glucose and mimic a disaccharide. This may explain the inhibitory effects of these two compounds [26,27] in a competitive manner. Therefore **1** and **2** may inhibit sucrase in a mixed competitive manner (Figure 3).

Some reports have shown that plants in the *Acer* genus contain galloyl derivatives, such as gallotannin and epigallocatechin gallate [28,29]. There have been some reports on the constituents of the genus. These have included flavonoid structures, such as quercetin [30], vitexin [31], afzelin, and quercitrin [32]. These constituents have an α-glucosidase-inhibitory effect [33,34] and some interesting bioactivities, such as anti-oxidative, anti-tumour, and anti-inflammatory effects. In one report on the constituents of *Aceraceae*, the contents of anthocyanins in the leaves were increased in spring and autumn. In particular, for *A. pycnanthum*, cyanidin 3-glucoside, 3-rutinoside, 3-galloylglucoside, 3-galloylrutinoside, and 3,5-diglucoside have been identified [35]. Cyandin 3-sambubioside [36] and cyanidin 3-galactoside [37] have been reported to have inhibitory effect of α-glucosidase. Therefore, these compounds may also contribute to the inhibitory effect of APM on α-glucosidase.

Conclusion

Acer pycnanthum has a potent anti-hyperglycaemic effect and an α-glucosidase inhibitory effect. The active

compounds were purified and identified as ginnalins B and C. It is possible that *A. pycnanthum* and its constituents may be useful for the prevention and treatment of diabetes mellitus.

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Declaration of interest

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