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The α-Glucosidase Inhibiting Isoflavones Isolated from *Belamcanda chinensis* Leaf Extract

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Abstract: The dried rhizome of *Belamcanda chinensis* is an important Chinese traditional medicine used for the treatment of inflammation and many other disorders. Previously, we reported the hypo- and antihyper-glycemic effects of the aqueous leaf extract of *B. chinensis* (BCL) and identified the isoflavones as its principal active fraction. In the present study, the α-glucosidase inhibitory effect of BCL and its rough isoflavone preparation (BIF) was tested *in vitro* and *in vivo*. Thirteen isoflavones were isolated from BCL and their α-glucosidase inhibitory activity was screened *in vitro*. The results showed that BCL (500 and 1000 mg/kg) and BIF (250 and 500 mg/kg) greatly inhibited the increase in blood glucose level after 5 g/kg starch loading in normal mice. Six out of the thirteen isoflavones (swertisin, 2”-O-rhamnosylswertisin, genistein, genistin, mangiferin and daidzin) exhibited strong α-glucosidase inhibitory activity *in vitro*. HPLC analysis showed that swertisin was the most abundant isoflavone in BCL accounting for 1.24% of BCL, 7.44% of BIF, and 11.24% of the total isoflavone fraction of BCL, respectively. These results demonstrate that BCL possesses significant α-glucosidase inhibitory activity and swertisin may be the principal active component of BCL in α-glucosidase inhibition.

Keywords: *Belamcanda chinensis*, diabetes, α-glucosidase inhibitor, swertisin, isoflavones, postprandial hyperglycemia

1. Introduction

Diabetes mellitus is one of the most prevalent diseases seriously threatening the health of humans. It is predicted that in 2010, the number of diabetic patients has soared to 285 million, accounting for

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approximately 6.4% of the world's adult population [1]. Each year, more than 3.8 million people die from diabetes-related causes [2]. The overwhelming prevalence of diabetes not only imposes a huge burden on global health care, costing at least 376 billion U.S. dollars or 12% of the total health care expenditures in 2010 [3], but also makes the searching for agents with antidiabetic activities one of the most urgent and an endless issue for pharmacologists all over the world.

In diabetes, the postprandial phase is characterized by a rapid and large increase in blood glucose levels, which is called postprandial hyperglycaemia or "hyperglycemic spikes". Recently, a growing body of evidence suggests that postprandial hyperglycaemia is an important and independent risk factor for atherosclerosis and some other diabetic complications with great effects than that of fasting hyperglycemia alone [4-6]. Effective management of postprandial hyperglycemia therefore involves not only the maintenance of normal blood glucose levels after meal but also the prevention of many other diabetic complications. In this realm, the α-glucosidase inhibitors attract most interest [7-9].

Alpha-glucosidase is a key enzyme in carbohydrate digestion. It catalyzes the hydrolysis of 1,4-α-glucosidic bonds within carbohydrates with release of α-glucose and promotes the increase of blood glucose levels after meal. Alpha-glucosidase inhibitors antagonize the activity of α-glucosidase, thereby delaying intestinal carbohydrate absorption and slowing the sharp rise in blood sugar levels that diabetic patients typically experience after meals [7]. For this reason, α-glucosidase inhibitors, such as acarbose and voglibose, are clinically used as oral antihyperglycemic agents [8-9]. However, they often cause severe gastrointestinal side effects such as flatulence and diarrhea. Therefore, search for new α-glucosidase inhibitors from natural resources has become an attractive approach for the treatment of postprandial hyperglycemia.

Belamcanda chinensis (L) DC belongs to the family Iridaceae. Its dried rhizome is an important Chinese traditional medicine used for the treatment of sore swollen throat, asthma, tonsillitis, and many other symptoms [10-11]. In our previous studies, we demonstrated the hypo- and antihyper-glycemic effects of the aqueous leaf extract of *B. chinensis* (BCL) in normal and STZ (Streptozocin)-induced diabetic rats [12-13]. Oral administration of BCL significantly inhibited the increase of blood glucose levels after administration of various carbohydrates. Interestingly, BCL was more effective for the carbohydrates with complicated structures such as starch and pulse flour [12], suggesting that BCL likely possess glycosidase inhibitory activities. In our subsequent investigations, we identified the isoflavones but not polysaccharides as the principal active fraction of BCL in diabetes treatment and isolated several isoflavones with known antidiabetic activities from this source [12-13], providing more detailed and solid evidence for the antidiabetic effects of the aqueous leaf extract of *B. chinensis* (BCL).

In the present study, the α-glucosidase inhibitory effects of the aqueous leaf extract of *B. chinensis* (BCL) were investigated *in vitro* and *in vivo.* Thirteen major isoflavones isolated from BCL were screened for the α-glucosidase inhibitory activity *in vitro*. Eventually, swertisin was identified as the principal active component of BCL in α-glucosidase inhibition.

2. Materials and Methods

2.1. Plant materials

2.1.1 Plant extracts preparation

Belamcanda chinensis (L) DC leaves were harvested from Hainan province, South China and authenticated by Dr. Hubiao Chen (Peking University Health Science Center, Beijing, China). A voucher specimen (No. H200507) has been deposited in the laboratory of the School of Life Sciences, Beijing Institute of Technology. The aqueous extract (BCL) and the crude isoflavone preparation (BIF) of *B. chinensis* leaves were prepared as previously reported [12-13].

2.1.2. Isolation and identification of compounds

The separation procedure and the amount of each isoflavone obtained were shown in Figure 1. Briefly, the dried powder of BIF (50 g) was dissolved in 300 mL of methanol, filtered and then methanol was evaporated out of the supernatant, which yielded about 41 g of methanol extract. The methanol extract was loaded onto a polyamide column (85 cm x 8 cm) and thoroughly eluted in steps with pure water, 40% and 95% ethanol. The pure water and 40% ethanol eluates were reloaded onto a separate Sephadex-LH20 column (21 cm x 2.5 cm) and sequentially eluted with pure water, 30%, 50% and 70% methanol (for the pure water eluate) or with 30%, 50%, 70% and 100% methanol (for the 40% ethanol eluate). The 95% ethanol eluate was reloaded onto an octadecyl silane (ODS) column (25 cm x 2.0 cm) and sequentially eluted with 40%, 55%, 70%, 80% and 100% methanol. All the subeluates obtained from the second cycle of column chromatography were individually loaded onto a preparative HPLC column and eluted with indicated concentrations of methanol.

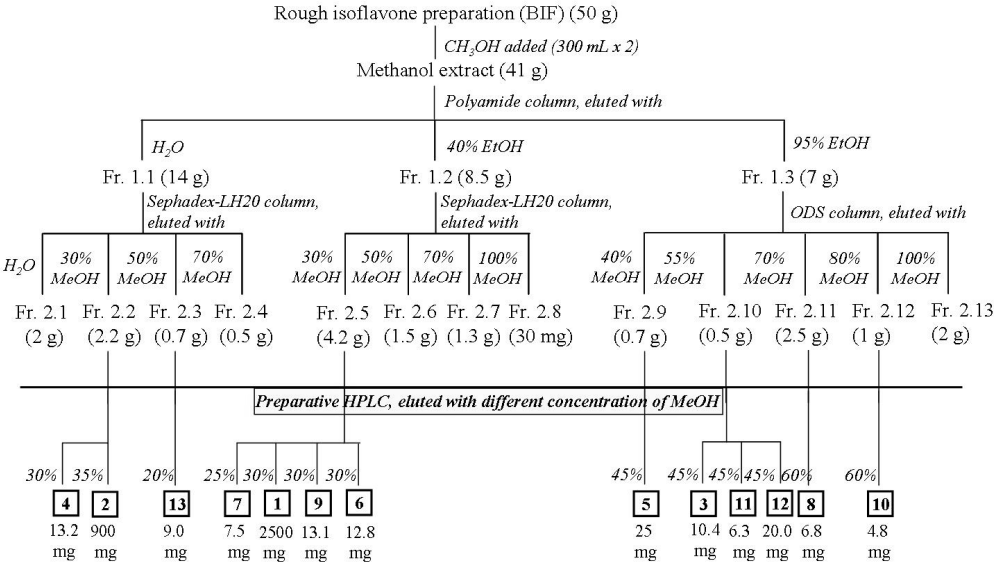


Figure 1. Isolation scheme of isoflavones (**1-13**) from the crude isoflavone preparation (BIF) of *B. chinensis* leaf extract (BCL). EtOH, ethanol; MeOH, methanol.

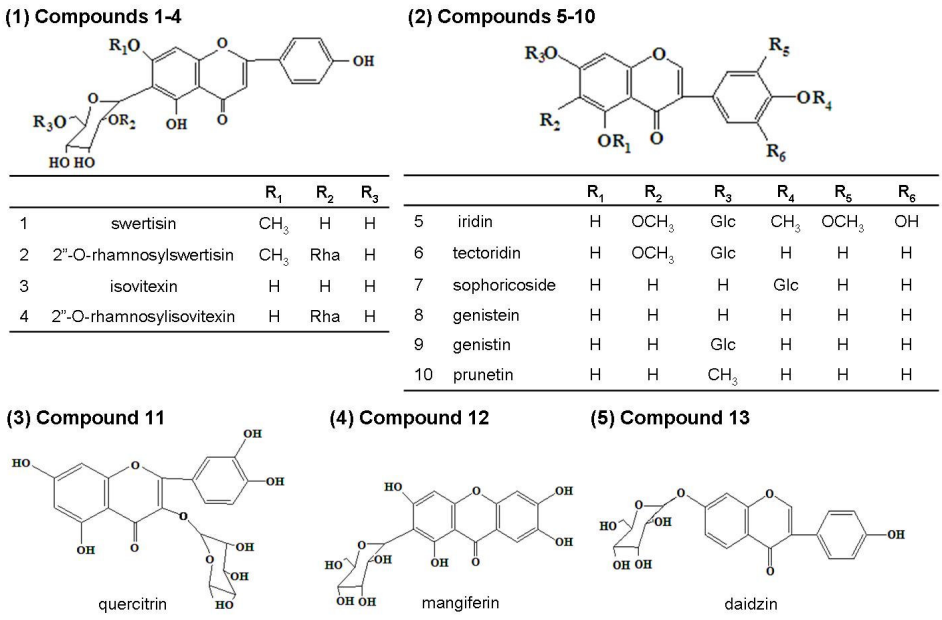
The NMR and MS data were obtained for each compound and compared with those reported in the literature. All the compounds are known chemicals and identified as **(1)** swertisin [14], **(2)** 2"-O-rhamnosylswertisin [15], **(3)** isovitexin [16], **(4)** 2"-O-rhamnosylisovitexin [17], **(5)** iridin [18], **(6)** tectoridin [18], **(7)** sophoricoside [19], **(8)** genistein [20], **(9)** genistin [21], **(10)** prunetin [22], **(11)** quercitrin [23], **(12)** mangiferin [24], and **(13)** daidzin [25]. The chemical structures of these thirteen isoflavones isolated from *B. chinensis* leaf extract are displayed in Figure 2.

2.1.3. HPLC and isoflavone content determination

0.15 g of *B. chinensis* leaf extract (BCL) was dissolved in 2 mL of deionized water and filtered through a 0.45 μm syringe filter. An aliquot of 5 μl solution was injected for HPLC analysis. An Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA USA) with an Agilent ODS column (250 mm×4.6 mm i.d., 5 μm) was used to acquire HPLC chromatograms. The mobile phase consisted of phosphoric acid solution (0.05% (v/v), pH 3.24, A) and acetonitril (B) using a linear gradient program of 5% B in 0-5 min, 5-15% B in 5-20 min, 15-20% B in 20-30 min, 20-30% B in 35-45 min, 30-100% B in 45-60 min. The flow rate was 1.0 mL/min. The DAD detector was set at 269 nm.

The contents of swertisin and 2”-O-rhamnosylswertisin in BIF were determined by comparing their peak areas in HPLC chromatographs with that obtained from a known weight of swertisin or 2”-O-rhamnosylswertisin, respectively. Briefly, serial dilutions (0.005, 0.010, 0.015, 0.020 and 0.025 mg/mL) of swertisin and 2”-O-rhamnosylswertisin were prepared in 50% methanol. An aliquot of 5 μl dilution was injected for HPLC analysis under the same HPLC conditions as described above. The peak areas were plotted against the concentration of swertisin or 2”-O-rhamnosylswertisin, and the standard curves were obtained. Thereafter, 0.75 g of BIF was dissolved in 25 mL of 50% methanol and filtered through a 0.45 μm syringe filter. An aliquot of 5 μl solution was injected for HPLC analysis under the same HPLC conditions as described above. The corresponding peak areas were obtained and the contents of swertisin or 2”-O-rhamnosylswertisin were calculated from the standard curves. Six BIF samples were analyzed under the same HPLC conditions.

The percent total isoflavone content in BIF fractions was determined by the absorption at 269 nm with 2”-O-rhamnosylswertisin as standard. A precise weight of 21.6 mg of 2”-O-rhamnosylswertisin was dissolved in 50% methanol to a final volume of 50 mL. Then, aliquots of 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.60 and 0.65 mL were taken and diluted to 10 mL with 50% methanol. The absorption of each aliquot at 269 nm was measured in a Beckman DU7400 spectrophotometer and plotted against the corresponding concentrations to obtain the standard curve. Thereafter, 0.75 g of BIF was dissolved in 25 mL of 50% methanol and filtered through a 0.45 μm syringe filter. The absorption at 269 nm was measured and the total isoflavone content was calculated from the standard curve. Six BIF samples were tested.



		R_1	R_2	R_3
1	swertisin	CH_3	H	H
2	2”-O-rhamnosylswertisin	CH_3	Rha	H
3	isovitexin	H	H	H
4	2”-O-rhamnosylisovitexin	H	Rha	H

		R_1	R_2	R_3	R_4	R_5	R_6
5	iridin	H	OCH_3	Glc	CH_3	OCH_3	OH
6	tectoridin	H	OCH_3	Glc	H	H	H
7	sophoricoside	H	H	H	Glc	H	H
8	genistein	H	H	H	H	H	H
9	genistin	H	H	Glc	H	H	H
10	prunetin	H	H	CH_3	H	H	H

Figure 2. Chemical structures of thirteen isoflavones isolated from *B. chinensis* leaf extract (BCL).

2.2. Animal experiment

Male Kunming mice (20-22 g each) were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences, Beijing, China. The study was carried out according to the " Principles of Laboratory Animal Care" [World Health Organization (WHO) Chronicle, 1985] and approved by the Animal Ethics Committee of our university. A standard pellet diet and water were given *ad libitum*. Animals were maintained under a constant 12-h light and dark cycle and an environmental temperature of 21-23 °C.

Animals were randomly divided into six groups with six mice in each group. Prior to the experiment, animals were fasted for eight hours then treated with corresponding agents orally. After another 15 min, each mouse was given 5 g/kg of starch (p.o.). The whole blood was collected from the tail veins at 0, 1 and 2 hours after starch administration, and the serum blood glucose concentration was measured by a glucose assay kit (BIOSINO Inc., Beijing, China) according to the vendor's instruction. Negative control group (NC) received distilled water only, whereas positive control group (PC) received acarbose (100 mg/kg, p.o.). BCL-L and BCL-H groups were treated with BCL (500 mg/kg and 1000 mg/kg respectively, p.o.). BIF-L and BIF-H groups were treated with BIF (250 mg/kg and 500 mg/kg respectively, p.o.).

2.3. Assessment of α-glucosidase inhibitory activity in vitro

Inhibition of α-glucosidase activity was performed according to the chromogenic method described by Watanabe [26], using 4-nitrophenyl-α-D-glucopyranoside (PNPG, Sigma, USA) as substrate. Briefly, BCL and Acarbose were dissolved in distilled water while BIF and all the isoflavone samples were dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) to a final concentration of 4 mg/mL. Yeast α-glucosidase (10 U/mL) and PNPG (10 mg/mL) were prepared in 50 mM potassium phosphate buffer (pH 6.8). Fifty μL of enzyme solution and 5, 10 or 20 μL of test materials were mixed in wells of a microplate. After incubation at room temperature for 15 min, PNPG solution was added to a final volume of 200 μL and incubated for an additional 15 min at room temperature. The absorbance of 405 nm was measured in a microplate reader (BIO-RAD, CA) after stopping the reaction by addition of 600 μL of 1M Na_2CO_3. The corresponding solvents (distilled water and DMSO) were used as blank and Acarbose was used as positive control. The α-glucosidase inhibitory activity was calculated as follows:

$$\alpha\text{-glucosidase inhibitory activity } (\%) = [A_{blank} - (A_{sample} - A_{background})] / A_{blank} \times 100$$

Where A_{blank}, A_{sample}, $A_{background}$ are defined as the absorbance of 100% enzyme activity (only the solvent with the enzyme), test sample with the enzyme and test sample without the enzyme, respectively. The IC_{50} values of compounds were calculated by the linear regression analysis.

2.4. Kinetics of enzyme inhibition

Inhibition assays were performed according to the above reaction conditions with inhibitors of various concentrations. The type of inhibition for the inhibitors were determined by double-reciprocal plot and its replot of slope versus the reciprocal of the substrate concentration.

2.5. Data analysis

The values were expressed as mean ± SD. Statistical significance was determined by One-way ANOVA followed by post-hoc Tukey test using SPSS 11.0 software. A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 The Inhibitory effects of BCL and BIF on postprandial hyperglycemia and α-glycosidase activity

As shown in Figure 3, oral treatment with BCL (500 and 1000 mg/kg) (Figure 3A) or its crude isoflavone preparation (BIF) (250 and 500 mg/kg) (Figure 3B) largely inhibited the increase of blood glucose levels in normal mice at 1 h after starch intake. The inhibitory effect of BCL and BIF on postprandial hyperglycemia was dose-dependent. At the doses used in this experiment, BCL and BIF were comparable to 100 mg/kg of Acarbose (Figure 3A and B).

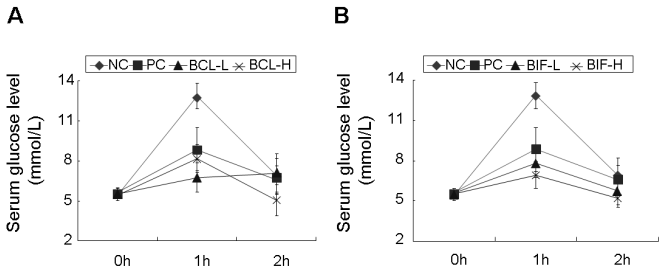


Figure 3. Effect of *B. chinensis* leaf extract (BCL) (A) and its crude isoflavone preparation (BIF) (B) on postprandial hyperglycaemia of normal mice induced by starch intake. NC, negative control treated with distilled water; PC, positive control treated with Acarbose (100 mg/kg, p.o.); BCL-L and –H were treated with BCL (500 and 1000 mg/kg respectively, p.o.); BIF-L and –H were treated with BIF (250 and 500 mg/kg respectively, p.o.).

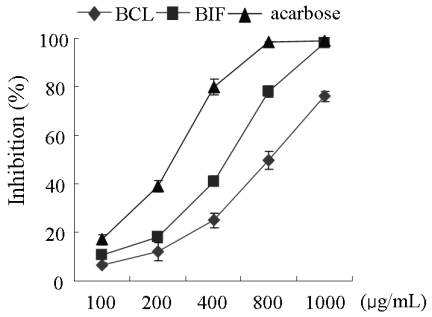


Figure 4. Inhibition of yeast α-glucosidase by *B. chinensis* leaf extract (BCL) and its crude isoflavone preparation (BIF) *in vitro*. Each result is expressed as the mean ± SD of three separate experiments. Acarbose was used as positive control.

The robust effects of BCL and BIF on postprandial hyperglycemia suggested that the aqueous extract of *B. chinensis* leaves has strong α-glycosidase inhibitory activity, and this hypothesis was confirmed by the *in vitro* α-glycosidase inhibition assay. BCL and BIF dose-dependently inhibited the activity of yeast α-glucosidase with IC_{50} values of about 800 μg/mL and 500 μg/mL, respectively, which were lower than that of acarbose (217 μg/mL) (Figure 4). The IC_{50} values of BCL and BIF was consistent with the results obtained from animal experiments, in which 500 mg/kg of BCL and 250 mg/kg of BIF displayed comparable inhibitory effects with 100 mg/kg of acarbose on postprandial hyperglycemia (Figure 3).

3.2 In vitro α-glucosidase inhibitory activity screening of isoflavones (1-13) isolated from BCL

To identify the active components of BCL in α-glucosidase inhibition, thirteen isoflavones were isolated from the crude isoflavone preparation (BIF) of BCL. The inhibitory effects of these thirteen isoflavones on α-glucosidase activity were assayed *in vitro*. As shown in Figure 5, six isoflavones i.e. swertisin **(1)**, 2”-O-rhamnosylswertisin **(2)**, genistein **(8)**, genistin **(9)**, mangiferin **(12)** and daidzin **(13)** exhibited strong inhibitory effect on α-glucosidase activity. The IC_{50} values of these isoflavones were listed in Table 1. All the compounds except 2”-O-rhamnosylswertisin have already been demonstrated to be strong α-glucosidase inhibitors in previous reports [26-28], which confirms our findings.

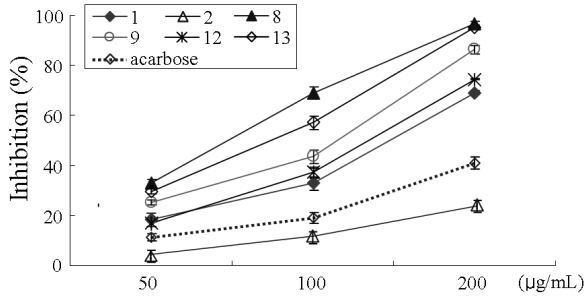


Figure 5. Inhibition of yeast α-glucosidase by isoflavones isolated from *B. chinensis* leaf extract *in vitro*. Each result is expressed as the mean ± SD of three distinct experiments. Acarbose was used as positive control. **(1)** swertisin; **(2)** 2”-O-rhamnosylswertisin; **(8)** genistein; **(9)** genistin; **(12)** mangiferin; **(13)** daidzin.

Figure 6 displayed the double-reciprocal plots of α-glucosidase kinetics with genistein, genistin, mangiferin and daidzin. Non-competitive inhibition was partially observed when genistein, genistin, mangiferin, and substrate were added simultaneously, showing Ki values of 32.4, 254.7 and 138.2 nmol/mL respectively. The double-reciprocal plots of α-glucosidase kinetics with daidzin showed a complex inhibition pattern, suggesting that daidzin may inhibit α-glucosidase in both competitive and non-competitive modes.

According to Choi et al. [26], the isoflavone glycosides exhibited a relatively poor inhibitory activity on α-glucosidase as compared with the corresponding isoflavone. Figure 5 and Table 1 showed that the α-glucosidase inhibitory effect of 2”-O-rhamnosylswertisin and genistin was significantly weaker than their less-glycosylated counterpart swertisin and genistein, which was in good accordance with Choi et al. These results also suggest that the aglycone moieties but not the glycone moieties of these isoflavones bind and inhibit α-glucosidase. However, experimental results are still needed to confirm this hypothesis.

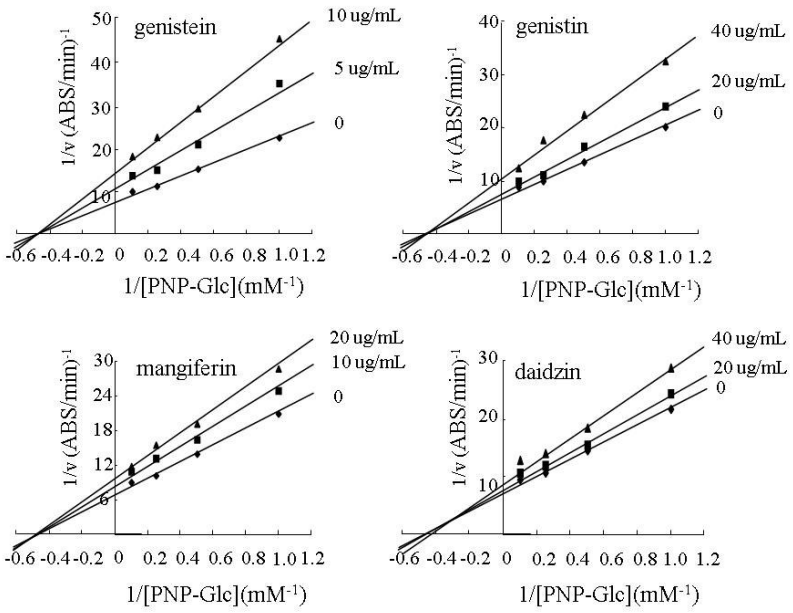


Figure 6. Double-reciprocal plots of the inhibition kinetics of yeast α-glucosidase by genistein, genistin, mangiferin and daidzin. α-Glucosidase (20 μl, 2 U/ml) was pre-incubated with each inhibitor for 15 min at room temperature before 4-nitrophenyl-α-D-glucopyranoside (PNPG) was added to initiate the reaction.

Table 1. α-glucosidase inhibitory effects of isoflavones (1-13) on α-glucosidase

Compounds	**IC_{50} (μg/mL)**
swertisin (**1**)	119
2''-O-rhamnosylswertisin (**2**)	333
isovitexin (**3**)	---
2''-O-rhamnosylisovitexin (**4**)	---
iridin (**5**)	---
tectoridin (**6**)	---
sophoricoside (**7**)	---
genistein (**8**)	74
genistin (**9**)	83
prunetin (**10**)	---
quercitrin (**11**)	---
mangiferin (**12**)	112
daidzin (**13**)	97
acarbose	217

IC_{50} represents the concentration of a compound required for 50% inhibition of α-glucosidase *in vitro*. Acarbose *was used as a positive control. "---" denotes no inhibitory effect.*

3.3 Swertisin may be the principal active component of BCL in α-glucosidase inhibition

Swertisin **(1)** and 2"-O-rhamnosylswertisin **(2)** were the most abundant compounds among those isolated from BCL. The amounts of swertisin and 2"-O-rhamnosylswertisin obtained from 50 g BIF were 2500 mg and 900 mg, respectively, much higher than the other isoflavone components (Figure 1 and Figure 6A). Quantitative analysis showed that swertisin accounted for 1.24% of BCL, 7.44% of BIF, and 11.24% of the total isoflavone fraction of BCL, whereas 2"-O-rhamnosylswertisin was approximately one-third of swertisin (Figure 6B). Based on its strong inhibitory effect on α-glucosidase activity and most abundance in BCL, swertisin might be the principal active component of BCL responsible for α-glucosidase inhibition.

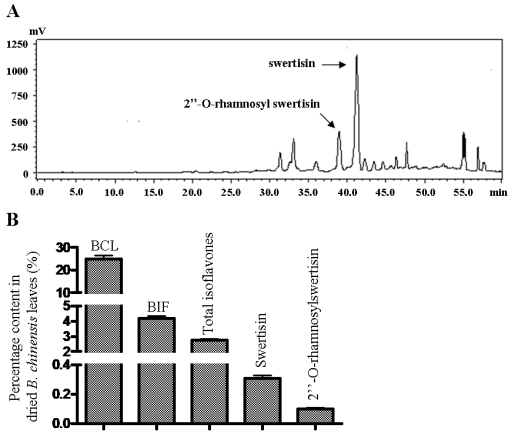


Figure 7. Swertisin is the most abundant isoflavone in *B. chinensis* leaf extract (BCL). (A) HPLC chromatogram of BCL. HPLC analysis was carried out on an Agilent 1100 series HPLC system with an Agilent ODS column (250 mm×4.6 mm i.d., 5 μm). A DAD230 diode array detector was used to monitor continuously at 269 nm. (B) The percent contents of BCL, crude isoflavone preparation (BIF), total isoflavone fraction, swertisin and 2"-O-rhamnosylswertisin in the dried leaves of *B. chinensis*.

Acknowledgments

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