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Glycobiology Insights

Synthesis of Glycosides of Resveratrol, Pterostilbene, and Piceatannol by Glucosyltransferase from *Phytolacca americana* Expressed in *Bacillus subtilis* and their Chemopreventive Activity Against Cancer, Allergic, and Alzheimer's Diseases

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ABSTRACT: Resveratrol was glucosylated to its 3- and 4′-β-glucosides by the glucosyltransferase *Pa*GT3 from *Phytolacca americana* expressed in *Bacillus subtilis*. *Pa*GT3 glucosylated pterostilbene to its 4′-β-glucoside. Piceatannol was converted into its 4′-β-glucoside by *Pa*GT3. Resveratrol 3-β-glucoside showed the inhibitory action of adhesion, migration, and invasion of KB cells. The resveratrol 3-β-glucoside would act as a chemopreventive agent against KB cells. Piceatannol 4′-β-glucoside showed the strongest inhibitory activity among the glycosides toward histamine release from rat peritoneal mast cells. The 4′-β-glucoside of piceatannol would be a potential therapeutic agent for allergic disease. Pterostilbene 4′-β-glucoside showed high phosphodiesterase (PDE) inhibitory activity. The 4′-β-glucoside of pterostilbene could be a potential chemopreventive agent for Alzheimer's disease.

KEYWORDS: glycoside, resveratrol, pterostilbene, piceatannol, glucosyltransferase, *Phytolacca americana*, *Bacillus subtilis*, therapeutic agents for cancer, allergic, Alzheimer's diseases

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Introduction

Resveratrol is one of the most important plant polyphenols and has attracted considerable pharmaceutical interest because of its diverse biological activities.[1] Pterostilbene, which is a dimethylether analog of resveratrol, has been found in blueberry and grapes. It has been shown to exhibit significant effects on cell proliferation, inflammation, and anti-oxidation, and to reverse the effects of aging in rats.[2] On the other hand, piceatannol, a naturally occurring hydroxylated analog of resveratrol, has been found in various plants, including grapes, passion fruit, white tea, and Japanese knotweed. It exhibits immunosuppressive, anti-oxidation activity, and antitumorigenic activities.[3,4] The water-insolubility of stilbenes such as resveratrol, pterostilbene, and piceatannol limits their further pharmacological exploitation. Glycosylation allows water-insoluble and unstable organic compounds to be converted into the corresponding water-soluble and stable compounds. Several attempts have been made to synthesize resveratrol glycosides by chemical methods, including tedious protection–deprotection procedures, but these resulted in low yields.[5] Plant cell cultures of *Phytolacca americana* would be useful for practical preparation of β-glucosides, due to high potential of plant glucosyltransferases to diastereoselectively produce β-glucosides through one-step enzymatic

glucosylation.[6] Here we report synthesis of β-glycosides of resveratrol, pterostilbene, and piceatannol by glucosyltransferase *Pa*GT3 from *P. americana* expressed in *B. subtilis* and their anticancer, anti-allergic, and anti-Alzheimer's diseases activities (inhibitory action on phosphodiesterase, PDE). In Parkinson's disease there is an alteration in cAMP/cGMP levels and it has been shown that the activity of PDE is increased in the 6-hydroxydopamine hemi-Parkinson model.[7] The PDE inhibitor reduced the severity levodopa-induced dyskinesias in this model. Therefore, PDE inhibitors show potential to exert a neuroprotective role, which could be particularly interesting for treatment of neurodegenerative disorders such as Alzheimer's disease.[7]

Experimental

General. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR), and heteronuclear multiple-bond correlation (HMBC) spectra were recorded in CD_3OD using a Varian XL-400 spectrometer (Varian Inc.). The chemical shifts were expressed in δ (ppm) referring to tetramethylsilane. The mass spectrometry (MS) spectra were measured using a JEOL MStation JMS-700 spectrometer (JEOL Ltd.). High performance liquid chromatography (HPLC) was carried out on Crestpak C18S column (4.6 × 150 mm, JASCO) [solvent: MeOH–H_2O (2:3, v/v); detection: UV (228 nm); flow rate: 1.0 mL/minute]. *B. subtilis* transformed with plasmid (pBSX1.101), which contains gene from *P. americana* encoding glucosyltransferase activity, was used as the source of the enzyme *Pa*GT3. *B. subtilis* was grown at 37°C in Luria–Bertani broth. The medium was sterilized in 1 L aliquots in 2 L conical flasks, supplemented with 5 μg mL^{-1} chloramphenicol, and inoculated with 0.5% fresh culture. Growth was carried out in an incubator with agitation (200 rpm) for 26 hours. Cells were lysed by sonication, and cellular debris was removed by centrifugation. The supernatant (20 mL) was applied to a His-accept column (1.6 × 30 cm) equilibrated with buffer A (50 mL) (15 mM potassium phosphate, 1 mM EDTA, 2 mM 2-mercaptoethanol). The column was washed with buffer A (50 mL) to remove impurities, and the bound enzyme was eluted with buffer A (100 mL) supplemented with 200 mM imidazole. The purified enzyme solution was dialyzed with 50 mM tris-HCl (pH 7.2) containing 5 mM dithiothreitol, and stored at −80°C.

Synthesis of glycosides of resveratrol, pterostilbene, and piceatannol by *Pa*GT3. Glucosylation reactions were performed at 37°C for 60 minutes in 1 mL of 50 mM potassium phosphate buffer (pH 7.2) supplemented with substrate (1 mg), UDP-glucose (15 mg), and enzyme (50 U). The incubation was stopped by adding 1.5% trifluoroacetic acid; the reaction mixture was analyzed by HPLC. The medium was applied to Diaion HP20, washed with water, and eluted with methanol. The methanol solution was analyzed by HPLC. For a large-scale experiment, the incubation was performed in 50 mL buffer containing 50 mg of substrate, UDP-glucose (750 mg), and enzyme (2000 U).

Effects of glycosides of resveratrol, pterostilbene, and piceatannol on KB cells. The effects of resveratrol, pterostilbene, piceatannol, and their glycosides on oral squamous carcinoma cell line KB cells were examined by a MTT colorimetric assay as follows.[8] Each well of a 96-well plate was coated with 10 μg matrigel. The plate was gently rocked until the matrigel evenly coated the inside of the insert. The inserts were aired in a laminar flow hood. The plate was then blocked with 20 μL of 2% BSA. Near-confluent KB cells were treated with 100 μmol sample for one hour and then harvested, resuspended in culture medium. Cell viability assay was performed according to the previously reported method.[8] KB cells were seeded in the wells at 5×10^4 (live cells)/well. The cells in the positive control group were treated with an equivalent volume of physiological buffered solution (PBS). Negative control (background) was the cells added to the uncoated (no matrigel) wells. The plates were incubated for two hours in RPMI 1640 medium at 37°C. Non-adherent cells were then rinsed off with three washes in warm PBS, and 10 μL MTT was added to each well. After a four-hour incubation, adhesion was estimated by measuring absorbance at 570 nm. The adhesion rate was determined by dividing the absorbance value of the experimental group by the value obtained for the negative control group. Cell migration assays were performed as follows.[8] Transwell chambers containing a polycarbonate membrane filter were used. The lower chamber contained serum-free NIH3T3 culture supernatant. Near-confluent KB cells were treated with 100 μmol sample for one hour and then harvested. Harvested cells were resuspended in culture medium. Cell viability assay was performed according to the previously reported method.[8] The cells were seeded in the chambers at 5×10^4 (live cells)/well. After incubation for eight hours, the cells on the top surface of the membrane were wiped off with cotton swabs, and the membrane was fixed and stained with Giemsa. Cells on the bottom surface of the membrane were examined with a light microscope. Cells from 10 random fields were imaged for counting purposes and the average number of invaded cells was used as a measure of invasion capacity. For cell invasion assays, a similar procedure was performed, except that the Transwell chambers were coated with matrigel before use.[8]

Effects of glycosides of resveratrol, pterostilbene, and piceatannol on histamine release from rat peritoneal mast cells. The effects of resveratrol, pterostilbene, piceatannol, and their glycosides on compound 48/80-induced histamine release from rat peritoneal mast cells were examined as follows. Peritoneal mast cells were collected from the abdominal cavities of rats (male Wistar rats, Nippon SLC) and purified to a level higher than 95% according to the method described previously.[9] The purified mast cells were suspended in a PBS containing 145 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2$, 5.6 mM glucose, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) to give

approximately 10^4 mast cells/mL. Cell viability was always greater than 90% as judged by the trypan blue exclusion test. Mast cells were preincubated with the test compound (1 μM (final conc.) dissolved in DMSO (20 μL)) for 15 minutes at 37°C, and subsequently exposed to compound 48/80 at 0.35 μg/mL. Control solution involved the same volume of DMSO (20 μL). Histamine release was determined by a fluorometric assay as described previously,[9] and was expressed as a percentage of total histamine. The amount of histamine in cells is displayed as 100%.

Effects of glycosides of resveratrol, pterostilbene, and piceatannol on PDE activity. BIOMOL Green, which is used for calculation of the amount of phosphate released, was purchased from Funakoshi Co. PDE activity was measured by using a cyclic nucleotide PDE assay kit from Enzo Life Sciences (BML-AK800) according to the manufacturer's instructions, except that PDE 100 mU per well were used. Stock solutions of inhibitors were prepared as 100 mM in DMSO, and diluted to the appropriate concentrations. In the assay, each inhibitor solution was added (final conc. of 10–900 μM) before the addition of PDE enzyme solution. 3-isobutyl-1-methylxanthine (IBMX) was used as a positive control. The PDE reaction was conducted at 37°C for 60 minutes and terminated by the addition of BIOMOL Green and further incubation at room temperature for 30 minutes. The color reaction was measured by reading OD at 620 nm with a Multiscan FC (Thermo Scientific).

Results and Discussions

After resveratrol (1) was incubated with the glucosyltransferase *Pa*GT3 from *P. americana* expressed in *B. subtilis*, the glycosylated products 2 and 3 were isolated. Based on the results of mass spectroscopy (MS) (Fig. 3), (^{1}H- and ^{13}C-NMR) (Fig. 2), and HMBC analyses, the products were determined to be resveratrol 3-β-glucopyranoside (2, 12%) and resveratrol 4′-glucoside (3, 76%). The MS spectrum of 2 showed a pseudomolecular ion $[M+Na]^+$ peak at *m/z* 413. The ^{13}C-NMR spectrum of 2 showed 20 carbon signals including the anomeric carbon signal at δ 100.5. Based on the coupling pattern of the proton signals and the chemical shifts of the carbon resonances because of the sugar moiety, the sugar component in 2 was concluded to be β-glucopyranose. Thus, the structure of 2 was determined to be resveratrol 3-β-glucopyranoside (glucoside). The MS spectrum of the product 3 showed a $[M+Na]^+$ peak at *m/z* 413. Based on the coupling pattern of the proton signals and the chemical shifts of the carbon resonances because of the sugar moiety, the sugar component in 3 was concluded to be β-glucopyranose. The analysis of ^{13}C-NMR data revealed anomeric carbon resonance at δ 100.2. Thus, compound 3 was determined to be resveratrol 4′-β-glucopyranoside (glucoside) (Fig. 1).

Pterostilbene (4) was subjected to the same glycosylation system using *Pa*GT3 expressed in *B. subtilis*. Glycoside product 5 was obtained. The product was identified as pterostilbene 4′-β-glucopyranoside (5, 50%). The MS spectrum of the product 5 showed a $[M+Na]^+$ peak at *m/z* 441. The sugar component in 5 was concluded to be β-glucopyranose based on the coupling pattern of the proton signals and the chemical sifts of the carbon resonances because of the sugar moiety. The ^{13}C-NMR spectroscopic data of 5 showed an anomeric carbon signal at δ 102.5. Thus, compound 5 was determined to be pterostilbene 4′-β-glucopyranoside (glucoside).

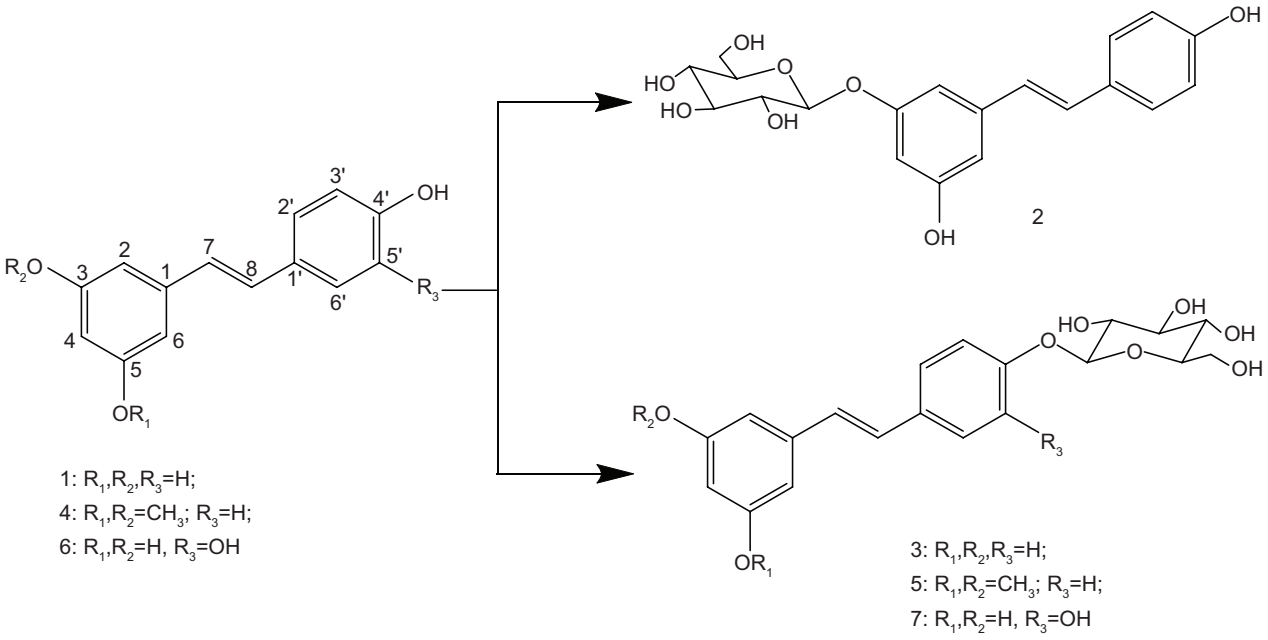

Figure 1. Glycosylation of stilbenes **1**, **4**, and **6** by *Pa*GT3 from *P. americana* expressed in *B. subtilis*.

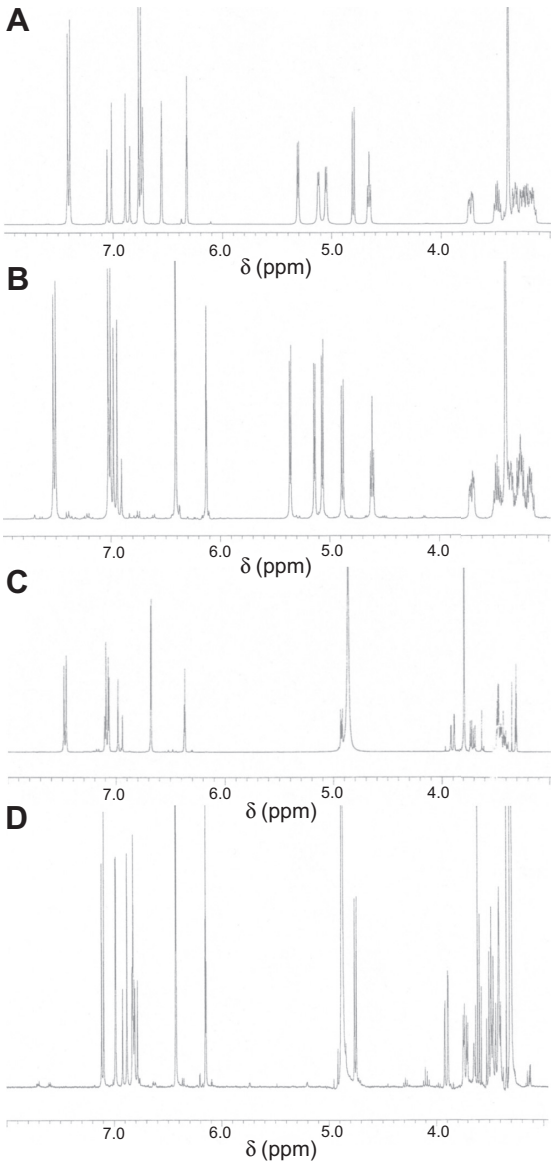

Figure 2. ^{1}H NMR spectra of (**A**) resveratrol 3-*β*-glucoside (**2**), (**B**) resveratrol 4′-*β*-glucoside (**3**), (**C**) pterostilbene 4′-*β*-glucoside (**5**), and (**D**) piceatannol 4′-*β*-glucoside (**7**).

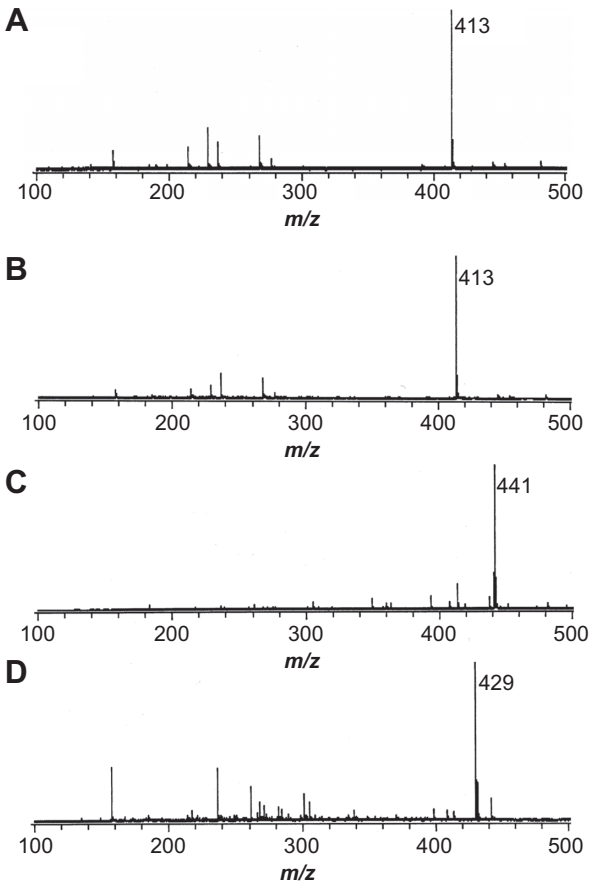

Figure 3. MS spectra of (**A**) resveratrol 3-*β*-glucoside (**2**), (**B**) resveratrol 4′-*β*-glucoside (**3**), (**C**) pterostilbene 4′-*β*-glucoside (**5**), and (**D**) piceatannol 4′-*β*-glucoside (**7**).

After piceatannol (6) was incubated with *Pa*GT3 expressed in *B. subtilis*, the product 7 was isolated. No additional conversion products were detected. Based on the MS, ^{1}H-, ^{13}C-NMR, and HMBC spectra, the product was identified as piceatannol 4′-*β*-glucopyranoside (7, 70%). The MS spectrum of 7 showed a pseudomolecular ion $[M+Na]^{+}$ peak at *m/z* 429. The ^{13}C-NMR spectrum of 7 showed 20 carbon signals including an anomeric carbon signal at δ 102.0. Based on the coupling pattern of the proton signals and the chemical shifts of the carbon resonances because of the sugar moiety, the sugar component in 7 was determined to be *β*-glucopyranose. Thus, the structure of 7 was determined to be piceatannol 4′-*β*-glucopyranoside (glucoside). The enzyme solution was stored at −80°C. After three months, the enzyme converted piceatannol (6) to the product 7 in 55% yield. On the other hand, *Pa*GT3 expressed in *Escherichia coli*[10] glucosylated piceatannol (6) to the product 7 in 72% yield under the same assay condition as described in section 2.2. After three months of storage at −80°C, this enzyme converted piceatannol (6) to the product 7 in 30% yield, indicating that *Pa*GT3 expressed in *B. subtilis* is more stable than *Pa*GT3 expressed in *E. coli*.

The effects of stilbenes and their glycosides 1–7 on KB cells were examined. To determine the effects of stilbene glycosides on KB cell adhesion, an in vitro adhesion assay was performed (Table 1). Among the glycosides tested, significant decreases in adhesion were observed with resveratrol 3-*β*-glucoside (2). Other glycosides 3, 5, and 7 showed little inhibitory effects. Adhesion was drastically decreased in cells treated with resveratrol (1), pterostilbene (4), and piceatannol (6). A transwell migration assay was performed to determine the effects of stilbenes and their glycosides 1–7 on KB cell migration (Table 2). Among the glycosides tested, significant decreases in migration were observed with resveratrol 3-*β*-glucoside (2). Other glycosides 3, 5, and 7 showed little inhibitory effects. Migration was drastically decreased in cells treated with resveratrol (1), pterostilbene (4), and piceatannol (6). A transwell migration assay was applied to determine

Table 1. Effects of compounds 1–7 on KB cell adhesion.

COMPOUND	RATE OF CELL ADHESION[a]
Control	75 ± 4.0
1	34 ± 4.2[b]
2	49 ± 2.0[b]
3	74 ± 3.8
4	38 ± 3.3[b]
5	71 ± 3.7
6	39 ± 2.5[b]
7	73 ± 2.2

Notes: [a]The results are shown as the mean ± standard deviation of five separate experiments. [b]Significantly decreased adhesion compared with control group ($P < 0.01$).

Table 3. Anti-allergic activities of compounds 1–7.

COMPOUND	HISTAMINE RELEASE (%)[a]
Control	55 ± 5.1
1	41 ± 5.5
2	40 ± 6.2
3	36 ± 3.9
4	43 ± 4.9
5	35 ± 3.5
6	5 ± 3.3[b]
7	17 ± 5.0[b]

Notes: [a]The results are shown as the mean ± standard deviation of five separate experiments. [b]Significantly decreased histamine release compared with control group ($P < 0.05$).

the effects of stilbenes and their glycosides 1–7 on invasion of KB cells (Table 2). Among the glycosides tested, resveratrol 3-β-glucoside (2) decreased KB cell invasion. Invasion was drastically decreased in cells treated with resveratrol (1), pterostilbene (4), and piceatannol (6). Other glycosides 3, 5, and 7 showed little inhibitory effects.

The effects of resveratrol (1), pterostilbene (4), piceatannol (6), and their glycosides 2, 3, 5, and 7 on compound 48/80-induced histamine release from rat peritoneal mast cells were examined (Table 3). Rat peritoneal mast cells released a high level of histamine (control, 52%) when stimulated with 0.35 μg/mL of compound 48/80. Piceatannol (6) effectively inhibited compound 48/80-induced histamine release from rat peritoneal mast cells. Thus, the anti-allergic action of stilbene compounds, ie, the ability to inhibit compound 48/80-induced histamine release from rat peritoneal mast cells, was examined. Piceatannol showed the highest anti-allergic activity among the stilbene compounds tested. On the other hand, piceatannol 4′-β-glucoside had anti-allergic activity. Resveratrol, pterostilbene, resveratrol 3-β-glucoside, resveratrol 4′-β-glucoside, and pterostilbene 4′-β-glucoside showed low inhibitory effects on histamine release.

Next, the inhibitory effects of compounds 1–7 on PDE activity were examined (Table 4). As a result, pterostilbene (4) and pterostilbene 4′-β-glucoside (5) showed high PDE inhibitory activity. On the other hand, resveratrol 3-β-glucoside (2), resveratrol 4′-β-glucoside (3), piceatannol (6), and piceatannol 4′-β-glucoside (7) had low inhibitory activity against PDE. Pterostilbene 4′-β-glucoside (5) could be a potential chemopreventive agent for Alzheimer's disease.

Thus, three stilbene compounds, ie, resveratrol, pterostilbene, and piceatannol, were transformed to their glucoside products by *Pa*GT3 expressed in *B. subtilis*. Resveratrol 3-β-glucoside showed the inhibitory action of adhesion, migration, and invasion of KB cells. Among the stilbene glycosides synthesized here, piceatannol 4′-β-glucoside inhibited histamine release from rat peritoneal mast cells more strongly than resveratrol 3-β-glucoside, resveratrol 4′-β-glucoside, and pterostilbene 4′-β-glucoside. Pterostilbene 4′-β-glucoside showed high PDE inhibitory activity.

Table 2. Effects of compounds 1–7 on KB cell migration and invasion.

COMPOUND	MIGRATORY CELLS[a]	INVASIVE CELLS[a]
Control	157 ± 9.0	70 ± 7.2
1	70 ± 10.0[b]	28 ± 5.1[b]
2	92 ± 6.2[b]	46 ± 5.0[b]
3	152 ± 9.1	69 ± 6.7
4	67 ± 8.6[b]	31 ± 4.5[b]
5	141 ± 8.9	67 ± 8.2
6	76 ± 9.4[b]	35 ± 4.1[b]
7	149 ± 8.7	68 ± 6.3

Notes: [a]The results are shown as the mean ± standard deviation of five separate experiments. [b]Significantly decreased migration or invasion compared with control group ($P < 0.01$).

Table 4. Inhibitory effects of compounds 1–7 on PDE activity.

COMPOUND	$IC_{50\ (\mu M)}$[a]
	INHIBITION OF PDE ACTIVITY
IBMX	28 ± 7
1	154 ± 10
2	136 ± 12
3	117 ± 9
4	63 ± 10
5	51 ± 7
6	77 ± 8
7	92 ± 9

Note: [a]The results are shown as the mean ± standard deviation of five separate experiments.

Author Contributions

Conceived and designed the experiments: HH, KS, NS, YS, MA. Analyzed the data: HH, KS, NS, YS, MA. Wrote the first draft of the manuscript: HH, KS, NS, YS, MA. Contributed to the writing of the manuscript: HH, KS, NS, YS, MA. Agree with manuscript results and conclusions: HH, KS, NS, YS, MA. Jointly developed the structure and arguments for the paper: HH, KS, NS, YS, MA. Made critical revisions and approved final version: HH, KS, NS, YS, MA. All authors reviewed and approved of the final manuscript.

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