



## Original Article

# $\alpha$ -Glucosidase and pancreatic lipase inhibitory activities and glucose uptake stimulatory effect of phenolic compounds from *Dendrobium formosum*



Prachyaporn Inthongkaew<sup>a</sup>, Nutputsorn Chatsumpun<sup>b</sup>, Chonlakan Supasuteekul<sup>a</sup>,  
Tharita Kitisripanya<sup>c</sup>, Waraporn Putalun<sup>c</sup>, Kittisak Likhitwitayawuid<sup>a</sup>, Boonchoo Sritularak<sup>a,\*</sup>

<sup>a</sup> Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

<sup>b</sup> Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand

<sup>c</sup> Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand

## ARTICLE INFO

## Article history:

Received 11 March 2017

Accepted 26 May 2017

Available online 11 July 2017

## Keywords:

*Dendrobium formosum*

Glucose uptake

$\alpha$ -Glucosidase

Pancreatic lipase

## ABSTRACT

A methanol extract from the whole plant of *Dendrobium formosum* Roxb. ex Lindl., Orchidaceae, showed inhibitory potential against  $\alpha$ -glucosidase and pancreatic lipase enzymes. Chromatographic separation of the extract resulted in the isolation of twelve phenolic compounds. The structures of these compounds were determined through analysis of NMR and HR-ESI-MS data. All of the isolates were evaluated for their  $\alpha$ -glucosidase and pancreatic lipase inhibitory activities, as well as glucose uptake stimulatory effect. Among the isolates, 5-methoxy-7-hydroxy-9,10-dihydro-1,4-phenanthrenequinone (**12**) showed the highest  $\alpha$ -glucosidase and pancreatic lipase inhibitory effects with an  $IC_{50}$  values of  $126.88 \pm 0.66 \mu\text{M}$  and  $69.45 \pm 10.14 \mu\text{M}$ , respectively. An enzyme kinetics study was conducted by the Lineweaver-Burk plot method. The kinetics studies revealed that compound **12** was a non-competitive inhibitor of  $\alpha$ -glucosidase and pancreatic lipase enzymes. Moreover, lusianthridin at 1 and  $10 \mu\text{g/ml}$  and moscatilin at  $100 \mu\text{g/ml}$  showed glucose uptake stimulatory effect without toxicity on L6 myotubes. This study is the first report on the phytochemical constituents and anti-diabetic and anti-obesity activities of *D. formosum*.

© 2017 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia that can cause further serious health problems such as neurological and cardiovascular complications (Peng et al., 2016). There are three main types of diabetes which are type I, type II, and gestational diabetes. Type II diabetes, which is due to insulin resistance, affects the majority (90–95%) of diabetic patients (American Diabetes Association, 2006; Rosak and Mertes, 2012).  $\alpha$ -Glucosidase is a carbohydrate-hydrolyzing enzyme secreted from the intestinal chorionic epithelium. Inhibition of this enzyme is one of the therapeutic approaches for type II diabetes since it can cause retardation of carbohydrate digestion, which leads to the prevention of excess glucose absorption (You et al., 2012; Peng et al., 2016). Insulin plays a key role in reduction of the glucose level by stimulating the glucose transport from blood into skeletal muscle

cells. It is well established that insulin-stimulated glucose uptake is impaired in type II diabetic patients (Yap et al., 2007; Choi et al., 2013). Therefore, searching for compounds that can enhance glucose uptake is an important approach to facilitate the development of new methods for insulin resistance treatment (Choi et al., 2013).

In addition, type II diabetes can be caused by the dysfunction of insulin-producing pancreatic  $\beta$  cells, which could be instigated by the excessive accumulation of lipids in the pancreas (Tushuizen et al., 2007; You et al., 2012). Pancreatic lipase is the key enzyme in lipid digestion, responsible for absorption of dietary fats through the breakdown of triacylglycerols into free fatty acids and monoacylglycerols in the intestinal lumen (Yang et al., 2014). Recently, inhibitors of pancreatic lipase have attracted much research interest due to their anti-obesity activity by delaying the lipolytic process. This action would lead to the decrease in lipid absorption and thus protect the pancreas, which will restore regular insulin production from the  $\beta$  cells (Tushuizen et al., 2007; You et al., 2012; Yang et al., 2014).

*Dendrobium* is a large genus in the Orchidaceae family which include about 1100 species, and 150 species have been identified

\* Corresponding author.

E-mail: [boonchoo.sr@chula.ac.th](mailto:boonchoo.sr@chula.ac.th) (B. Sritularak).

in Thailand (Lam et al., 2015; Limpanit et al., 2016). Several *Dendrobium* species are well-known for their traditional medicinal properties. The stems of several species of *Dendrobium* have been used in folk Chinese medicine called “Shi-Hu” as sources of tonic, antipyretic, astringent, and anti-inflammatory substances (Hu et al., 2008; Lam et al., 2015). Previous studies revealed that *Dendrobium* plants contain diverse groups of secondary metabolites and possess various biological activities, including cytotoxic, antioxidant, anticancer, antimalarial, antifibrotic, hypoglycemic, and neuroprotective activities (Lam et al., 2015). A number of phenolic compounds from *Dendrobium tortile*, such as 3,4-dihydroxy-5,4'-dimethoxybibenzyl, (2*S*)-eriodictyol and dendrofalconerol A, showed strong  $\alpha$ -glucosidase inhibitory activity in our recent investigation (Limpanit et al., 2016).

*Dendrobium formosum* Roxb. ex Lindl. is a rare orchid native to Himalayas and Indochina. It has one of the largest flowers among the dendrobies (Dohling et al., 2008). An earlier report of *D. formosum* described potential antitumor activity of its ethanolic extract (Prasad and Koch, 2014). However, prior to this study, there have been no reports of the phytochemical constituents and anti-diabetic and anti-obesity activities of this plant. As part of our ongoing research on bioactive constituents from *Dendrobium* species (Sukphan et al., 2014; Klengkumnuankarn et al., 2015), a methanol extract from the whole plant of *D. formosum* at a concentration of 50  $\mu$ g/ml was evaluated and found to exhibit 95% inhibition against both  $\alpha$ -glucosidase and pancreatic lipase enzymes. In this communication, we wish to report the first study on the chemical constituents of *D. formosum* and their  $\alpha$ -glucosidase and pancreatic lipase inhibitory activities, as well as their glucose uptake stimulatory potential.

## Material and methods

### General

Mass spectra were recorded on a Bruker micro TOF mass spectrometer (ESI-MS). NMR spectra were recorded on a Bruker Avance DPX-300 FT-NMR spectrometer. Microtiter plate reading was performed on a Perkin-Elmer Victor™ 1420 multilabel counter. Vacuum-liquid column chromatography (VLC) and column chromatography (CC) were performed on silica gel 60 (Merck, Kieselgel 60, 70–320  $\mu$ m), silica gel 60 (Merck, Kieselgel 60, 230–400  $\mu$ m) and Sephadex LH-20 (25–100  $\mu$ m, GE Healthcare).

### Chemicals

Alpha minimal essential medium ( $\alpha$ -MEM), fetal bovine serum (FBS) and penicillin-streptomycin (10000 IU/ml) were purchased from Thermo Fisher Scientific (Grand Island, NY, USA). Glucose Oxidase (GO) assay kit, sodium dodecyl sulfate (SDS), 3-(4,5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT),  $\alpha$ -glucosidase from *Saccharomyces cerevisiae*, lipase from porcine pancreas, 4-methylumbelliferyl oleate (4MUO), *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG), acarbose and orlistat were obtained from Sigma Aldrich (St Louis, MO, USA). Insulin (100 IU/ml) was obtained from Biocon (Bangalore, India). All other chemicals used were of analytical grade.

### Cell lines and culture medium

L6 (Rat skeletal muscle, ATCC® CRL-1458) cell culture was purchased from the American Type Culture Collection (Manassas, VA, USA). Stock cells of L6 were cultured in  $\alpha$ -MEM supplemented with 10% FBS, 1% penicillin-streptomycin, the growth medium, at 37 °C under 5% CO<sub>2</sub>.

### Plant material

The whole plant of *Dendrobium formosum* Roxb. ex Lindl., Orchidaceae, was purchased from Jatujak market, Bangkok, Thailand in September 2015. It was collected from Mae Sot district, Tak province, Thailand. Plant identification was performed by Prof. Thatri Phadungcharoen (Faculty of Pharmacy, Rangsit University). A voucher specimen (BS-DF-092558) is deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

### Extraction and isolation

The dried and powdered whole plant of *D. formosum* (2 kg) was extracted with MeOH (3  $\times$  10 l) at room temperature to give a viscous mass of dried extract (115 g) after removal of the solvent. This material was suspended in water and then partitioned with EtOAc and *n*-butanol to give an EtOAc extract (57 g), a butanol extract (25 g) and an aqueous extract (30 g), respectively, after evaporation of the solvent. The EtOAc extract was subjected to vacuum liquid chromatography (silica gel, EtOAc-hexane, gradient) to give eight fractions (A–H). Fraction F (6.8 g) was fractionated on a silica gel column (EtOAc-hexane, gradient) to give nine fractions (FI–FIX). Fraction FII (271 mg) was separated by column chromatography (CC) over silica gel, eluted with a CH<sub>2</sub>Cl<sub>2</sub>-hexane gradient to give four fractions (FII1–FII4). Confusarin (**1**) (3 mg) was obtained from fraction FII2. Hircinol (**2**) (15 mg) was obtained from fraction FII4 (21 mg) after purification on Sephadex LH-20 (MeOH). Purification of fraction FIII (85 mg) on Sephadex LH-20 (MeOH) gave erianthridin (**3**) (45 mg). Fraction FV (648 mg) was separated by CC (silica gel, CH<sub>2</sub>Cl<sub>2</sub>-hexane, gradient) to give six fractions (FV1–FV6). Gigantol (**4**) (94 mg) was obtained from fraction FV3. Fraction FV2 (17 mg) was further purified on Sephadex LH-20 (MeOH) to afford nudol (**5**) (10 mg). Fraction FV5 (193 mg) was subjected to CC (silica gel, EtOAc-hexane, gradient) and then purified on Sephadex LH-20 (MeOH) to yield lusianthridin (**6**) (8 mg). Fraction FVI (361 mg) was fractionated by CC (silica gel, CH<sub>2</sub>Cl<sub>2</sub>-hexane, gradient) to give six fractions (FVI1–FVI6). Coelonin (**7**) (75 mg) was obtained from fraction FVI2. Dihydroconiferyl dihydro-*p*-coumarate (**8**) (25 mg) and batatasin III (**9**) (18 mg) were yielded from fractions FVI4 (57 mg) and FVI5 (29 mg), respectively, after purification on Sephadex LH-20 (MeOH). Fraction FVIII (272 mg) was separated by CC (silica gel, EtOAc-CH<sub>2</sub>Cl<sub>2</sub>, gradient) and then purified on Sephadex LH-20 (MeOH) to afford 2,5,7-trihydroxy-4-methoxy-9,10-dihydrophenanthrene (**10**) (22 mg). Fraction G (10 g) was fractionated by CC over silica gel, eluted with an EtOAc-hexane gradient to give seven fractions (GI–GVII). Fraction GIII (508 mg) was separated on Sephadex LH-20 (MeOH) to give eight fractions (GIII1–GIII8). Fraction GIII2 (51 mg) was further purified by CC (silica gel, CH<sub>2</sub>Cl<sub>2</sub>-hexane, gradient) to yield moscatilin (**11**) (5 mg). 5-Methoxy-7-hydroxy-9,10-dihydro-1,4-phenanthrenequinone (**12**) (11 mg) was obtained from fraction GIII4 (52 mg) after purification by CC (silica gel, MeOH-CH<sub>2</sub>Cl<sub>2</sub>, gradient).

Confusarin (**1**): yellow amorphous solid; C<sub>17</sub>H<sub>16</sub>O<sub>5</sub>; HR-ESI-MS *m/z* 299.0919 [M–H]<sup>–</sup> (calc. for C<sub>17</sub>H<sub>15</sub>O<sub>5</sub> requires 299.0919). Its structure was identified by comparison of NMR data with published values (Majumder and Kar, 1987).

Hircinol (**2**): yellow amorphous solid; C<sub>15</sub>H<sub>14</sub>O<sub>3</sub>; HR-ESI-MS *m/z* 265.0847 [M+Na]<sup>+</sup> (calc. for C<sub>15</sub>H<sub>14</sub>O<sub>3</sub>Na requires 265.0840). Its structure was identified by comparison of NMR data with published values (Fisch et al., 1973).

Erianthridin (**3**): yellow amorphous solid; C<sub>16</sub>H<sub>16</sub>O<sub>4</sub>; HR-ESI-MS *m/z* 295.0949 [M+Na]<sup>+</sup> (calc. for C<sub>16</sub>H<sub>16</sub>O<sub>4</sub>Na requires 295.0946). Its structure was identified by comparison of NMR data with published values (Majumder and Joardar, 1985).

Gigantol (**4**): brown amorphous solid; C<sub>16</sub>H<sub>18</sub>O<sub>4</sub>; HR-ESI-MS *m/z* 297.1111 [M+Na]<sup>+</sup> (calc. for C<sub>16</sub>H<sub>18</sub>O<sub>4</sub>Na requires 297.1103). Its structure was identified by comparison of NMR data with published values (Chen et al., 2008).

Nudol (**5**): yellow amorphous solid; C<sub>16</sub>H<sub>14</sub>O<sub>4</sub>; HR-ESI-MS *m/z* 293.0793 [M+Na]<sup>+</sup> (calc. for C<sub>16</sub>H<sub>14</sub>O<sub>4</sub>Na requires 293.0790). Its structure was identified by comparison of NMR data with published values (Bhandari et al., 1985).

Lusianthridin (**6**): brown amorphous solid; C<sub>15</sub>H<sub>14</sub>O<sub>3</sub>; HR-ESI-MS *m/z* 265.0847 [M+Na]<sup>+</sup> (calc. for C<sub>15</sub>H<sub>14</sub>O<sub>3</sub>Na requires 265.0841). Its structure was identified by comparison of NMR data with published values (Guo et al., 2007).

Coelonin (**7**): brown amorphous solid; C<sub>15</sub>H<sub>14</sub>O<sub>3</sub>; HR-ESI-MS *m/z* 265.0845 [M+Na]<sup>+</sup> (calc. for C<sub>15</sub>H<sub>14</sub>O<sub>3</sub>Na requires 265.0841). Its structure was identified by comparison of NMR data with published values (Majumder et al., 1982).

Dihydroconiferyl dihydro-*p*-coumarate (**8**): yellow amorphous solid; C<sub>19</sub>H<sub>22</sub>O<sub>5</sub>; HR-ESI-MS *m/z* 353.1368 [M+Na]<sup>+</sup> (calc. for C<sub>19</sub>H<sub>22</sub>O<sub>5</sub>Na requires 353.1365). Its structure was identified by comparison of NMR data with published values (Zhang et al., 2006).

Batatasin III (**9**): brown amorphous solid; C<sub>15</sub>H<sub>16</sub>O<sub>3</sub>; HR-ESI-MS *m/z* 267.0995 [M+Na]<sup>+</sup> (calc. for 267.0997, C<sub>15</sub>H<sub>16</sub>O<sub>3</sub>Na). Its structure was identified by comparison of NMR data with published values (Sachdev and Kulshreshtha, 1986).

2,5,7-Trihydroxy-4-methoxy-9,10-dihydrophenanthrene (**10**): brown amorphous solid; C<sub>15</sub>H<sub>14</sub>O<sub>4</sub>; HR-ESI-MS *m/z* 281.0791 [M+Na]<sup>+</sup> (calc. for C<sub>15</sub>H<sub>14</sub>O<sub>4</sub>Na requires 281.0790). Its structure was identified by comparison of NMR data with published values (Hu et al., 2008).

Moscaticin (**11**): brown amorphous solid; C<sub>17</sub>H<sub>20</sub>O<sub>5</sub>; HR-ESI-MS *m/z* 327.1219 [M+Na]<sup>+</sup> (calc. for C<sub>17</sub>H<sub>20</sub>O<sub>5</sub>Na requires 327.1208). Its structure was identified by comparison of NMR data with published values (Majumder and Sen, 1987).

5-Methoxy-7-hydroxy-9,10-dihydro-1,4-phenanthrenequinone (**12**): red amorphous solid; C<sub>15</sub>H<sub>12</sub>O<sub>4</sub>; HR-ESI-MS *m/z* 279.0642 [M+Na]<sup>+</sup> (calc. for C<sub>15</sub>H<sub>12</sub>O<sub>4</sub>Na requires 279.0633). Its structure was identified by comparison of NMR data with published values (Sritularak et al., 2011).

#### Assay for $\alpha$ -glucosidase inhibitory activity

The assay was performed as described previously with a slight modification (Sun et al., 2014). The enzyme activity was assessed by monitoring the release of *p*-nitrophenol from the *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (pNPG) substrate. Each test sample was initially evaluated at a concentration of 50  $\mu$ g/ml, and then two-fold serial dilution was performed for IC<sub>50</sub> determination. In brief, 10  $\mu$ l of test sample (1.56–50  $\mu$ g/ml) and 40  $\mu$ l of 0.1 U/ml  $\alpha$ -glucosidase were mixed and allowed to react at 37 °C for 10 min in a 96-well microtiter plate. Then, 50  $\mu$ l of 2 mM pNPG was added and the reaction mixture was further incubated for 20 min. Finally, 100  $\mu$ l of 1 M Na<sub>2</sub>CO<sub>3</sub> solution was added to terminate the reaction. The absorption at 405 nm was then measured using a microplate reader. The percentage of  $\alpha$ -glucosidase inhibitory activity was calculated as follows:

$$\% \alpha\text{-glucosidase inhibitory activity} = \left[ \frac{A_c - A_s}{A_c} \right] \times 100$$

where  $A_c$  is the absorbance of the control and  $A_s$  is the absorbance of the sample. Acarbose (15.6–1000  $\mu$ g/ml) was used as a positive control and treated under the same conditions as the samples. Enzyme inhibition reactions for all samples were carried out in triplicate ( $n=3$ ), and each experiment consisted of three repetitions.

The enzyme kinetics study was performed using the double reciprocal Lineweaver–Burk plot. The experiment was conducted by varying the pNPG substrate concentration (0.125, 0.25, 1.0,

2.0 mM) in the absence and presence of different test sample concentrations (80 and 160  $\mu$ M).

#### Assay for pancreatic lipase inhibitory activity

Evaluation of pancreatic lipase inhibitory activity was done by measuring the release of 4-methylumbelliferone (4MU) from the substrate 4-methylumbelliferyl oleate (4MUO) (Sergent et al., 2012). Each test sample was initially evaluated at a concentration of 50  $\mu$ g/ml, and then two-fold serial dilution was performed for IC<sub>50</sub> determination. Briefly, 25  $\mu$ l of test sample (1.56–50  $\mu$ g/ml), 50  $\mu$ l of 0.25 mM 4MUO, and 25  $\mu$ l of 0.125 mg/ml pancreatic lipase were mixed and incubated at room temperature for 30 min in a 96-well microtiter plate. Then, 100  $\mu$ l of 0.1 M sodium citrate was added to stop the reaction. Fluorescence from the release of 4MU was measured using a microplate reader with excitation and emission wavelengths of 355 and 460 nm, respectively. The percentage of pancreatic lipase inhibitory activity was calculated as follows:

$$\% \text{ pancreatic lipase inhibitory activity} = \left[ \frac{A_c - A_s}{A_c} \right] \times 100$$

where  $A_c$  is the absorbance of the control and  $A_s$  is the absorbance of the sample. Orlistat (0.0008–50  $\mu$ g/ml) was used as a positive control and treated under the same conditions as the samples. Enzyme inhibition reactions for all samples were carried out in triplicate ( $n=3$ ), and each experiment consisted of three repetitions. The enzyme kinetics study was conducted using the double reciprocal Lineweaver–Burk analysis. The experiment was examined by varying the 4MUO substrate concentration (0.0625, 0.125, 0.25, 0.5, 1 mM) in the absence and presence of different test sample concentrations (40 and 80  $\mu$ M).

#### Glucose-uptake assay

The glucose-uptake assay was performed following the methods (Zhou et al., 2007; Jantaramanant et al., 2014) with some modification. Briefly, rat L6 myoblasts were maintained in  $\alpha$ -MEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C under 5% CO<sub>2</sub>. For treatment with test compounds, the cells were plated in 24-well plates at a density of  $2 \times 10^4$  cells/well. Once the cell reached 90% confluence, the media was switched to  $\alpha$ -MEM with 2% FBS and 1% penicillin-streptomycin (the differentiate medium). The cells were allowed to differentiate into myotubes for 5–7 days with media changed every other day. Then, the myotubes were incubated at 37 °C under 5% CO<sub>2</sub> for 24 h with the various concentrations (1, 10 and 100  $\mu$ g/ml) of test compound and 500 nM of insulin. The differentiate medium plus 0.1% DMSO was used as the diluent and negative control. Then, the medium was collected and analyzed for the glucose level using a glucose oxidase assay kit. The glucose-uptake was presented as the ratio of insulin relative which calculated as followed:

$$\begin{aligned} & \text{The ratio of insulin relative} \\ & = \frac{\% \text{ glucose uptake of the test compounds}}{\% \text{ glucose uptake of insulin}} \end{aligned}$$

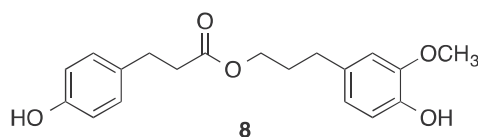
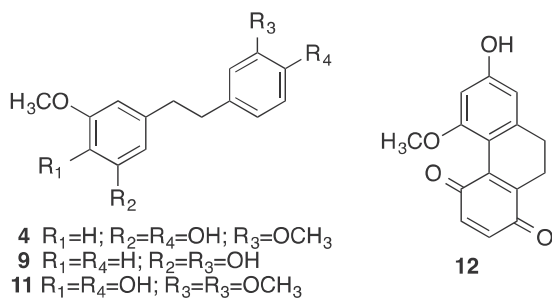
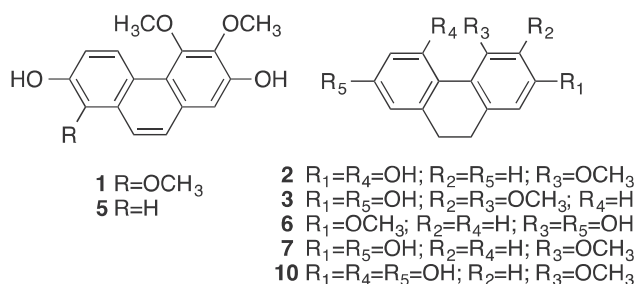
#### Cytotoxicity

Continuously, after 24 h of the cell treatment for the glucose determination, cytotoxicity test was performed following the method of Riss et al. with some modification (Riss et al., 2004). The medium was adjusted to 200  $\mu$ l per well. The cells were treated with 20  $\mu$ l of the MTT solution (5 mg/ml) and incubated at 37 °C under 5% CO<sub>2</sub> for 2 h. To dissolve the formazan crystal, each well was added 200  $\mu$ l of the solubilization solution (40%DMF, 2%

glacial acetic acid, 16%w/v SDS in distilled water) and shaken for 20 min. Then, the supernatants were collected and measured for absorbance at 595 nm using model 550 microplate reader (Biorad). The cytotoxicity was shown as the %cell viability.

## Results and discussion

As mentioned earlier, a MeOH extract prepared from the whole plant of *D. formosum*, at a concentration of 50 µg/ml, exhibited potent inhibition of 95% against both α-glucosidase and pancreatic lipase enzymes, respectively. The extract was then separated by solvent partition to give an EtOAc, a butanol and an aqueous extracts. These extracts were evaluated for their α-glucosidase and pancreatic lipase inhibitory activities. Only the EtOAc extract showed strong inhibitory effects at a concentration of 50 µg/ml, with 83% against pancreatic lipase and 96% against α-glucosidase enzymes while the butanol and an aqueous extract were inactive (less than 50% inhibition). Therefore, the EtOAc extract was selected for further chemical investigation. Through chromatographic separation, twelve phenolic compounds were identified.



Currently, only a few α-glucosidase inhibitors, such as acarbose and voglibose, have been approved for the treatment of diabetes mellitus, and their structures are mainly composed of sugar moieties (Yin et al., 2014). The production processes of these inhibitors are, however, rather complex and involve multistep procedures (Yin et al., 2014). With regard to pancreatic lipase enzyme, orlistat is a powerful inhibitor, clinically used for the treatment of obesity (Birari and Bhutani, 2007; Jang et al., 2008). In recent years, the numbers of reports on natural compounds with α-glucosidase and lipase inhibitory activities have continuously increased (Kim et al., 2000; Jang et al., 2008; Yin et al., 2014). Many researches have been focused on the search for alternative α-glucosidase inhibitors with non-sugar core structure, particularly the polyphenols due to their abundant availability in the nature and their promising biological activities (Yin et al., 2014).

**Table 1**

IC<sub>50</sub> values of compounds **1–12** isolated from *D. formosum* for α-glucosidase and pancreatic lipase inhibitory activities.

Compounds	α-Glucosidase (µM)	Pancreatic lipase (µM)
Confusarin ( <b>1</b> )	189.78 ± 1.11	154.61 ± 8.58
Hircinol ( <b>2</b> )	NA	NA
Erianthridin ( <b>3</b> )	NA	NA
Gigantol ( <b>4</b> )	NA	NA
Nudol ( <b>5</b> )	NA	NA
Lusianthridin ( <b>6</b> )	NA	NA
Coelonin ( <b>7</b> )	NA	NA
Dihydroconiferyl dihydro-p-coumarate ( <b>8</b> )	NA	NA
Batatasin III ( <b>9</b> )	NA	NA
2,5,7-Trihydroxy-4-methoxy-9,10-dihydrophenanthrene ( <b>10</b> )	NA	NA
Moscaticin ( <b>11</b> )	NA	NA
5-Methoxy-7-hydroxy-9,10-dihydro-1,4-phenanthrenequinone ( <b>12</b> )	126.88 ± 0.66	69.45 ± 10.14
Acarbose	745.9 ± 88.4	–
Orlistat	–	0.013 ± 0.004

NA means no inhibitory activity.

In this study, compounds **1–12** were evaluated for their α-glucosidase and pancreatic lipase inhibitory activities. Each compound was initially tested at a concentration of 50 µg/ml. Only compounds **1** and **12** showed >50% inhibition and were further analyzed to determine their IC<sub>50</sub> values (Table 1). Due to its high potency and availability, compound **12** was investigated for inhibition mechanisms against α-glucosidase and pancreatic lipase using the Lineweaver–Burk plots, and kinetic parameters with respect to the two enzymes were obtained, as listed in Table 2.

In the assay of α-glucosidase inhibitory activity with pNPG as the substrate, the maximum velocity ( $V_{max}$ ) value was determined as  $7.10 \times 10^{-3} \Delta A_{405}/\text{min}$ , and the Michaelis–Menten constant ( $K_m$ ) as 0.3 mM (Fig. 1A). The presence of compound **12** at difference concentrations (80 µM and 160 µM) reduced the  $V_{max}$  values to  $2.70 \times 10^{-3}$  and  $7.35 \times 10^{-4}$  respectively, but did not affect  $K_m$  of the enzyme. These results suggested that **12** is a non-competitive inhibitor of this enzyme.

In experiments on pancreatic lipase inhibitory activity with 4MUO as the substrate, the  $V_{max}$  value was found to be  $1.35 \times 10^5 \Delta A_{355,460}/\text{min}$ , and the  $K_m$  value was 0.4 mM (Fig. 1B). The presence of compound **12** at a concentration of 40 µM and 80 µM decreased the  $V_{max}$  values to  $9.60 \times 10^4$  and  $7.60 \times 10^4$ , respectively, but had no effect on  $K_m$ . These observations indicated that **12** is also a non-competitive inhibitor of pancreatic lipase.

The non-competitive mode of α-glucosidase and pancreatic lipase inhibitions obtained from the Lineweaver–Burk plots suggests that compound **12** does not compete with pNPG and 4MUO substrates for binding to the active site of enzymes, but it would rather bind to other sites of the enzymes to retard the carbohydrate and lipid digestion (Kazeem et al., 2013; Martinez-Gonzalez et al., 2017). The  $K_m$  values were unaffected because the inhibitor (**12**) did not cause any changes at the active site. The  $V_{max}$  of the reactions decreased because this non-competitive inhibitor (**12**) reduced the quantity of active enzymes (Balbaa and El Ashry, 2012; Kazeem et al., 2013).

Various classes of natural phenolic compounds exhibited the non-competitive type of inhibition on α-glucosidase and pancreatic lipase (Martinez-Gonzalez et al., 2017). Our recent studies have also been revealed the non-competitive α-glucosidase inhibition for dendrofalconerol A, which is a dimeric stillbene isolated from *D. tortile* (Limpanit et al., 2016). Non-competitive inhibitors offer the major advantage of not being affected by the higher



**Table 2**  
Kinetic parameters in of  $\alpha$ -glucosidase and pancreatic lipase in the presence of 5-methoxy-7-hydroxy-9,10-dihydro-1,4-phenanthrenequinone (**12**).

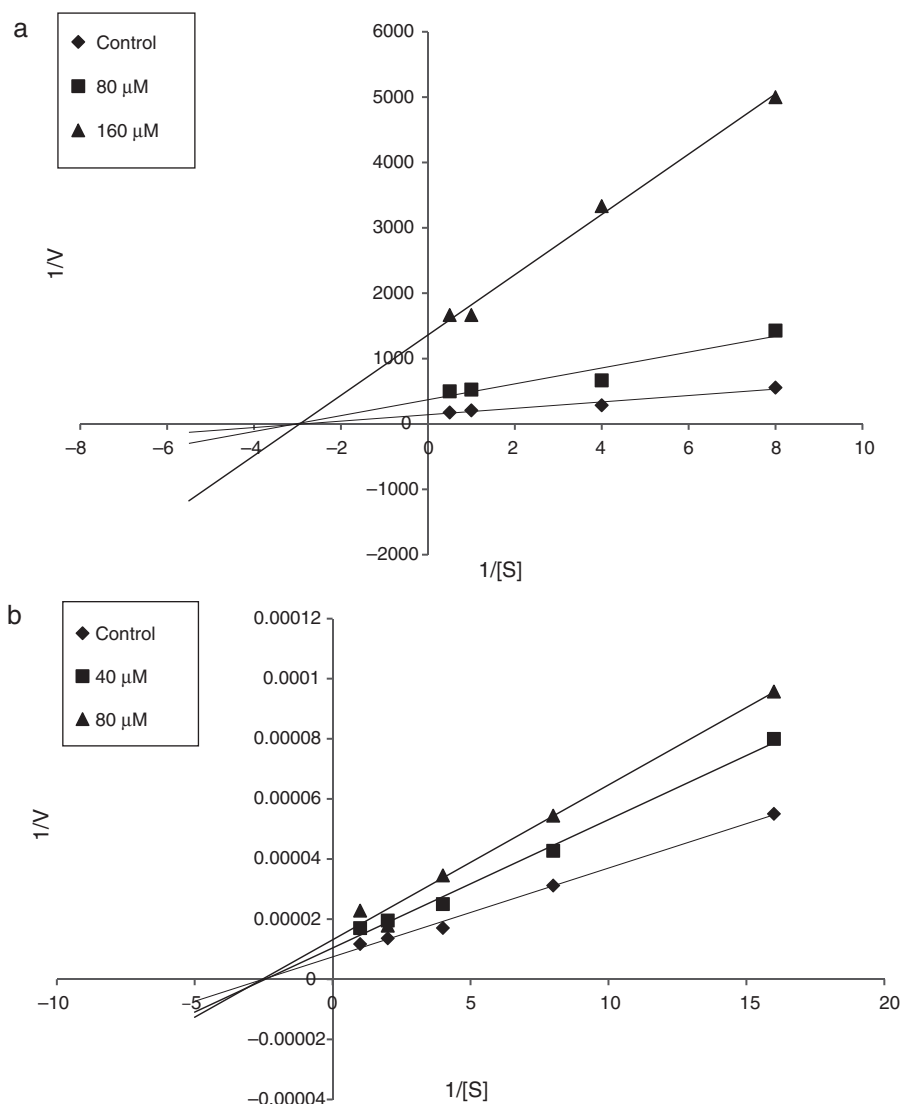
Inhibitors	$\alpha$ -Glucosidase			Pancreatic lipase		
	Dose ( $\mu$ M)	$V_{\max}$ ( $\Delta A_{405}/\text{min}$ )	$K_m$ (mM)	Dose ( $\mu$ M)	$V_{\max}$ ( $\Delta A_{355,460}/\text{min}$ )	$K_m$ (mM)
None	–	$7.10 \times 10^{-3}$	0.3	–	$1.35 \times 10^5$	0.4
Compound <b>12</b>	80	$2.70 \times 10^{-3}$	0.3	40	$9.60 \times 10^4$	0.4
	160	$7.35 \times 10^{-4}$	0.3	80	$7.60 \times 10^4$	0.4

concentrations of the substrate as compared to the competitive inhibitors such as acarbose and orlistat, which may require large amounts of inhibitors to compete with the substrate (Ghadyale et al., 2012).

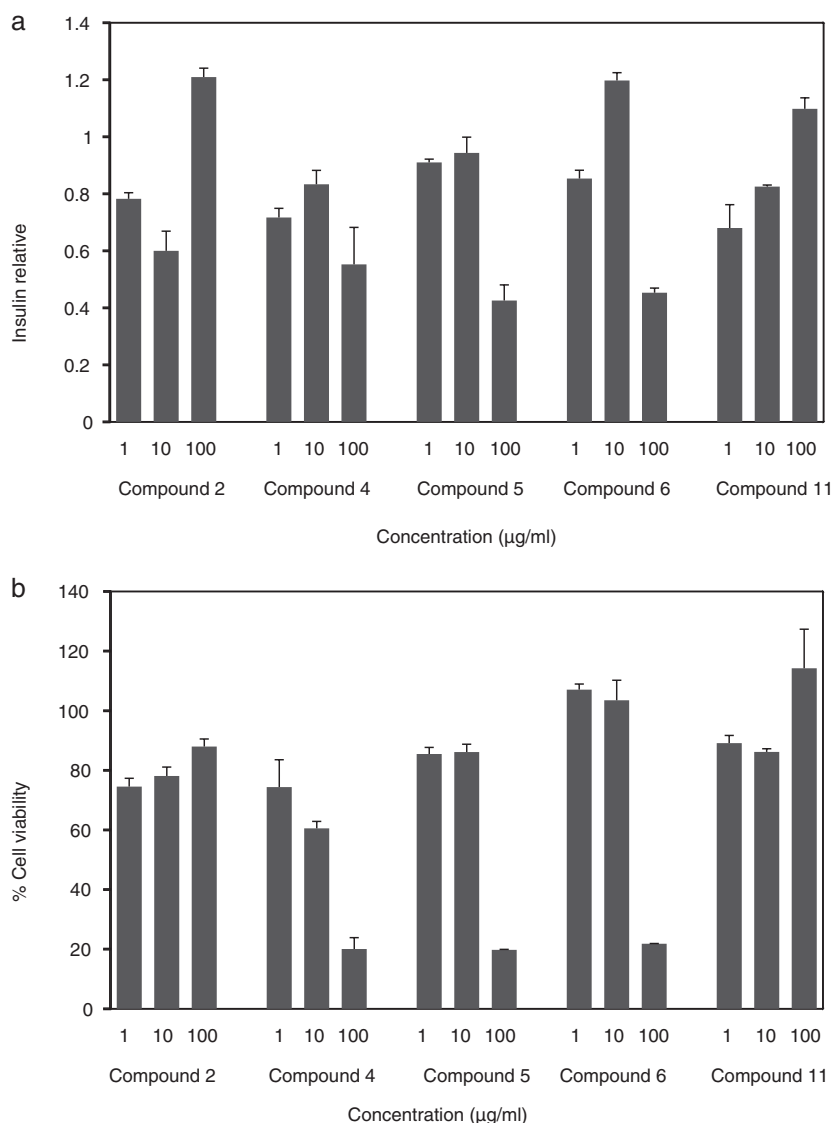
The treatment for patients with type II diabetes requires several types of medications to control the blood glucose level (Nathan et al., 2009). This implies that new glycemic control agents with novel mechanisms are indeed needed. Recent studies in L6 skeletal muscle cells showed that the stilbenoids resveratrol and piceatannol displayed antidiabetic activity by promoting glucose uptake (Breen et al., 2008; Minakawa et al., 2012). This led us to investigate the stilbene derivatives **1–12** in the hope of finding new glucose-uptake stimulators. Our preliminary evaluation of

these compounds revealed that hircinol (**2**), lusianthridin (**6**) and moscatilin (**11**) showed higher glucose uptake stimulation than insulin (insulin relative value  $>1.0$ ) (Fig. 2A). However, hircinol (**2**) displayed the activity at the concentration level that showed toxicity on L6 myotubes whereas lusianthridin (**6**) and moscatilin (**11**) did not (Fig. 2B). When we considered the glucose-uptake stimulation potencies of the test compounds at non-toxic concentration levels ( $\geq 100\%$  cell viability), we found that compound **6** at  $1 \mu\text{g}/\text{ml}$  exhibited recognizable glucose-uptake stimulatory activity, with insulin relative value  $\geq 0.8$ .

Maintaining the balance in blood glucose level is an important process in human physiology, and this is regulated by hormones such as insulin and glucagon (Hanhineva et al., 2010). Failure of



**Fig. 1.** Lineweaver–Burk plot of  $\alpha$ -glucosidase (A) and pancreatic lipase (B) in the presence and absence of 5-methoxy-7-hydroxy-9,10-dihydro-1,4-phenanthrenequinone (**12**).



**Fig. 2.** Stimulation of glucose uptake (A) and cytotoxicity (B) of L6 myotubes by hircinol (**2**), gigantol (**4**), nudol (**5**), lusianthridin (**6**) and moscatilin (**11**) at a different concentration in the range of 1–100 µg/ml.

this control can cause an incidence of metabolic syndrome (MetS), a cluster of metabolic abnormalities that include insulin resistance, hyperglycaemia or type II diabetes, obesity, hypertension, and dyslipidemia (Hanhineva et al., 2010; Li et al., 2013; Mohamed, 2014). MetS is known to increase the risk of atherosclerotic cardiovascular disease, eventually leading to morbidity and mortality. Numerous dietary components and over 800 plants are found to prevent or reduce MetS by assisting the carbohydrate metabolism and glucose homeostasis through the inhibition of carbohydrate digestion, stimulation of insulin secretion from the pancreatic  $\beta$ -cells, suppression of glucose release from the liver storage, activation of insulin receptors and improvement glucose uptake in the peripheral tissues (Hanhineva et al., 2010; Mohamed, 2014). In this study, the inhibition of carbohydrate and lipid hydrolyzing enzymes by compound **12** may reduce the rate of their cleavage, component release and absorption in the small intestine, and consequently suppress postprandial hyperglycemia and hyperlipidemia. Moreover, compounds **6** and **11** at non-toxic concentrations may find a role in helping to control glucose metabolism by stimulating glucose uptake in skeletal muscle, the largest site of glucose disposal, leading to the prevention of MetS

and type II diabetes. However, further investigations in animal models are still needed to confirm these suggestions.

## Conclusions

So far, there have been a few attempts to investigate the constituents of *Dendrobium* for  $\alpha$ -glucosidase and pancreatic lipase inhibitory activities, and glucose-lowering effects. In this study, the chromatographic separation of the methanolic extract from the whole plant of *D. formosum* led to the isolation and identification of twelve compounds, including confusarin (**1**), hircinol (**2**), erianthridin (**3**), gigantol (**4**), nudol (**5**), lusianthridin (**6**), coelonin (**7**), dihydroconiferyl dihydro-*p*-coumarate (**8**), batatasin III (**9**), 2,5,7-trihydroxy-4-methoxy-9,10-dihydrophenanthrene (**10**), moscatilin (**11**), and 5-methoxy-7-hydroxy-9,10-dihydro-1,4-phenanthrenequinone (**12**). Among these compounds, compounds **1** and **12** showed higher  $\alpha$ -glucosidase inhibitory activity than the drug acarbose, but none of the isolates displayed stronger lipase inhibitory activity than the positive control orlistat. The kinetics studies revealed that compound **12** was a non-competitive inhibitor of both  $\alpha$ -glucosidase and pancreatic lipase enzymes.

With regard to glucose-uptake stimulation effects, lusianthridin (**6**) and moscatilin (**11**) at non-toxic concentration (10 and 100 µg/ml, respectively) had higher activity than insulin. In addition, lusianthridin (**6**) at 1 µg/ml showed recognizable glucose uptake stimulation effect without toxicity on L6 myotubes. To the best of our knowledge, this is the first report on the phytochemical investigation and *in vitro* studies on glucose uptake stimulation, and α-glucosidase and pancreatic lipase inhibitory activities of this plant. The results from our investigation have formed a basis for the future development of anti-diabetic and anti-obesity drugs.

### Authors' contributions

PI contributed in isolation and purification of the compounds and running the laboratory work. NC and CS contribution included the analysis of the data and drafted the paper. TK and WP contributed in cell-based assay of glucose uptake and cytotoxicity. KL and BS contributed in supervision of the laboratory work and critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

### Conflicts of interest

The authors declare no conflicts of interest.

### Ethical disclosures

**Protection of human and animal subjects.** The authors declare that no experiments were performed on humans or animals for this study.

**Confidentiality of data.** The authors declare that no patient data appear in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

### Acknowledgements

This work was supported by a 2016 research grant from the National Research Council of Thailand (NRCT) through Chulalongkorn University (GB-A.60.018.33.03). We thank The Research Instrument Center of Faculty of Pharmaceutical Sciences, Chulalongkorn University, for providing research facilities and Prof. Thatree Phadungcharoen (Rangsit University, Bangkok, Thailand) for plant identification.

### References

American Diabetes Association, 2006. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 33, 62–69.

Balbaa, M., El Ashry, E.S.H., 2012. Enzyme inhibitors as therapeutic tools. *Biochem. Physiol.* 1, 1–8.

Bhandari, S.R., Kapadi, A.H., Majumder, P.L., Joardar, M., Shoolery, J.N., 1985. Nudol, a phenanthrene of the orchids *Eulophia nuda*, *Eria carinata* and *Eria stricta*. *Phytochemistry* 24, 801–804.

Birari, R.B., Bhutani, K.K., 2007. Pancreatic lipase inhibitors from natural sources: unexplored potential. *Drug Discov. Today* 12, 879–889.

Breen, D.M., Sanli, T., Giacca, A., Tsiani, E., 2008. Stimulation of muscle cell glucose uptake by resveratrol through sirtuins and AMPK. *Biochem. Biophys. Res. Commun.* 374, 117–122.

Chen, Y., Xu, J., Yu, H., Qing, C., Zhang, Y., Wang, L., Liu, Y., Wang, J., 2008. Cytotoxic phenolics from *Bulbophyllum odoratissimum*. *Food Chem.* 107, 169–173.

Choi, S.S., Cha, B.Y., Choi, B.K., Lee, Y.S., Yonezawa, T., Teruya, T., Nagai, K., Woo, J.T., 2013. Fargesin, a component of *Flos Magnoliae*, stimulates glucose uptake in L6 myotubes. *J. Nat. Med.* 67, 320–326.

Dohling, S., Kumaria, S., Tandon, P., 2008. Optimization of nutrient requirements for asymbiotic seed germination of *Dendrobium longicornu* Lindl. and *D. formosum* Roxb. *Proc. Indian Nat. Sci. Acad.* 74, 167–171.

Fisch, M.H., Flick, B.H., Arditti, J., 1973. Structure and antifungal activity of hircinol, loroglossol and orchinol. *Phytochemistry* 12, 437–441.

Ghadyale, V., Takalikar, S., Haldavnekar, V., Arvindekar, A., 2012. Effective control of postprandial glucose level through inhibition of intestinal alpha glucosidase by *Cymbopogon martinii* (Roxb.). *Evid. Based Complement. Alternat. Med.*, 6 p., Article ID 841752.

Guo, X.Y., Wang, J., Wang, N.L., Kitanaka, S., Yao, X.S., 2007. 9,10-Dihydrophenanthrene derivatives from *Pholidota yunnanensis* and scavenging activity on DPPH free radical. *J. Asian Nat. Prod. Res.* 9, 165–174.

Hanhineva, K., Törrönen, R., Bondia-Pons, I., Pekkinen, J., Kolehmainen, M., Mykkänen, H., Poutanen, K., 2010. Impact of dietary polyphenols on carbohydrate metabolism. *Int. J. Mol. Sci.* 11, 1365–1402.

Hu, J.M., Chen, J.J., Yu, H., Zhao, Y.X., Zhou, J., 2008. Five new compounds from *Dendrobium longicornu*. *Planta Med.* 74, 535–539.

Jang, D.S., Lee, G.Y., Kim, J., Lee, Y.M., Kim, J.M., Kim, Y.S., Kim, J.S., 2008. A new pancreatic lipase inhibitor isolated from the roots of *Actinidia arguta*. *Arch. Pharm. Res.* 31, 660–670.

Jantaramanant, P., Sermwittayawong, D., Noipha, K., Hutadilok-Towatana, N., Wititsuwannakul, R., 2014. β-Glucan-containing polysaccharide extract from the grey oyster mushroom [*Pleurotus sajor-caju* (Fr.) Sing.] stimulates glucose uptake by the L6 myotubes. *Int. Food Res. J.* 21, 779–784.

Kazeem, M.I., Adamson, J.O., Ogunwande, I.A., 2013. Modes of inhibition of α-amylase and α-glucosidase by aqueous extract of *Morinda lucida* Benth leaf. *Biomed Res. Int.*, <http://dx.doi.org/10.1155/2013/527570>.

Kim, J.S., Kwon, C.S., Son, K.H., 2000. Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. *Biosci. Biotechnol. Biochem.* 64, 2458–2461.

Klongkumnuankarn, P., Busaranon, K., Chanvorachote, P., Sritularak, B., Jongbunprasert, V., Likhitwitayawuid, K., 2015. Cytotoxic and antimigratory activities of phenolic compounds from *Dendrobium brymerianum*. *Evid. Based Complement. Alternat. Med.*, <http://dx.doi.org/10.1155/2015/350410>.

Lam, Y., Ng, T.B., Yao, R.M., Shi, J., Xu, K., Sze, S.C.W., Zhang, K.Y., 2015. Evaluation of chemical constituents and important mechanism of pharmacological biology in *Dendrobium* plants. *Evid. Based Complement. Alternat. Med.*, <http://dx.doi.org/10.1155/2015/841752>.

Li, M., Li, Y., Liu, J., 2013. Metabolic syndrome with hyperglycemia and the risk of ischemic stroke. *Yonsei Med. J.* 54, 283–287.

Limpanit, R., Chuanasa, T., Likhitwitayawuid, K., Jongbunprasert, V., Sritularak, B., 2016. α-Glucosidase inhibitors from *Dendrobium tortile*. *Rec. Nat. Prod.* 10, 609–616.

Majumder, P., Laha, S., Datta, N., 1982. Coelonin, a 9,10-dihydrophenanthrene from the orchids *Coelogyne ochracea* and *Coelogyne elata*. *Phytochemistry* 21, 478–480.

Majumder, P.L., Joardar, M., 1985. Erianthridin, a new 9,10-dihydrophenanthrene derivative from the orchids *Eria carinata* & *Eria stricta*. *Indian J. Chem.* 24, 1192–1194.

Majumder, P.L., Kar, A., 1987. Confusarin and confusarinidin, two phenanthrene derivatives of the orchid *Eria confusa*. *Phytochemistry* 26, 1127–1129.

Majumder, P.L., Sen, R.C., 1987. Moscatilin, a bibenzyl derivative from the orchid *Dendrobium moscatum*. *Phytochemistry* 26, 2121–2124.

Martinez-Gonzalez, A.I., Diaz-Sánchez, A.G., Rosa, L.A., Vargas-Requena, C.L., Bustos-Jaimes, I., Alvarez-Parrilla, E., 2017. Polyphenolic compounds and digestive enzymes: in vitro non-covalent interactions. *Molecules* 22, 1–27.

Minakawa, M., Miura, Y., Yagasaki, K., 2012. Piceatannol, a resveratrol derivative, promotes glucose uptake through glucose transporter 4 translocation to plasma membrane in L6 myocytes and suppresses blood glucose levels in type 2 diabetic model db/db mice. *Biochem. Biophys. Res. Commun.* 422, 469–475.

Mohamed, S., 2014. Functional foods against metabolic syndrome (obesity, diabetes, hypertension and dyslipidemia) and cardiovascular disease. *Trends Food Sci. Technol.* 35, 114–128.

Nathan, D.M., Buse, J.B., Davidson, M.B., Ferrannini, E., Holman, R.R., Sherwin, R., Zinman, B., 2009. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy. *Diabetes Care* 32, 193–203.

Peng, X., Zhang, G., Liao, Y., Gong, D., 2016. Inhibitory kinetics and mechanism of kaempferol on α-glucosidase. *Food Chem.* 190, 207–215.

Prasad, R., Koch, B., 2014. Antitumor activity of ethanolic extract of *Dendrobium formosum* in T-cell lymphoma: an in vitro and in vivo study. *Biomed Res. Int.* 2014, 1–11.

Riss, T.L., Moravec, R.A., Niles, A.L., Benink, H.A., Worzella, T.J., Minor, L., 2004. Cell Viability Assays. *Assay Guidance Manual*. Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Rosak, C., Mertes, G., 2012. Critical evaluation of the role of acarbose in the treatment of diabetes: patient considerations. *Diabetes Metab. Syndr. Obes.* 5, 357–367.

Sachdev, K., Kulshreshtha, D.K., 1986. Phenolic constituents of *Coelogyne ovalis*. *Phytochemistry* 25, 499–502.

Sergent, T., Vanderstraeten, J., Winand, J., Beguin, P., Schneider, Y.J., 2012. Phenolic compounds and plant extracts as potential natural anti-obesity substances. *Food Chem.* 135, 68–73.

Sritularak, B., Anuwat, M., Likhitwitayawuid, K., 2011. A new phenanthrenequinone from *Dendrobium draconis*. *J. Asian Nat. Prod. Res.* 13, 251–255.

Sukphan, P., Sritularak, B., Mekboonsonglarp, W., Lipipun, V., Likhitwitayawuid, K., 2014. Chemical constituents of *Dendrobium venustum* and their antimalarial and anti-herpetic properties. *Nat. Prod. Commun.* 9, 825–827.

Sun, J., Zhang, F., Yang, M., Zhang, J., Chen, L., Zhan, R., Li, L., Chen, Y., 2014. Isolation of α-glucosidase inhibitors including a new flavonol glycoside from *Dendrobium devonianum*. *Nat. Prod. Res.* 28, 1900–1905.

- Tushuizen, M.E., Bunck, M.C., Pouwels, P.J., Bontemps, S., Waesberghe, J.K.V., Schindhelm, R.K., Mari, A., Heine, R.J., Diamant, M., 2007. Pancreatic fat content and  $\beta$ -cell function in men with and without type 2 diabetes. *Diabetes Care* 30, 2916–2921.
- Yang, M.H., Chin, Y.W., Yoon, K.D., Kim, J., 2014. Phenolic compounds with pancreatic lipase inhibitory activity from Korean yam (*Dioscorea opposita*). *J. Enzyme Inhib. Med. Chem.* 29, 1–6.
- Yap, A., Nishiumi, S., Yoshida, K.I., Ashida, H., 2007. Rat L6 myotubes as an *in vitro* model system to study GLUT4-dependent glucose uptake stimulated by inositol derivatives. *Cytotechnology* 55, 103–108.
- Yin, Z., Zhang, W., Feng, F., Zhang, Y., Kanga, W., 2014.  $\alpha$ -Glucosidase inhibitors isolated from medicinal plants. *Food Sci. Hum. Wellness* 3, 136–174.
- You, Q., Chen, F., Wang, X., Jiang, Y., Lin, S., 2012. Anti-diabetic activities of phenolic compounds in muscadine against alpha-glucosidase and pancreatic lipase. *LWT – Food Sci. Technol.* 46, 164–168.
- Zhang, X., Gao, H., Wang, N., Yao, X., 2006. Phenolic components from *Dendrobium nobile*. *Chin. Trad. Herb. Drugs* 37, 652–655.
- Zhou, H., Zhang, T., Harmon, J.S., Bryan, J., Robertson, R.P., 2007. Zinc, not insulin, regulates the rat  $\alpha$ -cell response to hypoglycemia *in vivo*. *Diabetes* 56, 1107–1112.