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ARTICLE

Yeast α-Glucosidase Inhibition by Isoflavones from Plants of Leguminosae as an in Vitro Alternative to Acarbose

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In the course of searching for new classes of α-glucosidase inhibitors originated from natural resources, 11 kinds of isoflavones, i.e., medicarpin (**1**), formononetin (**2**), mucronulatol (**3**), (3*R*)-calussequinone (**5**), (3*R*)-5′-methoxyvestitol (**6**), tectorigenin (**7**), biochanin A (**8**), tuberosin (**9**), calycosin (**10**), daidzein (**11**), and genistein (**12**), as well as a flavone, liquritigenin (**4**), were isolated as active principles responsible for the yeast α-glucosidase inhibitory activity from two leguminous plant extracts, i.e., the heartwood extract of *Dalbergia odorifera* and the roots extract of *Pueraria thunbergiana*. Each components (**1**−**12**) demonstrated a significantly potent inhibition on yeast α-glucosidase in a dose dependent manner when the *p*-nitrophenyl-α-D-glucopyranoside was used as a substrate in vitro. The concentration required for 50% enzyme inhibition (IC_{50}) were calculated as 2.93 mM (**1**), 0.51 mM (**2**), 3.52 mM (**7**) 0.35 mM (**8**), 3.52 mM (**9**), 0.85 mM (**11**), and 0.15 mM (**12**) when that of reference drug acarbose was evaluated as 9.11 mM, in vitro. However, isoflavone glycosides, i.e., puerarin (**13**), daidzin (**14**), formononetin-7-*O*-*β*-glucopyranoside (**15**), and genistin (**16**), exhibited a relatively poor inhibitory activity on yeast α-glucosidase as compared with the corresponding isoflavone (**2**, **11**, **12**), respectively

KEYWORDS: *Dalbergia odorifera*; *Pueraria thunbergiana*; Leguminosae; yeast α-glucosidase; isoflavone

INTRODUCTION

Diabetes mellitus is one of the most common and serious metabolic disorders, characterized by high blood-glucose levels which resulted from defects in insulin secretion, or action, or both. Insulin enables cells to absorb glucose in order to turn it into energy. However, in diabetes, the body either does not respond properly to its own insulin, does not make enough insulin, or both. This causes glucose to accumulate in the blood, often leading to various complications, such as blindness, kidney failure, or microvascular disease, which could lead to atherosclerosis, strokes, and other coronary heart disease (*1*, *2*)

alpha-Glucosidase (α-glucosidase) is an enzyme that catalyzes the exohydrolysis of 1,4-α-glucosidic linkages with release of α-glucose, which decreases the absorption of carbohydrates from the digestive tract, thereby lowering the after-meal glucose levels. For this reason, the α-glucosidase inhibitors are clinically used as oral antihyperglycemic agents, delaying intestinal carbohydrate absorption and lessening postprandial increases in glucose levels (*3*). Some α-glucosidase inhibitors, such as acarbose and voglibose, are currently used in combination with either diet or other antidiabetic agents to control blood glucose levels of patients. However, they often cause severe gastrointestinal side effects such as flatulence and diarrhea (*4*).

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Hence, natural α-glucosidase inhibitors from natural resources, especially from food sources, have become an attractive therapeutic approach for the treatment of postprandial hyperglycemia (*5*−*7*).

For the purpose of searching for new classes of α-glucosidase inhibitors, particularly from natural resources, 900 methanol extracts prepared from Korean medicinal plants were evaluated for the inhibitory effect on α-glucosidase obtained from *Saccharomyces cerevisiae* (EC 3.2.1.20) by a spectrophotometric method (*8*), with *p*-nitrophenyl-α-D-glucopyranoside as a substrate in vitro. Among the tested materials, 72 kinds of plant extracts were found to exhibit a significant inhibition on yeast α-glucosidase in a dose-dependent manner and the concentration of tested materials required for 50% enzyme inhibition (IC_{50}) were calculated as below 50 mg/mL, when the IC_{50} value of acarbose was evaluated as 5.3 mg/mL. In particular, 12 kinds of plant extracts in Leguminosae including the heartwood extract of *Dalbergia odorifera* (IC_{50}; 19.5 mg/mL), the roots extract of *Pueraria thunbergiana* (IC_{50}; 21.8 mg/mL), the flower extract of *Sophora japonica* (IC_{50}; 26.5 mg/mL), and the roots extract of *Glycyrrhiza glabra* (IC_{50} ; 28.1 mg/mL) demonstrated a potent inhibition on yeast α-glucosidase.

The species *Dalbergia odorifera* is a perennial tree that mainly grows in East Asian countries, and the heartwood of the species is frequently used in Traditional Chinese Medicine (TCM) for the treatment of blood stagnation syndrome, ischemia, swelling, necrosis, and rheumatic pain in China and Korea (*9*). Previous

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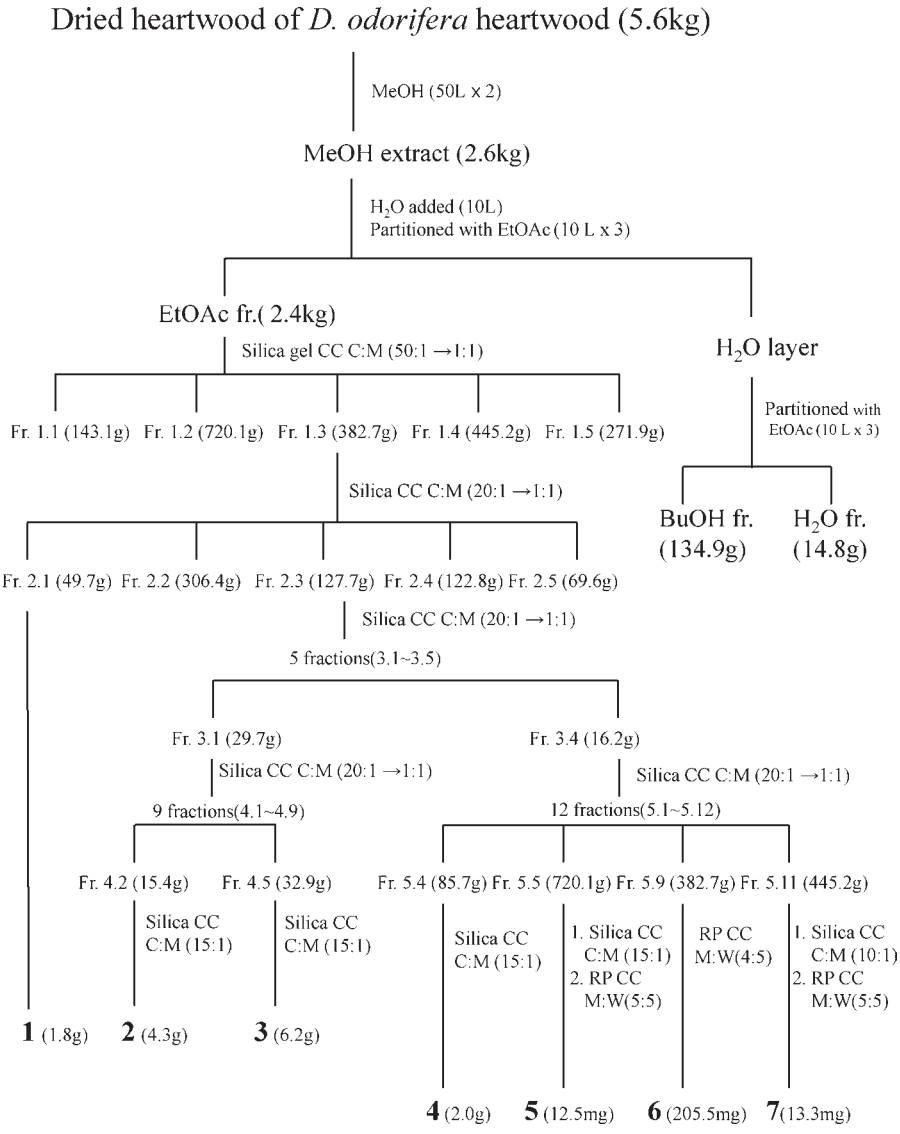


Figure 1. Isolation scheme of α-glucosidase inhibitors (**1**−**7**) from the heartwoods extract of *Dalbergia odorifera*. CC, column chromatography; RP, reversed phase; C, CH_2Cl_2; M, MeOH; W, H_2O.

phytochemical studies on the plant reported an isolation of flavonoids, quinones, and phenolic constituents (*10*−*12*). Some flavonoids found in the species have been reported to possess various pharmacological effects including anti-inflammatory, anticoagulant, antitumor, vasodilative, antihyperlipidemic, and antioxidant activities (*12*−*15*).

Another species, kudzu, *Pueraria thunbergiana*, is a climbing and trailing vine native to southeast China that spreads all over the world, and the roots of the species have been used as a famous food material and also as a traditional medicine in Korea, Japan, and China for the prevention and treatment of fevers, gastrointestinal disorders, skin problems, migraine headaches, lowering cholesterol, and treating chronic alcoholism in oriental medicine (*16*). It was also reported that some flavonoids of the species possessed antitumor, antioxidative, HMG-CoA reductase inhibitory, and estrogenic and antihelicobacter activity (*17*−*21*).

In the present study, we described the isolation of active principles (**1**−**12**) responsible for α-glucosidase inhibition from two leguminous plant extracts, i.e., the heartwood extract of *Dalbergia odorifera* and the roots extract of *Pueraria thunbergiana* as well as the inhibitory effect of them (**1**−**12**) and related glycoside compounds (**13**−**16**) on yeast α-glucosidase in vitro.

MATERIALS AND METHODS

General Procedures. MS spectra were measured on a Varian CP3800-1200 L (EI-MS), ^{1}H NMR (nuclear magnetic resonance), and ^{13}C NMR spectra were recorded on a Bruker (Rheinstetten, Germany) AM-300 and AMX 500 NMR spectrometer using tetramethylsilane (TMS) as an internal standard. Column chromatography was performed using a silica gel (Kieselgel 60, 70−230 mesh, Merck, Darmstadt, Germany), Sephadex LH-20 (25−100 mm, Pharmacia, Uppsala, Sweden), and Lichroprep RP-18 (40−63 mm, Merck). Thin layer chromatography (TLC) analysis was performed on Kieselgel 60 F_{254} plates (silica gel, 0.25 mm, Merck), and spots were detected under a UV lamp Spectroline Medel ENF-240 C/F (Spectronics Corporation, Westbury, NY) followed by 10% H_2SO_4 reagent. Acarbose (purity >95%) was purchased from Sigma. Solvents and reagents were obtained from commercial sources and used without further purification.

Plant Material. The heartwood of *D. odorifera* and the roots of *P. thunbergiana* was purchased from the TCM market in Daejeon, Korea, on September 2008. Voucher specimens (KR0035 and KR0262) were authenticated by Dr. Kyung Sik Hong and were deposited at the herbarium of Korea Research Institute of Chemical Technology, Korea.

Extraction and Isolation. The dried heartwood of *Dalbergia odorifera* (5.6 kg) were extracted twice with 10 volumes of MeOH by maceration at room temperature for 7 days. The MeOH solution was combined and evaporated to dryness to give 2.65 kg of dark syrupy extract, which was

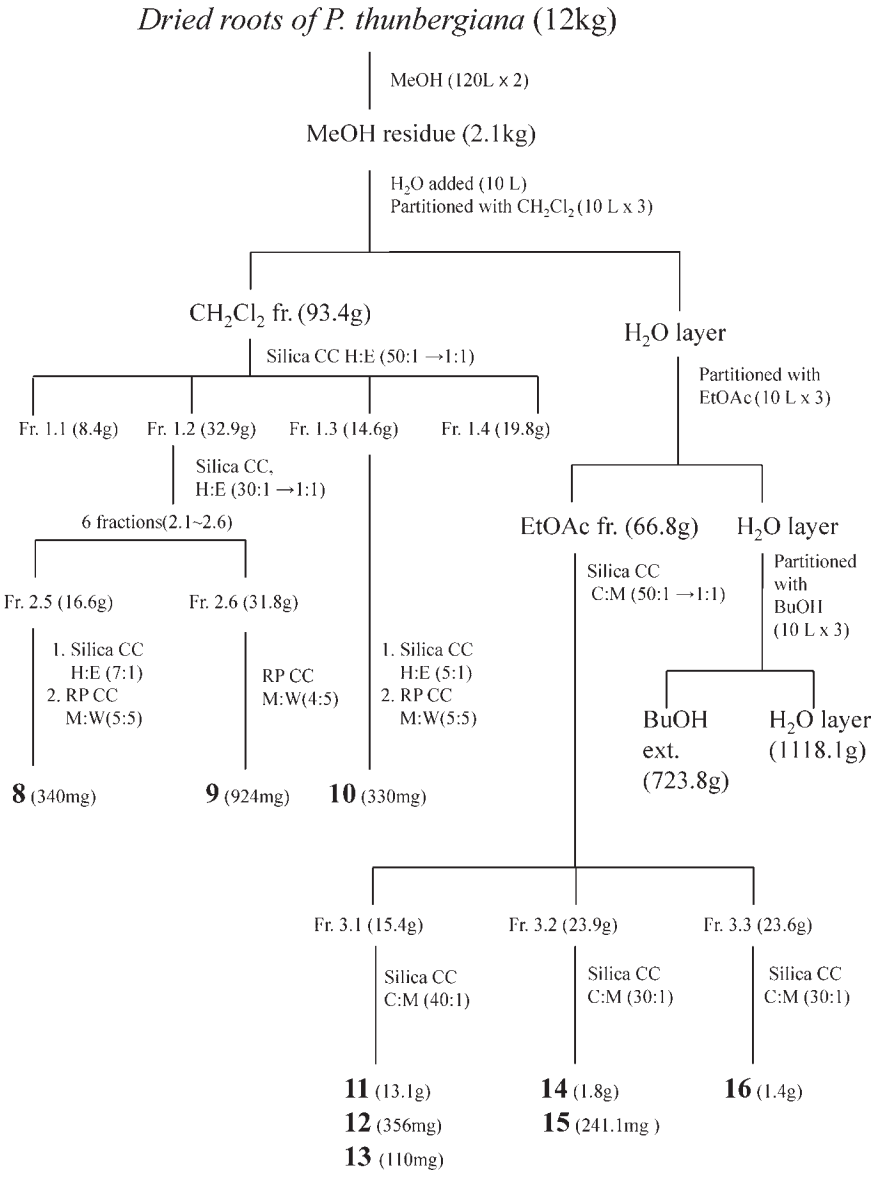


Figure 2. Isolation scheme of α-glucosidase inhibitors (**8**−**16**) from the roots extract of *Pueraria thunbergiana*. CC, column chromatography; RP, reversed phase; C, CH_2Cl_2; M, MeOH; W, H_2O.

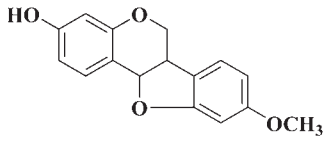
suspended in H_2O (10 L) and partitioned with an equal volume of EtOAc and *n*-BuOH, successively, which afforded 2.45 kg of EtOAc soluble fraction, 134.9 g of *n*-BuOH soluble fraction, and 14.8 g of aqueous fraction, respectively. The EtOAc soluble fraction (2450 g) was subjected to silica gel column chromatography (70−230 mesh), eluted with a gradient solvent system (MeOH in dichloromethane 1%/50%) to afford six fractions (Fr. 1.1−Fr. 1.6). The detailed purification process of active components (**1**−**7**) was performed by the flowchart scheme described in **Figure 1**.

Twelve kg of the dried roots of *P. thunbergiana* were extracted with MeOH by the same manner mentioned above, which resulted in 2.0 kg of MeOH extract. The MeOH extract was suspended in 10 L of H_2O and partitioned successively with an equal volume of dichloromethane, EtOAc, and *n*-BuOH, which afforded 93.4 g of dichloromethane soluble fraction, 66.8 g of EtOAc soluble fraction, 723.8 g of *n*-BuOH soluble fraction, and 1118.1 g of aqueous fraction, respectively. The dichloromethane soluble fraction (77 g) was subjected to silica gel (70−230 mesh) column chromatography, eluted with a stepwise gradient solvent system (MeOH in MC 1% to 50%) to afford four fractions (Fr. 1.1−Fr. 1.4). The EtOAc soluble fraction (50 g) was purified by repeated silica gel (70−230 mesh) column chromatography and eluted with a gradient solvent system (MeOH in MC 1% to 50%) and RP column chromatography according to the scheme described in **Figure 2**.

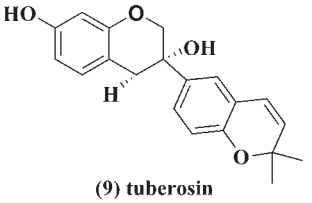
Assessment of α-Glucosidase Inhibitory Activity in Vitro. Inhibition assay of α-glucosidase was performed according to the chromogenic method described by Watanabe (*8*). Yeast α-glucosidase (0.7 U/mL, Sigma 9001-42-7, E.C 3.2.1.20) dissolved in 100 mM phosphate buffer (pH 7.0) containing 2 g/L bovine serum albumin and 0.2 g/L NaN_3 and 5 mM *p*-nitrophenyl-α-D-glucopyranoside (Fluka, BioChemika, Buchs, Switzerland) in the same buffer (pH 7.0) were used as an enzyme and a substrate solution, respectively. Then, 50 μL of enzyme solution and 10 μL of tested materials dissolved in dimethylsulfoxide were mixed in a microplate well. The absorbance of 405 nm at zero time was measured with a microplate reader (BIO-RAD, CA). After incubation for 5 min, 50 μL of substrate solution was added and incubated for an additional 5 min at room temperature. The increase in absorbance from zero time was measured. Each experiment was conducted in triplate. The IC_{50} values of compounds were calculated by the nonlinear regression analysis and expressed as the mean ± SD of three distinct experiments.

RESULTS AND DISCUSSION

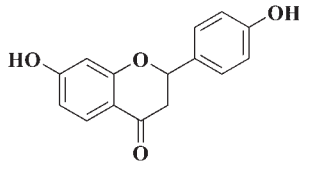
Isolation and Purification of α-Glucosidase Inhibitors from Plant Materials. The inhibitory effect on yeast α-glucosidase was evaluated according to the modified method of Watanabe (*8*) using *p*-nitrophenyl-α-D-glucopyranoside as a substrate, and acarbose,



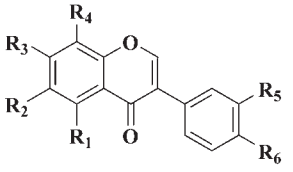
(1) medicarpin



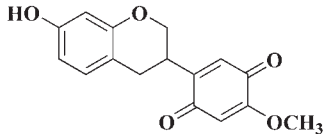
(9) tuberosin



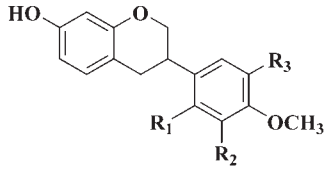
(4) liquritigenin



(2) formononetin
$R_1 = H, R_2 = H, R_3 = OH, R_4 = H, R_5 = H, R_6 = OCH_3$
(7) tectorigenin
$R_1 = OH, R_2 = OCH_3, R_3 = OH, R_4 = H, R_5 = H, R_6 = OH$
(8) biochanin A
$R_1 = OH, R_2 = H, R_3 = OH, R_4 = H, R_5 = H, R_6 = OCH_3$
(10) calycosin
$R_1 = H, R_2 = H, R_3 = OH, R_4 = H, R_5 = OH, R_6 = OCH_3$
(11) daidzein
$R_1 = H, R_2 = H, R_3 = OH, R_4 = H, R_5 = H, R_6 = OH$
(12) genitein
$R_1 = OH, R_2 = H, R_3 = OH, R_4 = H, R_5 = H, R_6 = OH$
(13) puerarin
$R_1 = H, R_2 = H, R_3 = OH, R_4 = C\text{-glu}, R_5 = H, R_6 = OH$
(14) daidzin
$R_1 = H, R_2 = H, R_3 = O\text{-glu}, R_4 = H, R_5 = H, R_6 = OH$
(15) formononetin-7-glucoside
$R_1 = H, R_2 = H, R_3 = O\text{-glu}, R_4 = H, R_5 = H, R_6 = OCH_3$
(16) genistin
$R_1 = OH, R_2 = H, R_3 = O\text{-glu}, R_4 = H, R_5 = H, R_6 = OH$



(5) (3R)-calussequinone



(3) mucronulatol
$R_1 = OCH_3, R_2 = OH, R_3 = H$
(6) (3R)-5'-methoxyvestitol
$R_1 = OH, R_2 = H, R_3 = OCH_3$

Figure 3. Structures of herbal α-glucosidase inhibitors isolated from two leguminous plants, *Dalbergia odrifera* and *Pueraria thunbergiana*.

a clinically used α-glucosidase inhibitor, was employed as a reference drug.

The heartwoods extract of *D. odorifera* (HDO) and the roots extract of *P. thunbergiana* (RPT) demonstrated a potent inhibition on yeast α-glucosidase in vitro, respectively. The concentration of each extract required for 50% inhibition (IC_{50}) of the enzyme activity was calculated as 19.5 and 21.8 mg/mL, respectively. However, among the three fractions obtained by serial solvent partition of HDO, i.e., the EtOAc soluble fraction, *n*-BuOH soluble fraction, and remaining aqueous fraction (**Figure 1**), only the EtOAc soluble fraction showed a potent inhibitory activity (IC_{50}; 12.4 mg/mL), whereas the other two fractions exhibited poor inhibitory effect on α-glucosidase (IC_{50} > 50 mg/mL). These results suggested that active components in HDO responsible for the α-glucosidase were exclusively concentrated in the EtOAc soluble fraction of HDO (**Figure 1**). On the other hand, both the dichloromethane soluble fraction and the EtOAc soluble fraction derived from the roots extract of *P. thunbergiana* (RPT) showed equipotent activity on α-glucosidase inhibition (IC_{50}; 15.4 and 15.8 mg/mL), whereas the *n*-BuOH fraction and remained water fraction demonstrated poor inhibitory effects (IC_{50} > 50 mg/mL) (**Figure 2**). The inhibitory activity of active fractions derived from HDO and RPT was almost comparable with that of reference drug, acarbose (9.11 ± 0.25 mM).

Thus, bioassay guided purification of three active fractions, i.e., the EtOAc soluble fraction of HDO, the dichloromethane, and the EtOAc soluble fractions of RPT, was conducted to purify the active principles responsible for the inhibition on yeast α-glucosidase followed by the process described in **Figures 1** and **2**, respectively.

Repeated column chromatography of the EtOAc soluble fraction of HDO yielded seven active components responsible for enzyme inhibition such as medicarpin (**1**), formononetin (**2**), mucronulatol (**3**), liquitigenin (**4**), (3*R*)-calussequinone (**5**), (3*R*)-5′-methoxyvestitol (**6**), and tectorigenin (**7**). By the similar chromatographic manner, the dichloromethane and the EtOAc soluble fractions of RPT afforded five active components, biochanin A (**8**), tuberosin (**9**), calycosin (**10**), daidzein (**11**), and genistein (**12**), together with four related isoflavone glycosides, i.e., puerarin (**13**), daidzin (**14**), formononetin-7-*O*-*β*-glucopyranoside (**15**), and genistin (**16**). The chemical structures of all isolated compounds (**1**−**16**) were identified by the comparison of their physicochemical and spectroscopic data with those reported in the literature (*22*−*24*) (**Figure 3**)

α-Glucosidase Inhibitory Effect of Isoflavones (1−12). All active components (**1**−**12**) isolated from two leguminous plant extracts demonstrated a significant inhibition on α-glucosidase in a dose-dependent manner. The α-glucosidase inhibitory effects of the

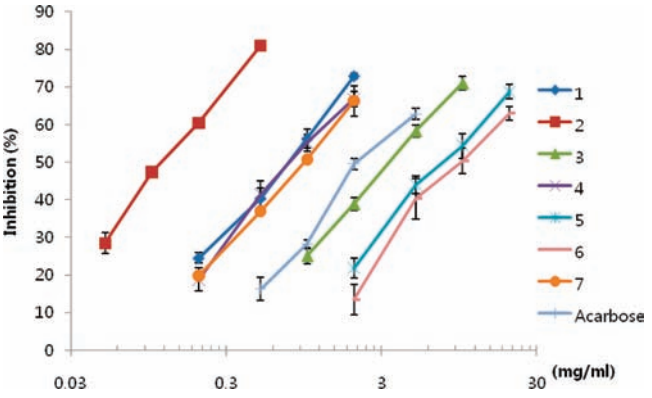


Figure 4. Inhibition of yeast α-glucosidase by **1**−**7** isolated from the heartwood extract of *Dalbergia odorifera* in vitro. Each result is expressed as the mean ± SD of three distinct experiments. Acarbose was used as positive references.

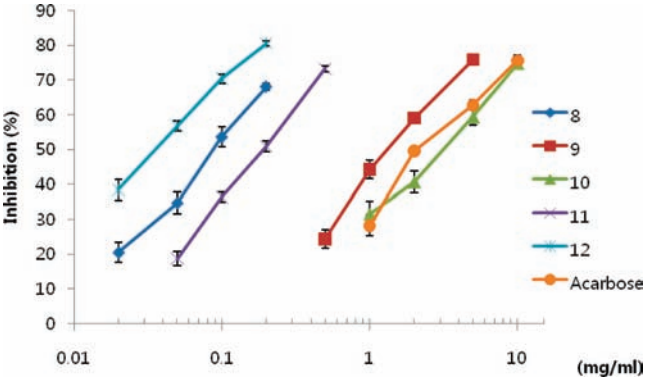


Figure 5. Inhibition of yeast α-glucosidase by **8**−**12** isolated from the roots extract of *Pueraria thunbergiana* in vitro. Each result is expressed as the mean ± SD of three distinct experiments. Acarbose was used as positive references.

seven active components (**1**−**7**) isolated from the HDO and five components (**8**−**12**) from RPT were depicted in **Figures 4** and **5**. The concentration of each compounds (**1**−**16**) for 50% inhibition of the enzyme activity (IC_{50}) were summarized in **Table 1**.

With an exception of a flavonoid component, liquilitigenin (**4**), all isolated active components (**1**−**12**) were categorized in a common structure, so-called isoflavone.

Isoflavone is known as a large and distinctive subclass of the flavonoid which was exclusively found in leguminous plants, particularly with the highest concentrations occurring in soy bean (*Glycine max*). It is also regarded as a group of phytoestrogens because of the unique chemical structure similar to that of estrogen and reported to provide various health benefits on diverse disorders such as prostate, breast, bowel, and other cancers, cardiovascular disease, brain function disorders, and osteoporosis (*25*).

Among the active components **1**−**12**, formononetin (**2**), biochanin A (**8**), daidzein (**11**), and genistein (**12**) demonstrated excellent inhibitions on yeast α-glucosidase with extremely high potency, and their IC_{50} values were calculated as 0.51 mM (**2**), 0.35 mM (**8**), 0.85 mM (**11**), and 0.15 mM (**12**), respectively, which were much lower than that of reference drug, acarbose (9.11 ± 0.25 mM).

Other active components, medicarpin (**1**), mucronulatol (**3**), (3*R*)-calussequinone (**5**), tectorigenin (**7**), and calycosin (**10**), also exhibited a potent inhibition with IC_{50} values of 2.93 mM (**1**), 12.53 mM (**3**), 29.38 mM (**5**), 3.52 mM (**7**), and 12.70 mM (**10**), which were much more potent or comparable to that of acarbose, respectively.

Table 1. α-Glucosidase Inhibitory Effects of Isoflavones (**1**−**16**) on α-Glucosidase

compd	IC_{50} (mM)[a]
medicarpin (**1**)	2.93 ± 1.02
formononetin (**2**)	0.51 ± 0.31
mucronulatol (**3**)	12.53 ± 0.58
liquitigenin (**4**)	3.61 ± 1.28
(3*R*)-calussequinone (**5**)	29.38 ± 0.99
(3*R*)-5′-methoxyvestitol (**6**)	>30
tectorigenin (**7**)	3.52 ± 0.89
biochanin A (**8**)	0.35 ± 0.36
tuberosin (**9**)	4.68 ± 0.73
calycosin (**10**)	12.70 ± 0.29
daidzein (**11**)	0.85 ± 0.67
genistein (**12**)	0.15 ± 0.16
puerarin (**13**)	>30
daidzin (**14**)	>30
formononetin-7-glucopyranoside (**15**)	>30
genistin (**16**)	>30
acarbose	9.11 ± 0.25

[a] IC_{50} represents the concentration of a compound required for 50% inhibition of α-glucosidase in vitro. Each data is expressed as the mean ± SD of three distinct experiments. Acarbose was used as a positive reference.

However, isoflavone glycosides, i.e., puerarin (**13**), daidzin (**14**), formononetin-7-*O*-*β*-glucopyranoside (**15**), and genistin (**16**) exhibited a relatively poor inhibitory activity as compared with corresponding aglycone compounds **2**, **11**, and **12**, respectively.

These results were well accorded with previous reports that soy isoflavonoids, daidzein (**11**) and genistein (**12**), exerted a potent inhibition upon yeast α-glucosidase, in the manner of a typical reversible and noncompetitive inhibition mode (*26*, *27*).

Soy isoflavones have been reported to decrease blood glucose levels and improve glucose tolerance in diabetic animal models (*28*, *29*). Moreover, it was reported that dietary supplementation with isoflavones reduced insulin resistance and improved glycemic control in T2DM patients and also lowered low density lipoprotein cholesterol, even though it was not clear whether these effects were associated with the α-glucosidase inhibitory activity (*30*). These results suggested the possibility that daidzein (**11**) and genistein (**12**), represented as typical soybean isoflavones, could be employed as a good α-glucosidase inhibitor of plant origin.

However, the α-glucosidase inhibitory effects of naturally occurring isoflavones were never investigated so far with an exception of two characteristic soybean isoflavone, daidzein (**11**) and genistein (**12**) (*27*, *28*, *31*).

In conclusion, our findings suggested that not only the soy isoflavones, daidzein (**11**) and genistein (**12**), but some structurally related isoflavones (**1**−**10**), which were abundant in two leguminous plants, *D. odorifera* and *P. thunbergiana*, also exhibited a potent inhibition on yeast α-glucosidase in vitro and these isoflavone compounds could be employed as a lead for the design of new potential α-glucosidase inhibitors.

However, the yeast α-glucosidase which was investigated in this report is known to be different from mammalian digestive enzymes, therefore ongoing experiments should be focused on the inhibitory activity of isoflavones (**1**−**12**) against mammalian intestinal α-glucosidase and on the mechanism and kinetic study of individual isoflavones, even though soy isoflavones **11** and **12** have already been reported to decrease blood glucose levels and improve glucose tolerance in diabetic animal models (*28*).

Supporting Information Available: Spectroscopic and physical data of compounds **1**−**16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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