Comparison of α-glucosidase inhibition by *Cudrania tricuspidata* according to harvesting time

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Abstract. *Cudrania tricuspidata* (CT) is a type of add-value beneficial plant. The aim of the present study was to determine the components of CT that inhibit α-glucosidase activity. Roots, leaves and stems of the plants were obtained and several subgroups were created according to harvesting time. Root extracts exhibited a 77% velocity inhibition at a concentration of 300 μg/ml and an inhibitory constant of 41.6 μg/ml. The inhibitory percentage of the positive control at 1 mM was ~67% of the enzymatic velocity with acarbose. According to the Michaelis-Menten equation, the type of inhibitory mechanism underlying the effects of the stem and root samples according to climate was competitive or non-competitive inhibition, suggesting that the extracts contain additional antidiabetic compounds produced during the growth period. Collectively, the results from our study suggested that stem and root extracts of CT serve as an antidiabetic biomaterial and contain a variety of antidiabetic compounds.

Introduction

Following food ingestion, carbohydrates are converted into glucose by several enzymes. Among these, α-glucosidase is produced by intestinal cells or tissues and cleaves glycosidic bonds in oligosaccharides during the last step of hydrolysis (1). Glucose is the main component of blood sugar. Therefore, α-glucosidase may be an ideal target for the treatment of type 2 diabetes mellitus (2). To investigate antidiabetic properties for nutraceutical purposes, this enzyme may also be used as a marker for *in vitro* assays, since the α-glucosidase inhibitor induces hypoglycemic symptoms less frequently compared to other oral glucose-lowering agents (3).

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Cudrania tricuspidata (CT) belongs to the Moraceae family and is distributed throughout Korea, Japan and China (4). Ethanol extracts of CT contain numerous compounds, including butyrospermyl acetate, glutinol, taraxerone, quercetin, kaempferol, isorhamnetin, orobol, 3'-O-methyorobol, 1,3,6,7-tetrahydroxyxanthone, taxifolin, naringenin, steppogenin and 5,7-dihydroxy chromone (5). Various effects of CT, such as tyrosinase inhibition (6), anti-oxidative activity (7) and anti-inflammatory activity (8) have been investigated. Its active ingredients have also been isolated and they include prenylated xanthones (mainly cudraxanthone) and cudraflavone (9). Although isolated CT compounds have already been proven to possess antidiabetic properties using α-glucosidase inhibitory assays (2), studies on the potential activity of CT from various sources have not yet been performed.

Recently, the consumption of crude CT extract in Korea was abruptly increased due to its potential benefits as a traditional complementary therapy. However, information regarding the functional activities of different plant components according to harvesting time has not yet been obtained. Therefore, additional studies are required to optimize the commercial preparation of CT extracts. In the present study, CT samples were divided according to plant component and harvesting period and extracts were prepared. The antidiabetic activities of the extracts were then analyzed using an α-glucosidase inhibitory assay.

Materials and methods

Reagents. α-glucosidase type 1 from baker's yeast (G5003; Sigma-Aldrich, St. Louis, MO, USA), *p*-nitrophenyl α-D-glucopyranoside (N1377, Sigma-Aldrich), sodium phosphate monobasic (S3139, Sigma-Aldrich), sodium phosphate dibasic (S5136, Sigma-Aldrich), filter paper (no. 1; Whatman Schleicher & Schuell, Keene, NH, USA), xanthone (95502; Fluka, Buchs St. Gallen, Switzerland) and acarbose (A8980, Sigma-Aldrich) were purchased for the purposes of this study.

Preparation of test material. The CT samples were obtained in Hampyeong, Korea, where the plants are collected on a monthly basis throughout the year. The plants were separated into leaves, roots and stems prior to being further

Table I. Determination of inhibitor constants for the *Cudrania tricuspidata* (CT) extracts.

Materials tested	K_m (mM)	K_m' (mM)	V_{max}	V_{max}'	K_i
CT 1	0.431	-	0.0805	0.0518	125.4±2.1
CT 3	0.489	0.884	0.0898	-	115.1±1.1
CT 4	0.376	-	0.0575	0.0416	221.3±4.7
CT 5	0.548	0.588	0.0716	-	1,694.1±12.5
CT 6	0.417	-	0.0717	0.0258	41.6±0.5
Acarbose	0.416	0.401	0.0716	0.1271	220.0±7.0

The inhibitor units were μg/ml for CT and mM for acarbose. K_m, Michaelis constant without inhibitor; K_m', Michaelis constant with 100 μg/ml CT extracts or 0.3 mM acarbose; V_{max}, maximum velocity of enzymatic activity without inhibitor; V_{max}', maximum velocity of enzymatic activity with 100 μg/ml CT extracts or 0.3 mM acarbose; K_i, inhibitory constants calculated with formulas derived from the Dixon plot analysis. Standard deviations for the K_i value were determined based on the intersection of the trend lines in the Dixon plot. Data were obtained from experiments performed in triplicate.

subdivided into groups according to harvesting time. The samples underwent aqueous extraction for 2.5 h, were filtered with filter paper and lyophilized with a freeze dryer (IlShin Biobase Co., Ltd., Dongducheon, Korea). The lyophilized samples were dissolved in distilled water as 100 mg/ml stock and diluted with distilled water prior to the experiment. Nine CT samples were prepared: stems from plants harvested between late April and early May, 2009 (CT 1); stems harvested in middle June (CT 2); stems harvested between late July and early August (CT 3); stems harvested in middle September (CT 4); stems harvested in middle January (CT 5); roots and stems harvested in middle January (CT 6); leaves harvested in middle June (CT 7); leaves harvested between late July and early August (CT 8); and leaves harvested in middle September (CT 9).

Concentration of α-glucosidase and substrate. Sodium phosphate buffer (0.1 M) was adjusted by 0.1 N HCl to pH 7.0 with a pH meter (Thermo Fisher Scientific Inc., Waltham, MA, USA). *p*-Nitrophenyl α-D-glucopyranoside (10 mM) and α-glucosidase solutions (1 U/ml) were solubilized in 0.1 M sodium phosphate buffer (pH 7.0). All the reagents were manufactured shortly before use and warmed to 37˚C in a water bath.

α-glucosidase inhibition assay. Sodium phosphate buffer (0.1 M, 158 μl per well) was added to a 96-well plate. α-Glucosidase (20 μl) and 2 μl of sample were added to 20 μl of *p*-nitrophenyl α-D-glucopyranoside. In the 200-μl final reaction volume (0.02 U/well, 0.1 U/ml) the substrate concentration was adjusted to 10 mM. The background signal due to the sample color was measured at 405 nm with the PerkinElmer Wallac Victor3 spectrophotometer (PerkinElmer, Waltham, MA, USA) prior to adding the enzyme. Immediately following α-glucosidase addition, absorbance was measured at 405 nm 8 times at 1-min intervals (10,11).

Creation of Lineweaver-Burk and Dixon plots. To predict whether the extracts contained similar patterns of compositions, a Lineweaver-Burk plot was created according to the Michaelis-Menten equation (12) together with a Dixon plot. This was performed instead of purifying, isolating and analyzing the active compounds by high-pressure liquid chromatography or thin layer chromatography. The protocol for obtaining data to create the Lineweaver-Burk plot was identical to that used for the α-glucosidase inhibitory assay. The α-glucosidase concentration was 0.1 U/ml, whereas the substrate was added at three concentrations: 0.1, 0.3, and 1.0 mM. The mode of inhibition was defined as competitive, non-competitive or mixed-type non-competitive, according to the Michaelis-Menten constants and the maximum velocity on the Lineweaver-Burk plot (13).

Results and Discussion

In the course of screening active antidiabetic agents, several medicinal plants were selected. The ability of CT extracts derived from nine different plant parts to inhibite α-glucosidase activity was investigated. As shown in Fig. 1, CT 1, CT 3 and CT 6 exhibited a significant inhibitory activity in a concentration-dependent manner. A kinetic study was performed to assess the effects of climate on the antidiabetic activity of the plant components, since functional food manufacturers are interested in identifying the active component concentrations during the growth of the leaves, stem and bark of the CT plant. To compare the inhibitory activities of the samples, acarbose was selected as positive control. With 1 mM of acarbose, the enzymatic velocity was decreased by ~67% (Fig. 1). Similar to acarbose, almost all the extracts were associated with the same pattern of enzymatic velocity decrement, which was concentration-dependent. The most effective sample was demonstrated to be CT 6 (derived from the root of plants collected in middle January). Specifically, 300 μg/ml of this sample inhibited the enzymatic velocity by 77% (Fig. 2). By contrast, leaf extracts (300 μg/ml) exhibited marginal to no inhibition of the enzymatic activity (22% for CT 7, 8% for CT 8 and 0% for CT 9; Fig. 2). In a recent study (12), the majority of xanthone derivatives were reported to have activities similar to that of α-glucosidase. However, it

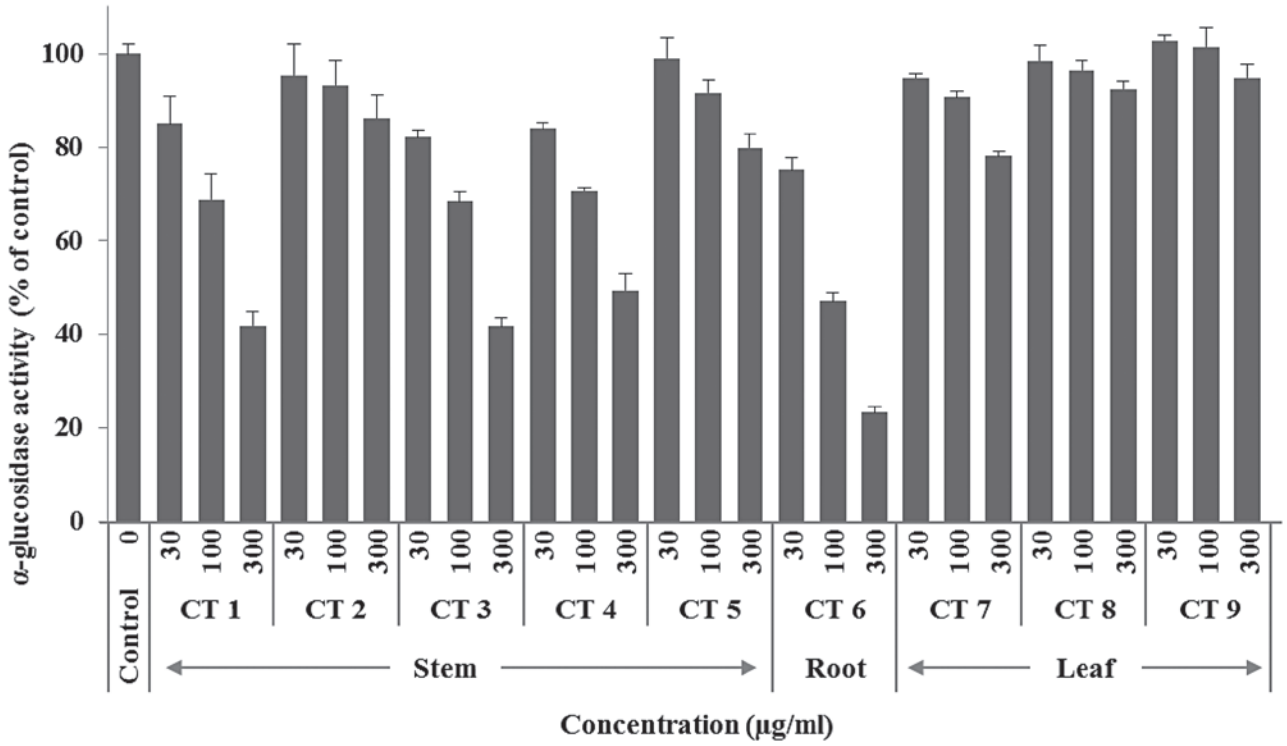

Figure 1. Comparison of α-glucosidase inhibition of the extracts according to harvesting time. Activities of the *Cudrania tricuspidata* (CT) extracts were measured according to the average increases of optical density. Data were calculated compared to the untreated sample and the experiment was performed in triplicate.

was observed that xanthone, an organic compound with the molecular formula $C_{13}H_8O_2$ that does not form derivatives, did not exert any obvious effect on α-glucosidase activity (data not shown).

The type of bioactive compounds present in the CT extracts and the composition changes in association with plant growth through the year were then determined. Five samples were selected and a Lineweaver-Burk plot was created based on the reciprocals of four different concentrations and the corresponding enzymatic velocities. As shown in Fig. 2, enzyme activities were reduced by the CT extracts in a dose-dependent manner and increased with substrate in a concentration-dependent manner. According to the Michaelis-Menten equation (1), the samples were classified according to the inhibition mode. The results were as follows (Fig. 3): CT 1, CT 4 and CT 6 as non-competitive inhibitors, CT 3 and CT 5 as competitive inhibitors and acarbose as a mixed-type non-competitive inhibitor. Our findings demonstrated that the stem extracts acted as non-competitive inhibitors, although one of them (CT 5) was classified as a competitive inhibitor. CT 6 exhibited the highest level of activity and was found to be a non-competitive inhibitor. Although the present data are not real activity values of CT, this approach is unique in assessing whether the extracts possess antidiabetic properties.

To analyze the mechanism underlying α-glucosidase inhibition, the inhibitor constant K_i was determined with a Dixon plot (Fig. 3). Based on the Michaelis-Menten equation, the Michaelis constant K_m value also was calculated. The inhibitory types were identified based on the Lineweaver-Burk plot. Inhibitor constants were represented by intersections of the lines as substrate condition on the Dixon plot (13). As shown in Table I, competitive inhibitors had only one V_{max} value whereas non-competitive inhibitors had one K_m value. The inhibitor constant of CT 6, the most effective inhibitor, was 41.6 μg/ml. The inhibitor constant of acarbose was 0.22 μM. A previous study by Seo *et al* (2) reported that xanthone derivatives isolated from CT exhibit potent α-glucosidase inhibitory activity. Hwang *et al* (9) also obtained xanthone derivatives from the root bark of CT. Those studies suggested that the potent inhibitory effect of the CT root is attributed to the abundant levels of xanthone derivatives. Acarbose was also reported to be a competitive inhibitor of α-glucosidase activity (3,14). However, the results of the present study demonstrated that acarbose acted as a mixed-type non-competitive inhibitor.

There were no differences observed in the inhibitory activities of CT 1-4 or CT 7-9 according to harvesting time. However, both stem and root extracts exerted potent inhibitory effects on α-glucosidase activity. Xanthone derivatives were previously isolated from the methanol fraction of the CT root and were demonstrated to inhibit α-glucosidase (2). In the present study, the aqueous fractions of the stem or root extracts were also found to possess potential inhibitory activities (data not shown). Our findings indicated that these fractions contain other compounds that are effective against α-glucosidase. In addition, the distribution of these compounds varied according to plant components and harvesting time. A previous study by Sen and Mukherji (15) reported that the carotenoid content of tomatoes undergoes

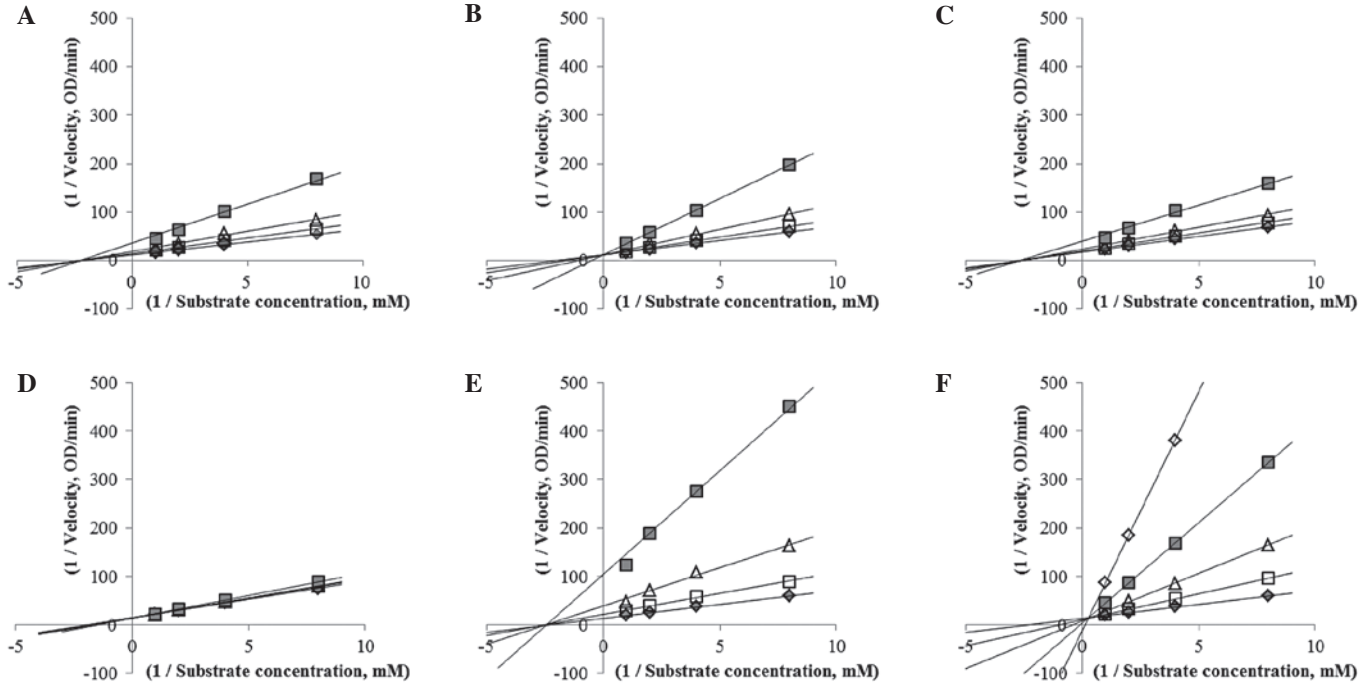

Figure 2. Comparison of α-glucosidase inhibition according to a Lineweaver-Burk plot. Plots were generated based on the Michaelis-Menten equation. (A) *Cudrania tricuspidata* (CT) 1, (B) CT 3, (C) CT 4, (D) CT 5, (E) CT 6, (F) acarbose. Concentrations of A-E (μg/ml): ■, 300; △, 100; □, 30; ◆, 0. Concentrations of acarbose (mM): ◊, 3; ■, 0.1; △, 0.3; □, 0.1; ◆, 0.

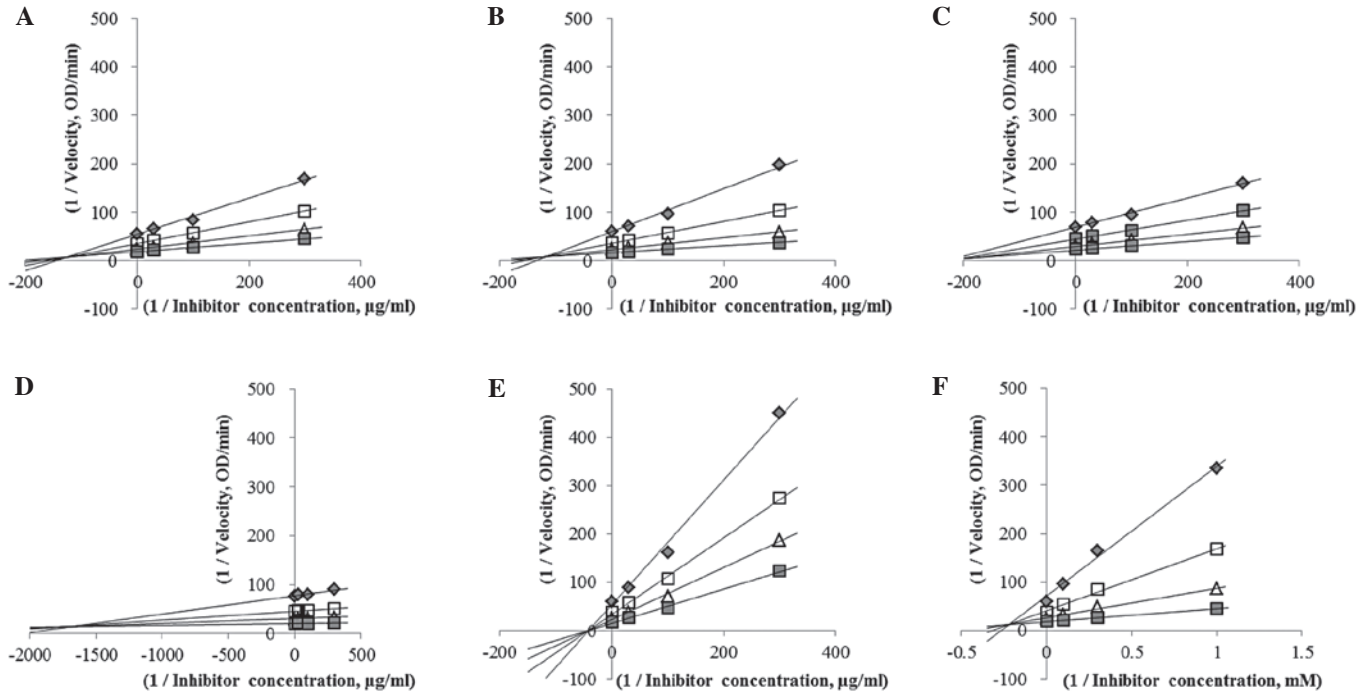

Figure 3. Comparison of α-glucosidase inhibition using a Dixon plot. The results are shown in the Dixon plot (Fig. 2). (A) *Cudrania tricuspidata* (CT) 1, (B) CT 3, (C) CT 4, (D) CT 5, (E) CT 6, (F) acarbose. Concentration of substrate (mM): ◆, 0.125; □, 0.25; △, 0.5; ■, 1.

seasonal variation. The results of their investigation demonstrated that carotenoid contents are highest in the winter and lowest during the rainy season. Therefore, it was hypothesized that CT 2, CT 8 and CT 9, which exhibited the lowest inhibitory activities among the stem and leaf samples, were affected by climatic conditions (Fig. 1). Since CT extract production in Korea is currently indiscriminate and in need of certification, our results may help define optimum conditions for the production of CT-containing foods or health beverages for antidiabetic purposes.

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