α-Glucosidase Inhibitory and Glucose Uptake Stimulatory Effects of Phenolic Compounds From Dendrobium christyanum

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Abstract

A methanolic extract from the dried root of Dendrobium christyanum Rchb.f. (Orchidaceae) exhibited α-glucosidase inhibitory activity and glucose uptake stimulatory effect. Chromatographic separation of the extract led to the isolation of 13 phenolic compounds (1-13). Their structures were determined by spectroscopic analysis. The isolates were then evaluated for in vitro α-glucosidase inhibitory and glucose uptake stimulatory activities. Methyl haematommate (1), methyl 2,4-dihydroxy-3,6-dimethylbenzoate (3), n-docosyl 4-hydroxy-trans-cinnamate (4), coniferyl aldehyde (6), 4,5-dihydroxy-2-methoxy-9,10-dihydrophenanthrene (7), gigantol (10), and diorcinolic acid (13) showed higher α-glucosidase inhibitory activity than the drug acarbose. Moreover, n-docosyl 4-hydroxy-trans-cinnamate (4), vanillin (5), and coniferyl aldehyde (6) could enhance glucose uptake by L6 myotubes. Compounds 4 and 6 appear to be potential hypoglycemic agents since they possess both α-glucosidase inhibitory and glucose uptake stimulatory activities. This study is the first report on the chemical constituents and antidiabetic activity of D. christyanum.

Keywords
phenolics, antidiabetic, L6 myotubes, Dendrobium christyanum, α-glucosidase

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Diabetes mellitus (DM) is a disease of abnormal glucose metabolism caused by impaired insulin secretion and/or inefficient utilization of insulin. The hormone insulin is secreted from the β cells of the islets of Langerhans in the pancreas. It plays an essential role in blood glucose homeostasis by facilitating cellular glucose uptake and regulating the metabolism of protein, lipid, and carbohydrate. Untreated DM can produce several complications, such as diabetic nephropathy, neuropathy, and retinopathy, resulting in premature death. In type II DM, the most common form of diabetes, impaired glucose tolerance leads to insulin resistance, a condition of decreased responsiveness of target tissues (liver, muscles, and adipose tissues) to regular circulating insulin, and results in postprandial hyperglycemia.

At present, several types of oral antidiabetic drugs with different mechanisms of action are available. Our study focuses on α-glucosidase inhibiting and glucose-uptake stimulating drugs.

α-Glucosidase inhibitors (AGIs) were discovered to control postprandial hyperglycemia in the 1970s and became a new class of antidiabetic drugs in the 1980s, but so far, only a few are commercially available. Examples are voglibose and acarbose. They can decrease the absorption of carbohydrates from the small intestine by competitively inhibiting the α-glucosidase enzyme in the brush border and thus lowering the postprandial blood glucose level. Acarbose is a complex oligosaccharide obtained from the microbial origin, and its recommended doses are 50-200 mg 3 times daily. Studies showed that 56%-76% of patients who took AGIs suffered from the side effects related to the digestive system, such as bloating due to indigestible glucose, flatulence, diarrhea, and nausea. Apart from the above drawbacks, the structures of these AGIs mainly

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comprise sugar units, and their production processes are complicated. In recent years, researchers have turned their attention to finding AGI drugs from alternative sources, such as medicinal plants. There are several classes of compounds from medicinal plants that have been reported as AGIs, such as terpenoids, flavonoids, and phenylpropanoids. The investigation for new AGIs from medicinal plants still remain attractive because of the finding of active compounds would be helpful for the development of dietary supplements.

Insulin maintains the glucose homeostasis after a meal by stimulating glucose uptake into skeletal muscles. In type II DM, peripheral insulin resistance in skeletal muscles or adipose tissues leads to defects in insulin-mediated glucose uptake and utilization and then results in hyperglycemia. Insulin sensitizers can lower the blood glucose level by increasing the glucose uptake in muscles and reducing the hepatic glucoseogenesis and lipogenesis. The most frequently used drug in this class is metformin. Although the side effects of metformin, such as diarrhea and flatulence, are well tolerated, there are serious concerns over the long-term use of this drug due to some side effects, such as the decrease in vitamin B12 absorption, lactic acidosis, and macrocytic anemia. Thus, alternative glucose-uptake enhancing drugs are still needed.

Dendrobium is one of the large genera in the Orchidaceae family. The genus comprises about 1100 species, and in Thailand, 150 species have been identified. In traditional Chinese medicine, several members of Dendrobium known as “Shihu” have been used to increase body fluid production, relieve indigestion, and improve eyesight. Previous studies have shown that Dendrobium plants produce many classes of secondary metabolites with interesting biological activities, including antioxidant, anticancer, antimicrobial, antifungal, antiherpetic, anti-diabetic, anti-inflammatory, antimalarial, neuroprotective, immunomodulatory, and antiplatelet aggregation activities, as well as inhibition of lymphocyte infiltration and apoptosis in Sjogren’s syndrome.

Over the years, we have been working on several plants in the genus Dendrobium and found that some possess outstanding α-glucosidase inhibitory activity, for instance, lod digno nols G-J from Dendrobium loddigesi, 5-methoxy-7-hydroxy-9,10-dihydro-1,4-phenanthrenequinone from Dendrobium formosum, and dendrofalconerol A from Dendrobium tortile. Besides, some phenolic compounds with glucose uptake stimulatory activity from D. formosum have been reported. Dendrobium christyanum Rchb.f. is a small epiphytic plant, widely distributed in Thailand, Vietnam, and Southwest China. “Uaeng Sao Phukradung” is the name in Thai. The occurrence of D. christyanum is more abundant than at higher elevations, usually above 5900 ft. Before the present investigation, neither chemical nor biological studies have been reported for this plant.

In the preliminary evaluation, a methanolic extract of D. christyanum at 100 µg/mL exhibited α-glucosidase inhibitory activity by more than 70%. At 10 µg/mL and 100 µg/mL, it enhanced glucose uptake by L6 myotubes by 34.5% ± 51.7% and 56.4% ± 19.7%, respectively. These observations encouraged us to perform a detailed investigation of this plant to find the compounds responsible for the biological activities. The results from this study constitute the first report of glucosidase inhibitory and glucose uptake stimulatory activities of D. christyanum and its active principles.

Materials and Methods

Isolation and Structural Analysis

General experimental procedures. Vacuum-liquid column chromatography (VLC) and column chromatography were conducted on silica gel 60 (Merck, Kieselgel 60, 70-320 µm and 230-400 µm). Gel filtration chromatography was performed on Sephadex LH-20 (25-100 µm, GE Healthcare). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DPX-300 FT-NMR spectrometer or a Bruker Avance III HD 500 spectrometer (Bruker Biosciences Corporation, Billerica, MA, USA). Mass spectral data were analyzed using a Bruker micro TOF electro spray ionization-mass spectrometer (ESI-MS).

Plant material. Samples of D. christyanum were purchased from Jatuchak market, Bangkok, in July 2015. Plant identification was done by one of the authors (B. Sritularak) and compared with the database of the Botanical Garden Organization. A voucher specimen (BS-DC-072558) has been deposited at the herbarium of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Extraction and isolation. The root parts of air-dried plants (0.5 kg) were chopped and extracted with MeOH (3 × 6 L) at room temperature, and then, the organic solvent was evaporated under reduced pressure to give a dried mass (36.1 g). The MeOH extract was fractionated by VLC on silica gel (hexane–dichloromethane [CH2Cl2] and CH2Cl2–MeOH, gradient) to give 5 fractions (A–E). Fraction A (479 mg) was separated by column chromatography (CC, silica gel hexane-acetone, gradient) to give 5 fractions (AI–AV). Fraction AII (155.2 mg) was separated by Sephadex LH-20 (CH2Cl2) and then by CC (silica gel, hexane-acetone, gradient) to obtain methyl haematoside (I) (4 mg). Fractions AIII (138.9 mg) and AIV (38.6 mg) were purified on Sephadex LH-20 (acetone) to yield α-eicosyl trans-ferulate (2) (109 mg) and methyl 2,4-dihydroxy-3,6-dimethylbenzoate (3) (5.9 mg), respectively. Fraction B (2.4 g) was fractionated by CC (silica gel, hexane-acetone, gradient) to give 17 fractions (BI–BXVII). Fraction BIV (348.8 mg) was purified on Sephadex LH-20 (CH2Cl2) to furnish α-docosyl 4-hydroxy-trans-cinnamate (4) (40.6 mg). Fraction BVIII (179.7 mg) was separated on Sephadex LH-20 (CH2Cl2) to get vanillin (5) (29.1 mg). Fraction BIX (198.5 mg) was subjected...
to Sephadex LH-20 (CH₂Cl₂) to give 12 fractions (BIX1–BIX12). Fraction BIX7 (17.7 mg) was purified by CC (silica gel; hexane–acetone, gradient) to give coniferyl aldehyde (E) (13.7 mg). 4,5-Dihydroxy-2-methoxy-9,10-dihydrophenanthrene (7) (20.8 mg) was obtained from fraction BIX10 (190.5 mg) after purification on Sephadex LH-20 (CH₂Cl₂). Fraction BX (556.7 mg) was separated on Sephadex LH-20 (CH₂Cl₂) to give 9 fractions (BX1–BX9). Moscatilin (8) (25.6 mg) was obtained from fraction BX2 (90.8 mg) after separation by CC (silica gel, hexane-acetone, gradient). Aloifol I (9) (215.6 mg) was isolated from fraction BX3 (361 mg) by CC (silica gel; hexane-acetone, gradient). Fraction C (1.7 g) was fractionated by CC (silica gel; dichloromethane-acetone, gradient) to yield 10 fractions (CI–CX). Fraction CVI (50.9 mg) was subjected to CC (silica gel, hexane–acetone, gradient) to yield gigantol (10) (3.3 mg). Fraction CIX (260 mg) was separated on Sephadex LH-20 (acetone) and then further purified by CC (silica gel, hexane–acetone, gradient) to give batatasin III (11) (49.8 mg). Fraction CX (884.9 mg) was fractionated by CC (silica gel, hexane–acetone, gradient) to give 13 fractions (CX1–CX13). Fraction CX9 (57.7 mg) was further purified by CC (silica gel, hexane-acetone, gradient) and then by Sephadex LH-20 (acetone) to afford dendrosinen B (12) (19.1 mg). Fraction E (15.2 g) was separated on Diaion HP-20 (water (H₂O)–MeOH, gradient) to give 5 fractions (EI–EV). Fraction EII (1.47 g) was separated (C18, acetonitrile–H₂O, gradient) and then further purified by CC (silica gel, hexane-acetone, gradient) to give dioricnolic acid (13) (7.1 mg).

α-Glucosidase Inhibition Assay

α-Glucosidase inhibition assay was carried out by following the method in the previous report by Inthongkaew et al.16 α-Glucosidase inhibition was determined by observing the release of p-nitrophenol from the substrate p-nitrophenol-α-D-glucopyranoside. Acarbose was used as a positive control.

Glucose Uptake Stimulation Assay

Glucose uptake stimulation assay was performed according to the method by Inthongkaew et al.16 Insulin (500 nM) and metformin (2 mM) were used as positive controls, and the differentiation medium containing 0.1% dimethyl sulfoxide was used as a negative control.

Cell Viability Assay

Cell viability in each well was evaluated promptly after the determination of glucose uptake in L6 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay method was used to determine the cytotoxicity of each test compound.20

Statistical Analysis

The results of glucose uptake stimulation and cytotoxicity assays were described as the mean ± standard deviation. Analysis of variance was performed using the GraphPad Prism Version 7.00 for Windows (GraphPad Software, Inc., San Diego, CA, USA). The statistical analysis for evaluating the significance of the difference between means is performed by the incorrect Fisher’s least significant difference post hoc test. A P value <0.05 was considered statistically significant.

Results and Discussion

Isolation, Characterization, and Chemical Structures of Isolated Compounds

In this study, chromatographic separation of the MeOH extract of D. christyanum root led to the isolation of 13 compounds which included methyl haematommate (1),21 n-eicosyl trans-ferulate (2),22 methyl 2,4-dihydroxy-3,6-dimethylbenzoate (3),23 n-docosyl 4-hydroxy-trans-cinnamate (4),24 vanillin (5),25 coniferyl aldehyde (6),26 4,5-dihydroxy-2-methoxy-9,10-dihydrophenanthrene (7),27 moscatilin (8),28 aloifol I (9),29 gigantol (10),30 batatasin III (11),31 dendrosinen B (12),32 and dioricnolic acid (13)33 (Figure 1). The structures of these isolates were determined by comparing their ¹H NMR, ¹³C NMR, and MS data with literature values (see supplemental material).

Compounds 1, 3, and 13 were frequently found in lichens,34,35 but 3 was also obtained from a higher plant named Pygeum africantu.36 The phenolics 2 and 4 were esters of ferulic acid and cinnamic acid, respectively. Compounds 8–12 were identified as stilbenes and compound 7 as a phenanthrene derivative. All of the isolates 1–13 were then evaluated for α-glucosidase inhibition and glucose uptake stimulation activities.

α-Glucosidase Inhibitory Activity of the Compounds

In the assays for α-glucosidase inhibitory activity, we evaluated each compound at 100 µg/mL. Only 1, 3, 4, 6, 7, 10, and 13 exhibited more than 70% inhibition and, thus, were further analyzed for half-maximal inhibitory concentration (IC₅₀) values. All of them showed stronger activity than acarbose, as evidenced by their lower IC₅₀ values (Table 1). The α-glucosidase inhibitory activities of methyl haematoominate (1) and dioricnolic acid (13) were reported for the first time in this study whereas those of 3, 4, 6, 7, and 10 were described earlier.17,37

Compound 4 displayed potent α-glucosidase inhibitory activity, but 2 was devoid of such activity, despite their structural similarity. Other research groups have also reported several esters of trans-cinnamic acid derivatives as strong AGIs.39,40 On the other hand, studies revealed that trans-tetracosylferulate and cis-docosylferulate and cis-tetracosylferulate from D. tortile and D. scarbrilingue17,37 did not possess α-glucosidase inhibitory activity. It appears that the presence of the MeO group in the aromatic ring might cause the loss of activity in the ferulic acid esters.
Glucose Uptake Stimulatory Activity of the Compounds

In the study for the glucose uptake stimulatory effect, we evaluated all compounds, except for 1 and 10 because of their inadequate quantity, using rat L6 myotubes as the test model. Each compound was first screened for cytotoxicity at 3 different concentrations (1, 10, and 100 µg/mL) (Figure 2(a)). Percentages of cell viability above 80% were considered as non-cytotoxicity. The sample was then evaluated for glucose uptake stimulatory activity at non-toxic concentrations, as shown in Table 2. When tested at 100 µg/mL, n-docosyl 4-hydroxy-trans-cinnamate (4) (0.212 mM), vanillin (5) (0.657 mM), and coniferyl aldehyde (6) (0.561 mM) enhanced glucose uptake by L6 myotubes by 31.6% ± 4.4%, 97.1% ± 8.7%, and 56.4% ± 2.5%, respectively, without toxicity. Furthermore, aloifol (9) (0.036 mM) and batatasin III (11) at 10 µg/mL (0.041 mM) showed enhancement of 11.3% ± 2.5% and 30.2% ± 6.7%, respectively. (Figure 2(b)). In a previous report, gigantol (10) showed noticeable glucose uptake stimulation activity but it was cytotoxic to the cells.

The hypoglycemic potentials of vanillic acid and coniferyl aldehyde warrant close attention. In an earlier report, vanillic acid showed enhancement of glucose uptake by L6 myotubes and displayed synergistic effects with metformin and 2,4-thiazolidinedione. In a separate study, coniferyl aldehyde could reduce the plasma glucose concentration in streptozotocin-induced male diabetic Wistar rats. Interestingly,
Table 1. IC₅₀ Values of Compounds 1-13 Against α-Glucosidase Enzyme.

| Compounds                              | IC₅₀ (µM)       | IC₅₀ (µg/mL) |
|----------------------------------------|----------------|-------------|
| Methyl haematommate (1)                | 18.67 ± 2.06   | 3.925 ± 0.43|
| n-Eicosyl trans-ferulate (2)           | NA             | NA          |
| Methyl 2,4-dihydroxy-3,6-dimethylbenzoate (3) | 47.76 ± 3.33   | 9.37 ± 0.65 |
| n-Docosyl 4-hydroxy-trans-cinnamate (4) | 4.61 ± 0.24    | 2.18 ± 0.11 |
| Vanillin (5)                           | NA             | NA          |
| Coniferyl aldehyde (6)                 | 66.39 ± 4.7    | 11.83 ± 0.85|
| 4,5-Dihydroxy-2-methoxy-9,10-dihydrophenanthrene (7) | 133.11 ± 10.82 | 32.25 ± 2.62|
| Moscatilin (8)                         | NA             | NA          |
| Alofol I (9)                           | NA             | NA          |
| Gigantol (10)                          | 79.87 ± 14.20  | 21.91 ± 3.89|
| Batatasin III (11)                     | NA             | NA          |
| Dendrosinen B (12)                     | NA             | NA          |
| Diorcinolic acid (13)                  | 31.79 ± 2.42   | 10.12 ± 0.77|
| Acarbose (positive control)            | 724.74 ± 46    | 467.90 ± 29.72|

Abbreviation: IC₅₀, half-maximal inhibitory concentration.
NA means no inhibitory activity.

Figure 2. Cytotoxicity (a) and glucose uptake stimulatory activity (b) of extract and isolated compounds.
Table 2. Glucose Uptake Stimulation of *Dendrobium christyanum*.

| Component                          | Glucose uptake (%) | Enhancement (%) |
|------------------------------------|--------------------|-----------------|
| DMSO                               | 100                | 0               |
| Metformin (2 mM)                   | 207.3 ± 9.1        | 107.3 ± 9.1     |
| Insulin (500 nM)                   | 192.7 ± 13.1       | 92.7 ± 13.1     |
| MeOH extract 1 µg/mL               | 105.5 ± 11.5       | NA              |
| MeOH extract 10 µg/mL              | 134.5 ± 51.7       | 34.5 ± 51.7     |
| MeOH extract 100 µg/mL             | 156.4 ± 19.7       | 56.4 ± 19.7     |
| 1α-Eicosyl trans-ferulate (2)      |                    |                 |
| 1 µg/mL (0.002 mM)                 | 88.0 ± 24.3        | NA              |
| 10 µg/mL (0.021 mM)                | 109.8 ± 4.4        | NA              |
| 100 µg/mL (0.211 mM)               | 117.1 ± 5.0        | NA              |
| Methyl 2,4-dihydroxy-3,6- dimethylbenzoate (3) |              |                 |
| 1 µg/mL (0.005 mM)                 | 64.7 ± 6.7         | NA              |
| 10 µg/mL (0.051 mM)                | 82.2 ± 14.0        | NA              |
| 100 µg/mL (0.510 mM)               | NT                 | NA              |
| 1α-Docosyl 4-hydroxy-trans-cinnamate (4) |            |                 |
| 1 µg/mL (0.002 mM)                 | 32.7 ± 2.5         | NA              |
| 10 µg/mL (0.021 mM)                | 56.0 ± 19.7        | NA              |
| 100 µg/mL (0.212 mM)               | 131.6 ± 4.4        | 31.6 ± 4.4      |
| Vanillin (5)                       |                    |                 |
| 1 µg/mL (0.006 mM)                 | 77.8 ± 27.7        | NA              |
| 10 µg/mL (0.066 mM)                | 92.4 ± 7.6         | NA              |
| 100 µg/mL (0.657 mM)               | 197.1 ± 8.7        | 97.1 ± 8.7      |
| Coniferyl aldehyde (6)             |                    |                 |
| 1 µg/mL (0.005 mM)                 | 88.0 ± 7.6         | NA              |
| 10 µg/mL (0.056 mM)                | 90.9 ± 20.2        | NA              |
| 100 µg/mL (0.561 mM)               | 156.4 ± 2.5        | 56.4 ± 2.5      |
| 4,5-Dihydroxy-2-methoxy -9,10-dihydrophenanthrene (7) |              |                 |
| 1 µg/mL (0.004 mM)                 | 72.0 ± 9.1         | NA              |
| 10 µg/mL (0.041 mM)                | 79.3 ± 4.4         | NA              |
| 100 µg/mL (0.413 mM)               | 118.5 ± 15.1       | NA              |
| Moscatilin (8)                     |                    |                 |
| 1 µg/mL (0.003 mM)                 | 76.4 ± 13.3        | NA              |
| 10 µg/mL (0.033 mM)                | 79.3 ± 4.4         | NA              |
| 100 µg/mL (0.329 mM)               | 108.4 ± 2.5        | NA              |
| Aloifol I (9)                      |                    |                 |
| 1 µg/mL (0.003 mM)                 | 66.2 ± 20.0        | NA              |
| 10 µg/mL (0.036 mM)                | 111.3 ± 2.5        | 11.3 ± 2.5      |
| 100 µg/mL (0.365 mM)               | NT                 | NA              |
| Bataterin III (11)                 |                    |                 |
| 1 µg/mL (0.004 mM)                 | 117.1 ± 18.2       | NA              |
| 10 µg/mL (0.041 mM)                | 130.2 ± 6.7        | 30.2 ± 6.7      |
| 100 µg/mL (0.409 mM)               | NT                 | NA              |
| Dendrosinen B (12)                 |                    |                 |
| 1 µg/mL (0.004 mM)                 | 44.4 ± 0.0         | NA              |
| 10 µg/mL (0.038 mM)                | 54.5 ± 2.5         | NA              |
| 100 µg/mL (0.384 mM)               | NT                 | NA              |
| Diocrinolic acid (13)              |                    |                 |
| 1 µg/mL (0.003 mM)                 | 69.1 ± 6.7         | NA              |
| 10 µg/mL (0.031 mM)                | 70.5 ± 34.9        | NA              |
| 100 µg/mL (0.314 mM)               | 74.9 ± 11.5        | NA              |

Abbreviations: NT, not tested due to toxicity; DMSO, dimethyl sulfoxide; NA, not applicable.

*P < 0.05 significantly different when compared with the control (DMSO).
in the current investigation, vanillin (5) and coniferyl aldehyde (6) showed potent glucose uptake stimulatory activity. The findings in this study may help to understand the mechanisms of action underlying the hypoglycemic effects expressed by these phenolic compounds. Experiments in animals are suggested to confirm this postulation.

Conclusion
Chromatographic separation of D. christyanum root extract led to the isolation of 13 compounds (1-13). n-Docosyl 4-hydroxy-trans-cinnamate (4) exhibited potent α-glucosidase inhibitory activity with an IC₅₀ value of 4.61 ± 0.24 μM. Moreover, 6 other compounds also showed higher α-glucosidase inhibitory activity than the drug acarbose (724.74 ± 46 μM): methyl haematommate (1) (18.67 ± 2.06 μM), methyl 2,4-dihydroxy-3,6-dimethylbenzoate (3) (47.76 ± 3.33 μM), coniferyl aldehyde (6) (66.39 ± 4.7 μM), 4,5-dihydroxy-2-methoxy-9,10-dihydrophenanthrene (7) (133.11 ± 10.82 μM), gigan- tol (10) (79.87 ± 14.20 μM), and diorcinolic acid (13) (31.79 ± 2.42 μM). Furthermore, at the concentration of 100 μg/mL, n-docosyl 4-hydroxy-trans-cinnamate (4) (0.212 mM), vanillin (5) (0.657 mM), and coniferyl aldehyde (6) (0.561 mM) could enhance the glucose uptake by rat L6 myotubes by 31.6% ± n-Docosyl 4-hydroxy-trans-cinnamate. These findings in this study may help to understand the mechanisms of action underlying the hypoglycemic effects expressed by these phenolic compounds. Experiments in animals are suggested to confirm this postulation.

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