Effects of iriflophenone 3-C-β-glucoside on fasting blood glucose level and glucose uptake

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ABSTRACT

Background: One of the biological activities of agar wood (*Aquilaria sinensis* Lour., Thymelaeaceae), is anti-hyperglycemic activity. The methanolic extract (ME) was proven to possess the fasting blood glucose activity in rat and glucose uptake transportation by rat adipocytes. **Objective:** To determine the decreasing fasting blood glucose level of constituents affordable for *in vivo* test. If the test was positive, the mechanism which is positive to the ME, glucose transportation, will be performed. **Materials and Methods:** The ME was separated by column chromatography and identified by spectroscopic methods. Mice was used as an animal model (*in vivo*), and rat adipocytes were used for the glucose transportation activity (*in vitro*). **Result:** Iriflophenone 3-C-β-glucoside (IPG) was the main constituent, 3.17%, and tested for the activities. Insulin and the ME were used as positive controls. The ME, IPG and insulin lowered blood glucose levels by 40.3, 46.4 and 41.5%, respectively, and enhanced glucose uptake by 152, 153, and 183%, respectively. **Conclusion:** These findings suggest that IPG is active in lowering fasting blood glucose with potency comparable to that of insulin.

Key words: *Aquilaria sinensis*, glucose uptake activity, iriflophenone 3-C-β-glucoside, lowering fasting blood glucose, rat adipocytes

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INTRODUCTION

Herbs are currently used to protect or treat type 2 diabetes.[1,2] The organ systems involved in glucose metabolism include the gastrointestinal tract, the endocrine-pancreatic system, the hepatic system and the central nervous system as well as musculoskeletal and adipose tissues.[3]

Aquilaria, or agarwood (*Aquilaria sinensis* Lour., Thymelaeaceae), leaves have been found to exhibit various types of biological activity. Genkwanin 5-O-β-primeveroside, a compound isolated from agarwood leaves, was shown to exert the laxative effect in mice.[4] An ethanolic extract of *A. sinensis* leaves was found to have analgesic and anti-inflammatory activities.[5] Feng *et al.*[6] used an α-glucosidase inhibition assay as a guide to isolate active compounds from *A. sinensis*. Eight compounds were isolated which are aquilarisinin, aquilarisin, aquilarixanthone, hypolaetin 5-O-β-D-glucuronopyranoside, iriflophenone 3-C-β-D-glucoside, iriflophenone 3, 5-C-β-diglucopyranoside, iriflophenone 2-O-α-L-rhamnopyranoside, and mangiferin. All of the isolated compounds exhibited the α-glucosidase inhibition activity stronger than acarbose, the positive drug control.[6] Many polyphenolic compounds inhibit α-glucosidase, preventing the digestion and absorption of carbohydrates. Such activity has no effect on the glucose that has already been absorbed into the body. However, the activity of these compounds, except mangiferin, on the regulation of glucose disposition in peripheral tissues has not been investigated. The activity is one of the action of anti-diabetics.

In our previous study, *A. sinensis* leaves were sequentially extracted with hexane, ethylacetate (EtOAc) and methanol (MeOH). The methanolic extract (ME) was found to exert the greatest effect in lowering fasting blood glucose level in streptozotocin (STZ)-induced diabetic rats (*in vivo*). This extract was also most active in the enhancement of glucose uptake into rat adipocytes (*in vitro*).[7] In this work, constituents in the ME were isolated. One of the separated compounds had

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never been tested for the effects described above, and it was investigated in both *in vivo* and *in vitro* experiments. Mice were used instead of rats for the *in vivo* experiment in this study because of the limited availability of the compound derived from the ME.

MATERIALS AND METHODS

The solvents used for the extraction and isolation were reagent grade (Labscan, Thailand). Silica gel 60 (0.040-0.063 mm) was used for column chromatography and silica gel GF 254 precoated-plates for thin-layer chromatography (TLC) were purchased from Merck, KGaA, Darmstadt, Germany. Bovine serum albumin fraction V, collagenase type 1, cytochalasin B, and *N*-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and STZ was from Sigma-Aldrich (Chemie GmbH, Germany). 2-Deoxy-D-[U-^{14}C] glucose (^{14}C-2-DG) and aqueous counting scintillant (ACS II®) were from Amersham Pharmacia Biotech (GE Healthcare Bio-Sciences GmbH, Freiburg, Germany). Insulin (Mixtard 30) was purchased from Novo Nordisk Pharma Ltd (Thailand).

Instruments

Spectroscopic data were acquired using a Fourier transform infrared spectrometer (Perkin Elmer® Series 1600, California, USA) and nuclear magnetic resonance (NMR) spectrophotometer, 400MHz (Bruker Corporation, Fällenden, Switzerland). Mass spectrometric data were obtained on a Bruker MicroTOF mass spectrometer (Bruker Corporation, Massachusetts, USA). Melting ranges were determined using a differential scanning calorimeter (DSC, Mettler 822e, Mettler-Toledo, Ohio, USA). The Liquid Scintillation Analyzer used was a TRI-Carb 2900 TR from Perkin Elmer Inc. (Massachusetts, USA).

Isolation and structure elucidation

Leaves of *A. sinensis* were obtained from Mr. Nuthawongse Siriyodaroon in Nakhon Ratchasima Province, Thailand. The plants were grown in the area for oleoresin from the trunks of the plants. The plant voucher (Number NAT001-002) was deposited at the Faculty of Pharmaceutical Sciences, Khon Kaen University. It was validated by comparison with the voucher specimen, BKF number 140455, in the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand.

Silica gel GF 254 precoated plate was used as a stationary phase, a mixture of toluene, EtOAc, MeOH, and acetic acid (1:2:1:0.04) was used as a mobile phase. Chromatograms were visualized under ultraviolet light at 254 nm and 366 nm or sprayed with either vanillin-sulphuric acid reagent or 0.1M diphenyl-(2,4,6-trinitrophenyl) iminoazanium (DPPH).

Two kilograms of dried leaf powder was extracted sequentially with hexane, EtOAc and MeOH. The yields were 0.9%, 0.9% and 7.95%, respectively. The ME was separated by column chromatography using gradient mixtures of hexane, EtOAc and MeOH. Eluates (200 mL) were collected over 55 fractions. Similar fractions were combined to give six fractions: MF, MF1, MF2, MF3, MF4 and MF5, as shown in Figure 1. MF and MF1 were combined and crystallized in hexane to afford yellowish crystals (Cpd 1). MF2 was crystallized in ~50% CH_2Cl_2 in hexane to give yellowish crystals (Cpd 2). MF4 was dissolved in EtOAc, hexane was added until the solution became very slightly clouded. EtOAc was added slowly until the cloudiness disappeared. The solution was left at room temperature for crystallization, and the crystals were collected by filtration (Cpd 3). Cpd 5 was crystallized from MF5 in a mixture of 50% MeOH in EtOAc. MF3 was chromatographed again on a silica column using CH_2Cl_2 and gradient mixtures of MeOH in CH_2Cl_2 as eluents. Cpd 4 was eluted with 15% MeOH in CH_2Cl_2. The fraction was crystallized in ~10% hexane in EtOAc.

Lowering fasting blood glucose level activity in an animal model

Streptozotocin-diabetes induction in mice

Imprinting control regions (ICR) mice (2-month-old, 25-35 g) were purchased from the National Laboratory Animal Center, Mahidol University (Nakhon Pathom, Thailand). The mice were maintained in an air-conditioned room (25°C ± 1°C) with a 12 h light-dark cycle. Food (C.P. mouse feed, Bangkok, Thailand) is fully provided, and water can be access all the time. All procedures complied with the Guidelines for the Care and Use of Laboratory Animals (Thailand) which follow the American Guidelines. The laboratory is accredited by the National Research Council of Thailand. The experiment was approved by the Animal Ethics Committee of Khon Kaen University (Record Number: AEKKU 33/2554). Diabetes was induced by three intraperitoneal injections of STZ at doses of 100, 50 and 50 mg/kg body weight (BW) on days 1, 3 and 6, respectively. STZ was delivered as a solution in 0.1M citrate buffer (pH 4.5). Fasting blood glucose levels, collected from the tail vein, were checked after day 7 of the last dose. Mice with fasting blood glucose levels over 200 mg/dL were used. After the experiment, the animals were terminated by intra-peritoneal injections of sodium pentobarbital (100 mg/kg BW).

Experimental design for the animal model

Seven groups of mice, six each, were designed for: Group I: Normal mice, 2 mL of distilled water was

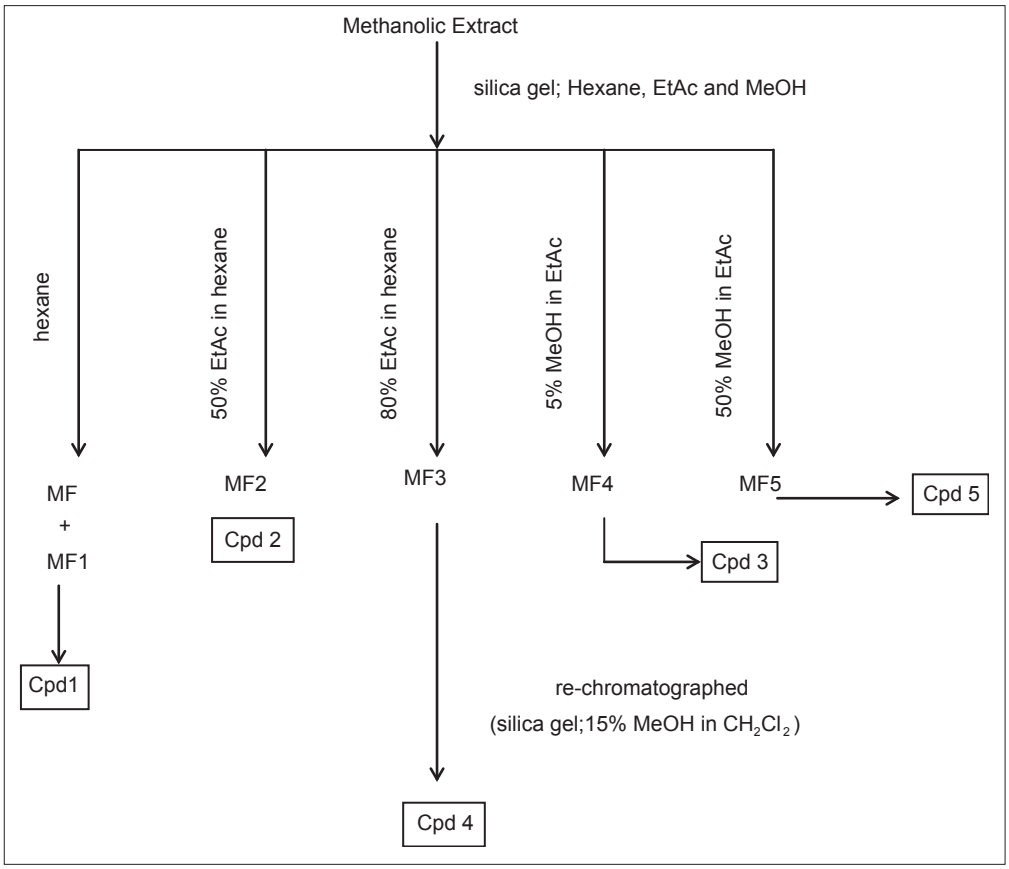


Figure 1: Scheme of the extraction and isolation of ME of *A. sinensis* leaf

orally administered (normal control); Group II: Diabetic mice, 2 mL of distilled water, oral (negative control); Group III: Diabetic mice, 8 U/kg insulin, subcutaneous with (positive control); Group IV: Diabetic mice 1 g/kg ME, oral. Group V: Diabetic mice, 0.47 g/kg iriflophenone 3-C-β-glucoside (IPG) oral. Group VI: Diabetic mice, 0.1 g/kg of ME oral; and Group VII: Diabetic mice, 0.047 g/kg of IPG oral. Treatments were administered daily between 4 pm and 5 pm for 3 weeks. The animals were weighed and fasting blood glucose levels were monitored weekly between 7 am and 8 am by a glucometer (Accu-Check AdvantageII, Roche Diagnostics, Mannheim, Germany). During fasting, mice were deprived of food overnight (12 h) with free access to water.

Test for glucose uptake into rat adipocytes

Preparation of adipocytes

White adipocytes from epididymal fat pads were isolated from normal rats using the method as described by Rodbell.[8] The fat pad, it was digested with collagenase type 1 in Krebs-Ringer bicarbonate buffer (KRBB, pH 7.4) containing 25 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid, 1% w/v bovine serum albumin and 1.11 mM glucose. The suspension of fat cells was obtained, filtered and washed with KRBB, and centrifuged. Fat cells which accumulated on the top of the medium were suspended to give a 40% packed cell volume (approximately 10^5-10^6 cells/mL) in KRBB without glucose. Glucose uptake was determined by using 2-deoxy-D-[U-^{14}C] glucose (^{14}C-2-DG) as a tracer.

Viability of the cells was determined by 0.4% trypan blue exclusion test. Cell viability for the experiment has to be more than 90%. The effects of different concentrations of IPG (0.1-10 μg/mL or 0.25-25 μM) on fat cell viability were examined prior to the glucose uptake test, and no significant effect on cell viability was observed.

Experimental design

One milligram of IPG was dissolved in 1 mL of KRBB without glucose. This solution was serially diluted to various concentrations in the same solvent.

Cell suspension, 200 μL, and 50 μL of KRBB without glucose was mixed in a microtube for each test. The solution was incubated at 37°C for 15 min in a shaking water bath. Then, 50 μL of insulin solution (to achieve a final concentration of 1.5 nM) or 50 μL of a serially diluted IPG solution (to achieve final concentrations of 0.25, 2.45, 12.2, and 24.5 μM) was added separately to each microtube of the incubated mixture. Finally, 50 μL of ^{14}C-2-DG

(0.4 μCi/mL in 1.11 mM glucose in KRBB) was added to each tube. Each treatment was performed in triplicate.

After incubation at 37°C for 15 min, 10 μL of 20 μM cytochalasin B was added and incubated at 4°C for 10 min to terminate the reaction. The fat cells were washed three times with 1.5 mL of cold KRBB without glucose, filtered through Whatman® microfiber filters and placed in scintillation vials filled with 3 mL of an aqueous scintillation cocktail. Radioactivity was measured using a liquid scintillation analyzer. Glucose uptake activity was corrected by subtracting nonspecific uptake activity, which was determined by pretreatment of the adipocyte cells with 20 μM cytochalasin B.

Statistical analysis

All results are expressed as the mean values ± standard error of the mean. Comparisons between baseline fasting blood glucose levels and levels after treatment were performed using Student's paired *t*-test. For the glucose uptake determination, results among groups were compared and tested by analysis of variance followed by the Student-Newman-Keuls test for specific group differences. A $P < 0.05$ was accepted as statistically significance.

RESULTS

Isolation and structure elucidation

Five compounds were obtained from the extraction procedure. Cpd 1 is 5-hydroxy- 7,4'-dimethoxyflavone (0.22% of dried leaf powder); Cpd 2 is 5,4'-dihydroxy-7-methoxyflavone, or genkwanin (0.15%); Cpd 3 is protocatechuic acid (0.32%); Cpd 4 is IPG (3.17%); and Cpd 5 is mangiferin (0.24%). Compound identities were elucidated from the spectroscopic data and confirmed by comparison with data reported in the literature.[9-13] The structures of the compounds are shown in Figure 2. The yield of Cpd 4 was 47% of the ME, and this compound was selected for investigation of *in vivo* anti-hyperglycemic activity and enhancement of glucose uptake by adipocytes. The physicochemical characteristics and spectroscopic details of Cpd 4, or IPG, described below.

Cpd4 (IPG): IPG. A yellowish crystalline powder (~10% hexane in EtOAc); Rf, 0.39; mp, 272.66-278.08°C. IR (KBr) ν_{max}/cm: 3339, 1607, 1511, 1448, 1317, 1275, 1166, 1109, 1075, 1020, 897, 818, 754, 605, 523. Electrospray ionization-mass spectrometry (MS), quasi-molecular ion m/z 431.0923 (M + Na) (calcd. 431.10 for $C_{19}H_{20}NaO_{10}$). ^{1}H NMR (acetone-d_6) δ: 3.46 (1H, m, glc-5), 3.6 (1H, m, glc-3), 3.7 (1H, *dd, j* = 8.81, 8.93), 3.7 (1H, m, glc-2), 3.84 (2H, *d, j* = 3.27), 4.92 (1H, *d, j* = 9.75), 5.99 (1H, *s*), 6.85 (2H, *d, j* = 8.7), 7.65 (2H, *d, j* = 8.7). ^{13}C-NMR (acetone-d_6) δ: 61.66 (glc-6), 70.55 (glc-4), 74.46 (glc-2), 76.58 (glc-1), 79.25 (glc-3), 82.09 (glc-5), 96.82 (C-3), 104.6 (C-1'), 106.72 (C-5), 115.28 (C-3',5'), 132.57 (C-2',6'), 133.21 (C-1'), 160.57 (C-2), 161.34 (C-4'), 162.03 (C-6), 162.54 (C-4), and 197.9 (C-7).

Lowering fasting blood glucose activity of the methanolic extract and Iriflophenone 3-C-β-glucoside

Body weight changes of the mice are shown in Table 1. The STZ-induced diabetic mice that were treated with distilled water lost significant weight (12.8% ± 2.3%), which is a sign of diabetes. The STZ-induced diabetic mice that were treated with IPG and insulin did not show significantly changes in weight from the beginning of the treatment. On the other hand, treatment with the ME exerted a drastic weight loss of −24.7% ± 2.6%.

The effects of the ME and IPG on fasting blood glucose levels are shown in Table 2. The fasting blood glucose levels of the groups treated with 1.0 g/kg ME and 0.47 g/kg IPG

Table 1: Effects of the ME and IPG on the BW of STZ-mice

Treatment	Mean of BW (g)[a]				Percentage change[b]
	Week 0	Week 1	Week 2	Week 3	
Control					
Normal mice+DW	39.6±0.6	39.9±0.6	39.9±0.7	37.0±0.7[a]	−6.6±0.7
STZ-mice+DW	36.6±0.9	37.4±0.9	36.8±0.9	32.0±1.0[a]	−12.9±2.3
STZ-mice+ins 8 U/kg BW	38.8±1.1	38.8±1.2	40.0±1.2	40.6±0.9	+5.0±4.7[c]
High dose					
STZ-mice+ME 1 g/kg BW	35.0±0.7	30.0±0.8[a]	30.0±0.7[a]	26.2±0.5[a]	−24.8±2.6[bc]
STZ-mice+IPG 0.47 g/kg BW	36.6±1.1	37.4±0.9[a]	35.7±0.9	36.1±0.9	−1.0±1.9[c]
Low dose					
STZ-mice+ME 0.1 g/kg BW	34.9±1.0	31.8±1.5[a]	31.2±1.1[a]	27.8±1.2[a]	−20.2±2.3[b]
STZ-mice+IPG 0.047 g/kg BW	36.6±1.3	38.1±0.8	38.0±0.7	37.7±0.8	+3.9±2.9[c]

ANOVA: Analysis of variance; Ins: Insulin; ME: Methanolic extract; IPG: Iriflophenone 3-*D*-*β*-glucoside; STZ: Streptozotocin; BW: Body weight; DW: Distilled water. [a]P<0.001 by paired *t*-test comparing to week 0; [b]P<0.05 by ANOVA test and followed by student-Newman-Keuls test to compare with the normal control group; [c]P<0.05 by ANOVA test and followed by Student-Newman-Keuls test to compare with the STZ-mice control group. n=6

Table 2: Effects of ME and IPG on fasting blood glucose levels in STZ-mice

Treatment	Mean of fasting blood glucose (mg/dL)				Percentage change
	Week 0	Week 1	Week 2	Week 3	
Control					
Normal mice+DW	70.4±1.7	66.4±3.2	71.4±6.3	67.2±1.6[a]	−4.5±0.5
STZ-mice+DW	365.8±42.0	403.8±44.1	417.2±46.4	403.7±46.0	+12.6±9.7
STZ-mice+Ins 8 U/kg BW	330.6±40.4	257.4±21.0	236.2±19.2[a]	190.8±30.0[a]	−41.5±8.0[b]
High dose					
STZ-mice+ME 1 g/kg BW	366.9±37.0	284.8±23.5[a]	254.8±29.4[a]	210±21.3[a]	−40.3±5.7[b]
STZ-mice+IPG 0.47 g/kg BW	289.2±25.7	271.7±42.1	204.8±22.5[a]	150.7±12.8[a]	−46.4±5.6[b]
Low dose					
STZ-mice+ME 0.1 g/kg BW	433.1±52.5	420.6±50.2	413.4±56.9	412.9±57.7	−10.7±13.6
STZ-mice+IPG 0.047 g/kg BW	306.1±38.0	342.7±29.5	324.1±31.2	343.7±30.4	+18.6±9.6

ANOVA: Analysis of variance; STZ: Streptozotocin; BW: Body weight; Ins: Insulin; ME: Methanolic extract; IPG: Iriflophenone 3-C-β-glucoside; DW: Distilled water. [a]*P*<0.001 by paired *t*-test comparing to week 0; [b]*P*<0.05 by ANOVA followed by the student-Newman-Keuls test to compare with STZ-mice. *n*=6

were (reduced 40.3% and 46.4%) significantly ($P < 0.05$) and were similar to those of the group treated with 8 U/kg insulin (41.5%).

Effects of Iriflophenone 3-C-β-glucoside on glucose uptake into rat adipocytes

The enhancement of glucose uptake into rat adipocytes by treatment with 0.25 and 2.5 μM IPG was 153.3% and 154.6%, respectively. These enhancements were about the same degree as that resulting from treatment with 1 mg/L of ME (152%), while the effect shown by treatment with 1.5 nM insulin was 183%. At IPG doses of 12.5 and 25 μM, glucose uptake was increased to approximately 114-117% [Table 3], which was 11-14% above that of the negative control. Thus, the minimum concentration of IPG that exerts the highest effect is 0.25 μM.

Table 3: Effect of IPG on glucose uptake by rat adipocytes

Treatment	2-deoxyglucose (% of control)
KRBB buffer (control)	100±8.0
Insulin 1.5 nM	183.1±17.9*
ME 1 mg/L	152±7.0*
IPG 0.25 μM	153.5±18.0*
IPG 2.5 μM	154.6±11.2*
IPG 12.5 μM	114.0±15.2
IPG 25 μM	111.7±7.4

ANOVA: Analysis of variance; SEM: Standard error of the mean; IPG: Iriflophenone 3-C-β-glucoside; KRBB: Krebs-Ringer bicarbonate buffer; ME: Methanolic extract. Results are expressed as the mean±SEM of four independent experiments, each of which was performed in triplicate. *P<0.05 by ANOVA followed by the student-Newman-Keuls test by comparing to buffer control group

DISCUSSION

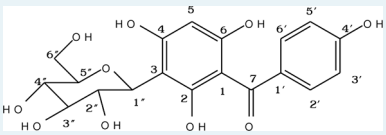
In this study, five pure compounds were isolated from the ME of the leaves of *A. sinensis*. Cpd 1 was 5-hydroxy-7,4'-dimethoxyflavone and its yield was 0.22% of dried leaf powder. Cpd2 was identified as 5,4'-dihydroxy-7-methoxyflavone, or genkwanin, and was isolated in 0.15% yield. Cpd 3, protocatechuic acid, was isolated in 0.32% yield. Cpd 4, IPG, was isolated in 3.17% yield and represented 47% of the ME. Cpd 5 was found to be mangiferin and was recovered in 0.24% yield. This is the first report of the isolation of protocatechuic acid from *A. sinensis* leaves.

Cpd 4, IPG, was crystallized from 10% hexane in EtOAc as a yellowish crystalline powder. The yield of Cpd 4 was approximately 47% of the ME, accounting for nearly half of the extract. The IR spectrum revealed a broad O-H stretching band at 3339/cm and the C = O stretching vibration of a ketone at 1607/cm. The electrospray-MS spectrum of Cpd 4 shows a quasi-molecular ion at m/z 431.0923 [M + Na] (calculated 431.10 for $C_{19}H_{20}O_{10}Na$). The ^{1}H and ^{13}C NMR peaks were assigned as listed in Table 4. The DEPT, COSY, HMQC and HMB NMR spectra show complete correlation between the protons and carbons. The structure of Cpd 4 was elucidated to be identical to that of IPG, and our data for this compound agree with those described in the literature.[12] Cpd 4 has also been found in *Hypodematium fauriei* Yagawa and *Hypodematium crenatum* Kuhn[12] and in the leaves of *A. sinensis*[6] and *Mangiferin indica* L.[14]

All of the isolated compounds are polyphenols. Cpd 1 and Cpd 2 are flavones, some of which have been found to inhibit glucose transportation.[15] These compounds were not isolated in quantities sufficient to permit *in vivo* studies, and so no effort was made to determine their activity on lowering fasting blood glucose. Protocatechuic acid (Cpd 3) was found to enhance glucose uptake in human omental adipocytes and a murine adipocyte (3T3-L1) cell line by 40% at 100 μM.[16] Administration of 1 μM mangiferin (Cpd 5), a xanthone, to the muscle cell line (L6-myotubes) led to the enhancement of glucose uptake by 59.2%.[1] Mangiferin was found to possess anti-diabetic activity comparable to that of glibenclamide when dosed at 40 mg/kg BW in rats.[17]

Blood glucose levels can be lowered by several mechanisms, one of which involves the availability of insulin. Insulin can be thought of as opening a gate to utilization of glucose by cells. In this experiment, STZ was injected to damage β-cells of the pancreas. The primary aim of the experiment was to determine whether IPG can be used to replace the insulin.

Table 4: ^{1}H and ^{13}C NMR spectral data (δ, ppm) for Cpd 4 (IPG)



Position	$^{\delta}H^{a}$	$^{\delta}C^{b}$
1	-	104.6 qC
2	-	160.57 qC
3	-	96.82 qC
4	-	162.54 qC
5	5.99 (1H, *s*)	106.72 CH
6	-	162.03 qC
7	-	197.9 qC
1'	-	133.21 qC
2', 6'	7.65 (2H, *d, j*=8.7)	132.57 CH
3', 5'	6.85 (2H, *d, j*=8.7)	115.28 CH
4'	-	161.34 qC
Glc-1	4.92 (1H, *d, j*=9.75)	76.58 CH
Glc-2	3.7 (1H, *m*)	74.46 CH
Glc-3	3.60 (1H, *m*)	79.25 CH
Glc-4	3.7 (1H, *dd, j*=8.81, 8.93)	70.55 CH
Glc-5	3.46 (1H, *m*)	82.09 CH
Glc-6	3.84 (2H, *d, j*=3.27)	61.66 CH_2

[a]Figures in parentheses are coupling constants in Hz; [b]Multiplicities were determined by analysis of the DEPT spectrum. DEPT: 135 and DEPT 90; IPG: Iriflophenone 3-C-β-glucoside

Iriflophenone 3-C-β-glucoside (Cpd 4) is a compound with four hydroxyl substituents distributed over two benzene rings that are linked through a carbonyl group. The compound was detected on TLC in the isolation process by spraying with DPPH; the color changed rapidly from violet to yellow, indicating that IPG is a good radical scavenger. IPG is a 3-*C-b-glucoside* and was the most abundant compound isolated in this work (47% of the ME). IPG has not been investigated previously for activity on lowering blood glucose level or glucose transportation. The experiments described in this study are the first *in vivo* and *in vitro* investigations into the activity of IPG. The properties of this compound were examined at high and low doses. The experiments were run in parallel with treatment by ME.

Due to the limited quantities of IPG available, ICR mice were employed as an animal model instead of rats that had been used in previous studies. ICR mice were selected because they are readily available from the standard and recognized Animal Center of Thailand. A review of the literature indicated that ICR mice had also been used as a model for diabetes.[18] In our experiment, mice were initially induced by intra-peritoneal injection with STZ at 100 mg/kg BW, but the effect was not prolonged and

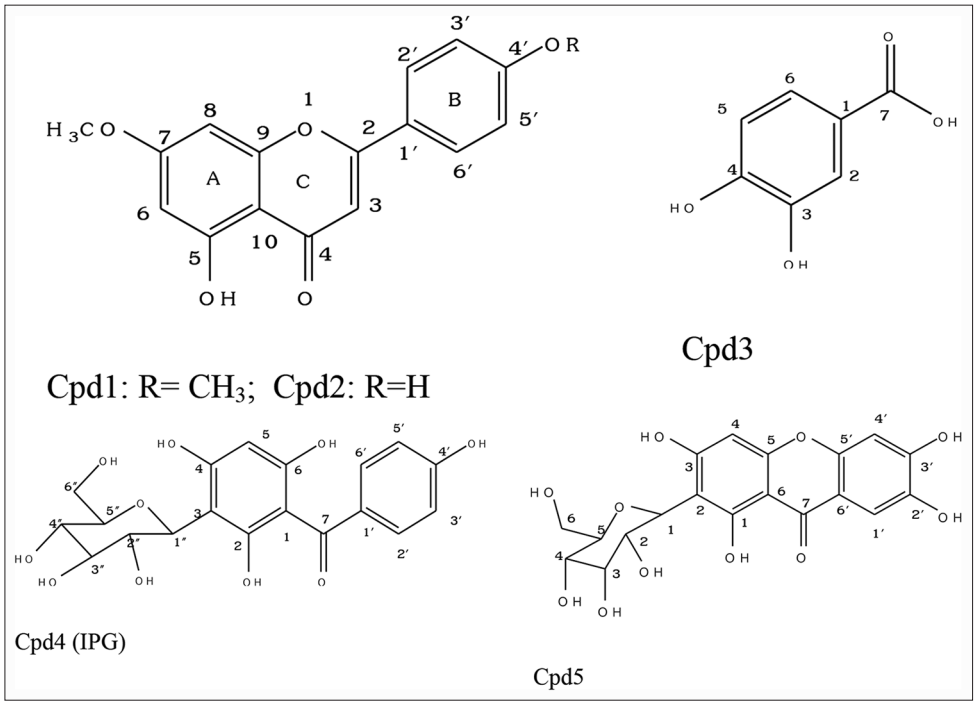


Figure 2: Structures of Cpds 1-5 isolated from methanolic extract. Cpd1: 5-hydroxy-7,4'-hydroxyflavones; Cpd2: 7-methoxy-5,4'-hydroxyflavone or genkwanin; Cpd3: protocatechuic acid; Cpd4 : iriflophenone 3-*C-β*-glucoside (IPG); and Cpd5: mangiferin

varied between mice. Thus, two additional injections of STZ at 50 mg/kg BW were used to boost the effect. Our findings agreed with reports in the literature that intra-peritoneal doses below 150 mg/kg may be insufficient to induce diabetes in mice.[19] Various doses were employed in mice, including 100-200 mg/kg by the intravenous (IV) route or multiple doses of 40 mg/kg IV for 5 days.[18,19] In this study, only fasting blood glucose was measured to monitor the effect. The STZ-induced diabetic mouse model has a limitation with respect to blood collection. Determination of fasting blood glucose levels can be performed easily using very small amounts of blood, and only small volumes of blood can be withdrawn from a tiny tail vein. Although the glucose tolerance test is the gold standard for measuring anti-hyperglycemic effects, this assay requires that large amounts of blood withdrawn from frequent vein punctures, which may cause excessive trauma to the animals.

In our preliminary study, 4 U/kg BW of insulin was employed as a positive control. This was the same dose as had been used in rats, but blood glucose levels were not decreased in mice. The dose was then doubled. In this study, ME and IPG were evaluated at high and low doses. These doses were 1 and 0.1 g/kg BW for ME and 0.47 and 0.047 g/kg BW of IPG (the compound that constituted approximately 47% of ME). Blood glucose levels were decreased by 46.4% at the 0.47 g/kg dose of IPG, a level similar to those obtained with 8 U/kg insulin (41.5%) and 1 g/kg ME (40.3%), as shown in Table 2. No effect was observed at low doses of IPG or ME, which were 0.047 and 0.1 g/kg, respectively [Table 2]. Weight loss [Table 1] and liquid stool were not observed with IPG treatment, but were observed at both doses of the ME treatment. The effect is most likely attributable to genkwanin (Cpd 2), one of the constituents in the ME, which is a laxative.[4]

It was found in the *in vivo* study that β-cells producing insulin were affected by STZ. The effects of STZ on insulin activity were investigated, and one property was found to be insulin mimicking activity.

Iriflophenone 3-C-β-glucoside significantly increased glucose uptake to 153-154% of control at 0.25 and 2.5 μM, which may be the maximum effect of IPG in the experiment as it was designed. At concentrations as high as 12.5 and 25 μM, the enhancement efficacy was lost and was not significantly different from the control.

Polyphenols influence glucose transportation in pathways that are either insulin-dependent (through glucose transporter type 4 [GLUT4]) or insulin-independent.[16] Insulin stimulates glucose uptake in adipose tissue by eliciting the translocation of GLUT4 from the intracellular region to the plasma membrane and through adiponectin secretion. Our finding that the enhancement of glucose transportation declined at high concentrations of IPG may be attributable to several possible factors. One possibility is that GLUT4 was present in a limited number of the adipocytes used in the experiment, such that additional IPG would not exert any further effect. This can be seen from the activity of IPG at 0.25 and 2.5 μM. Alternatively, at very high concentrations (12.5 and 25 μM) the glucose transportation may be lost. As IPG is a glycoside containing a molecule of glucose, it might act as a competitive inhibitor or obstruct the transportation of 2-deoxyglucose. IPG might also affect other factors involved in the mechanism, that is, PPARY, or adiponectins that influence GLUT4 transporters. However, these assumptions have yet to be verified at the molecular level.

All of the compounds isolated from the ME in our study are polyphenols. Two of these compounds, mangiferin and protocatechuic acid, have been shown to enhance glucose uptake activity.[1,16] In these previous studies, the glucose uptake enhancement mediated by these two compounds was 59% (0.4 μg/mL or 0.95 μM) and 40% (100 μM), respectively. In our study, IPG (0.25 μM) enhanced glucose uptake by 53%, whereas the effect from 1 mg/L ME was an enhancement of 52%.[7] Thus, the three effective components in the ME, namely mangiferin, protocatechuic acid and IPG, did not exert a combined or synergistic effect in enhancing glucose uptake. Furthermore, blood glucose levels were not significantly different after administration of 1 g/kg ME (containing approximately 0.47 g of IPG) or of 0.47 g/kg IPG alone. These results imply that there was also no synergistic effect from the other phenolic compounds in the ME. However, two flavones are present in the ME (Cpd 1 and 2). Strobel *et al*.[15] found that some flavones inhibit the transportation of methylglucose by GLUT4. Further experiments are needed to investigate the possibility that a combination of these compounds could have the same effect or counteract activity.

Many polyphenols have been investigated for the prevention of diabetes. The activities of such compounds include influencing β-cell function by increasing insulin secretion and mimicking insulin activity by stimulating the translocation of GLUT4 to the plasma membrane. Cyanidin-3-O-γ-glucoside and protocatechuic acid increase glucose uptake and increase GLUT4 membrane translocation by exerting their insulin-like activity through peroxisome proliferator-activated receptor-γ activation. Polyphenolic compounds are good radical scavengers and prevent oxidized low density lipoprotein generation.[16,20,21] Structure activity relationships are well established for the inhibition of the enzyme α-glucosidase, which leads to an anti-hyperglycemic effect.[21,22]

In this study, IPG, a polyphenolic compound containing a C-β-glycoside, was isolated in abundance from *A. sinensis* leaves. IPG was demonstrated to have anti-hyperglycemic activity in STZ-induced mice and to enhance glucose transportation into rat adipocytes.

CONCLUSION

This is the first report of the activity of IPG in lowering fasting blood glucose. The results of *in vivo* and *in vitro* experiments demonstrate that IPG has potency in decreasing the fasting blood glucose levels in STZ-induced diabetic mice and for enhancing glucose uptake into adipocytes. Other researchers are investigating the leaf extracts of this plant for their anti-hyperglycemic activity should be aware of their laxative effects. This finding provides important evidence in the search for new antidiabetic agents from plants. IPG was present in significant quantities and was easily isolated from the plant.

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