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New type of anti-diabetic compounds from the processed leaves of *Hydrangea macrophylla* var. *thunbergii* (Hydrangeae Dulcis Folium)

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Abstract—Two 3-phenyldihydroisocoumarins (hydrangenol and phyllodulcin), a 3-phenylisocoumarin (thunberginol A), and a stilbene (hydrangeaic acid) from the processed leaves of *Hydrangea macrophylla* var. *thunbergii* (Hydrangeae Dulcis Folium) promoted adipogenesis of 3T3-L1 cells. Hydrangenol, a principal constituent, significantly increased the amount of adiponectin released into the medium and mRNA levels of adiponectin, peroxisome proliferator-activated receptor γ2 (PPARγ2), and glucose transporter 4 (GLUT4), while it decreased the expression of interleukin 6 (IL-6) mRNA. Furthermore, hydrangenol significantly lowered blood glucose and free fatty acid levels 2 weeks after its administration at a dose of 200 mg/kg/d in KK-A^y mice.

In modern society, diabetes mellitus is a chronic and often undiagnosed or inadequately treated disease. The prevalence of diabetes is growing at an alarming rate. There are more than 125 million people with the disease in the world today, and by 2010 this number is expected to approach 220 million, with more than 90% of affected individuals having type 2 diabetes mellitus, which is characterized by reduced insulin action in the liver, adipose tissue, and skeletal muscle, and progressive loss of pancreatic β-cell function. Furthermore, type 2 diabetes is closely associated with other metabolic diseases, such as hypertension, cardiovascular diseases, and atherosclerosis. Insulin resistance is an important marker for developing type 2 diabetes.[1] Therefore, more effective and safe medicines are required.

Thiazolidinedione compounds (TZDs) such as pioglitazone are potent insulin sensitizers and currently used clinically to treat type 2 diabetes. TZDs were originally identified based on their antihyperglycemic activity, but they are also able to improve other abnormalities associated with type 2 diabetes, such as hyperlipidemia, atherosclerosis, hypertension, and chronic inflammation. TZDs are potent ligands of peroxisome proliferator-activated receptor γ (PPARγ) and improve insulin sensitivity by increasing the mRNA and protein levels of adiponectin, an important adipocytokine associated with insulin sensitivity in adipose tissue, and by decreasing free fatty acid (FFA) and inflammatory factor TNF-α levels in diabetic subjects and animal models in vivo and in adipocytes such as 3T3-L1 cells in vitro.[2–4] It is well established that PPARγ agonists such as TZDs promote the adipogenesis of 3T3-L1 cells, and so the cells have been used for the development of anti-diabetic compounds.[5]

In the course of searching for anti-diabetic constituents from natural medicines,[6] we found that the ethyl acetate fraction of the processed leaves of *Hydrangea macrophylla* var. *thunbergii* (Hydrangeae Dulcis Folium), which is listed in Japanese Pharmacopoeia XV and used as a sweetening agent for diabetic patients, promoted the accumulation of triglyceride in 3T3-L1 cells [increment: 23.4 ± 4.1% ($p < 0.01$) at 10 μg/mL]. Anti-microbial and anti-allergic effects of the constituents of this herb have been reported before,[7] however, anti-diabetic effects have not. Therefore, isolated constituents [hydrangenol (**1**, yield: 2.35% from the herb), phyllodulcin (**5**, 1.99%), thunberginols A (**8**, 0.0086%), B (**9**, 0.0016%),

Keywords: Hydrangenol; Anti-diabetic compound; 3T3-L1 cell; Adiponectin; KK-A^y mouse; *Hydrangea macrophylla* var. *thunbergii*.
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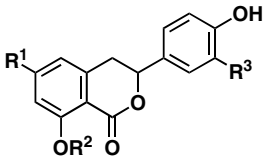
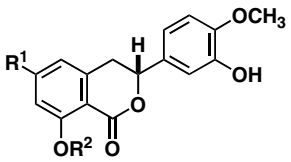
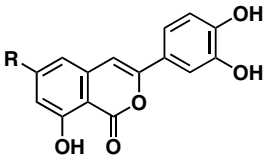


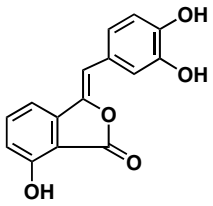
hydrangenol (**1**): R^1=H, R^2=H, R^3=H
hydrangenol 8-*O*-glucoside (**2**):R^1=H, R^2=Glc, R^3=H
thunberginol C (**3**) : R^1=OH, R^2=H, R^3=H
thunberginol D (**4**) : R^1=OH, R^2=H, R^3=OH



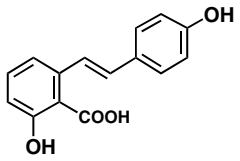
phyllodulcin (**5**): R^1=H, R^2=H
phyllodulcin 8-*O*-glucoside (**6**): R^1=H, R^2=Glc
thunberginol E (**7**): R^1=OH, R^2=H



thunberginol A (**8**) : R=H
thunberginol B (**9**) : R=OH



thunberginol F (**10**)



hydrangeaic acid (**11**)

Glc: β-D-glucopyranosyl

Figure 1. Chemical structures of **1**–**11** from the processed or un-processed leaves of *Hydrangea macrophylla* var. *thunbergii*.

Table 1. Effects of the isolated constituents (**1**–**11**) from the processed and un-processed leaves of *Hydrangea macrophylla* var. *thunbergii* and troglitazone on TG levels in 3T3-L1 cells

	Increase (%) in TG levels Concentration (μM)					
	0	1	3	10	30	100
Hydrangenol (**1**)	0.0 ± 1.3	14.4 ± 2.2**	19.2 ± 4.4**	17.7 ± 4.0**	29.9 ± 5.5**	41.3 ± 5.3**
Hydrangenol 8-*O*-glucoside (**2**)	0.0 ± 1.7	11.1 ± 4.7	11.2 ± 4.6	1.2 ± 3.6	10.5 ± 2.5	9.2 ± 3.2
Thunberginol C (**3**)	0.0 ± 3.9	−9.3 ± 2.3	−7.5 ± 3.3	−11.1 ± 2.0*	−17.6 ± 4.7*	1.4 ± 2.5
Thunberginol D (**4**)	0.0 ± 1.8	−1.2 ± 1.3	2.8 ± 3.1	−0.8 ± 1.2	4.0 ± 2.4	12.5 ± 1.4*
Phyllodulcin (**5**)	0.0 ± 0.8	−0.7 ± 1.7	12.8 ± 3.0*	9.8 ± 2.1	23.8 ± 3.3**	20.3 ± 4.0**
Phyllodulcin 8-*O*-glucoside (**6**)	0.0 ± 1.8	−3.6 ± 4.4	−8.7 ± 2.7	−1.5 ± 1.7	−3.0 ± 5.7	−6.6 ± 4.2
Thunberginol E (**7**)	0.0 ± 5.7	−6.3 ± 5.0	−7.5 ± 4.2	−8.0 ± 2.2	3.4 ± 2.0	−4.0 ± 3.1
Thunberginol A (**8**)	0.0 ± 3.0	8.0 ± 2.6	17.5 ± 3.1**	19.6 ± 3.7**	15.7 ± 3.5**	−27.6 ± 1.3**
Thunberginol B (**9**)	0.0 ± 4.4	−3.8 ± 2.0	−8.0 ± 5.3	−7.8 ± 4.4	7.2 ± 4.7	−54.1 ± 0.8**
Thunberginol F (**10**)	0.0 ± 4.1	2.1 ± 4.4	6.2 ± 6.2	6.2 ± 2.2	8.3 ± 6.7	−54.8 ± 2.0**
Hydrangeaic acid (**11**)	0.0 ± 3.8	2.2 ± 1.4	11.7 ± 3.4	11.1 ± 7.3	27.3 ± 4.7**	41.2 ± 2.6**
Troglitazone	0.0 ± 1.6	33.8 ± 1.6**	40.5 ± 1.6**	39.6 ± 1.8**	—	—

Murine 3T3-L1 cells [Cell No. IFO50416 obtained from Health Science Research Resources Bank (Osaka, Japan)] (5.0×10^4 cells/well) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS were seeded into a 48-well multiplate. After 24 h, differentiation was induced by changing the medium to a differentiation medium [DMEM (high glucose) supplemented with 10% FCS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 μg/mL insulin]. After 3 d, the differentiation medium was replaced with a maintenance medium [DMEM (high glucose) supplemented with 10% FCS and 5 μg/mL insulin]. After 4 d (on day 8), the medium was removed and H_2O (200 μL/well) was added to each well, and then the cells were sonicated. The triglyceride (TG) level in the sonicate was determined with a commercial kit (Triglyceride E-test Wako, Wako Chemical Industries). Troglitazone was used as a reference compound. The test compound dissolved in DMSO was added to the differentiation and maintenance media (final DMSO concn was 0.1%). Values represent means ± SEM of % increase in TG levels (n = 4). Significantly different from the control group, $^*p < 0.05$, $^{**}p < 0.01$.[9]

Table 2. Effect of hydrangenol (**1**) on release of adiponectin in the medium

	Concentration (μM)	Adiponectin (ng/mL)	
		On day 4	On day 6
Control (DMSO)	—	21.7 ± 0.02	65.7 ± 0.03
Hydrangenol (**1**)	3	22.3 ± 0.01	68.2 ± 0.07
	10	22.8 ± 0.07	84.2 ± 0.05
	30	27.4 ± 0.02**	94.0 ± 0.05**
	100	33.0 ± 0.04**	99.8 ± 0.07**
Troglitazone	3	43.0 ± 0.05**	101.2 ± 0.06**

3T3-L1 cells (5.0×10^4 cells/well) in DMEM supplemented with 10% FCS were seeded into a 48-well multiplate. After the initial differentiation (on day 4) or 2 d after the replacement with the maintenance medium (on day 6), adiponectin concentrations in the medium were measured using a ELISA kit (R&D systems) according to the manufacturer's instructions. Troglitazone was used as a reference compound. The test compound dissolved in DMSO was added to the differentiation and/or maintenance media (final DMSO concn was 0.1%). Values represent means ± SEM (n = 3). Significantly different from the control group, $^{**}p < 0.01$.[9]

C (**3**, 0.0038%), D (**4**, 0.0012%), E (**7**, 0.0045%), and F (**10**, 0.0028%), and hydrangeaic acid (**11**, 0.0024%)][7a,b] and hydrangenol 8-*O*-glucoside (**2**, 0.55%) and phyllodulcin 8-*O*-glucoside (**6**, 1.40%) from the un-processed leaves[8] were examined for effects on adipogenesis in 3T3-L1 cells (Fig. 1).

As shown in Table 1, hydrangenol (**1**), phyllodulcin (**5**), thunberginol A (**8**), and hydrangeaic acid (**11**) promoted the accumulation of TG in the cells in a concentration-dependent manner, with **1** and **11** exhibiting stronger activity. Compounds **8**–**10** reduced TG levels at a high concentration (100 μM), suggesting cytotoxic effects at high concentrations. With regard to the structural requirements for the activity, a hydroxyl group at the 6-position and the 8-*O*-glucopyranosyl moiety reduced the activity [inhibition at 30 μM: **1** (29.9%) > **2** (10.5%) and **3** (−17.6%); **5** (23.8%) > **6** (−3.0%) and **7** (3.4%); **8** (15.7%) > **9** (7.2%)].

Next, effects of hydrangenol (**1**) on the release of adiponectin into the medium, the gene expression of adiponectin, PPARγ2, interleukin 6 (IL-6), and glucose transporter 4 (GLUT4), and the uptake of 2-deoxyglucose were examined. As shown in Tables 2 and 3, **1** caused a concentration-dependent increase in the release of adiponectin in the medium and expression of adiponectin, PPARγ2, and GLUT4 mRNA, while **1** decreased the expression of IL-6 mRNA which was associated with insulin resistance. On the other hand, the reference

Table 3. Effects of hydrangenol (**1**) and troglitazone on gene expression of adiponectin, PPARγ2, IL-6, and GLUT4

	Concentration (μM)	Ratio (target gene/β-actin mRNA)			
		Adiponectin	PPARγ2	IL-6	GLUT4
Control (DMSO)	—	1.00 ± 0.09	1.00 ± 0.05	1.00 ± 0.02	1.00 ± 0.10
Hydrangenol (**1**)	30	1.98 ± 0.06**	1.92 ± 0.03**	0.78 ± 0.03**	1.12 ± 0.07
	100	2.46 ± 0.09**	2.65 ± 0.08**	0.51 ± 0.05**	1.38 ± 0.04**
Troglitazone	3	3.01 ± 0.11**	1.09 ± 0.02	0.89 ± 0.02	2.92 ± 0.11**

3T3-L1 cells (1.0×10^6 cells/well) in DMEM supplemented with 10% FCS were seeded into a 6-well multiplate. After the initial differentiation, the medium was replaced with the maintenance medium. After 2 d (on day 6), total RNA was extracted using RNeasy™ Mini Kit (Qiagen) according to the manufacturer's instructions. The total RNA was reverse transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad). Then a real time PCR was carried out on a MiniOpticon real-time machine (Bio-Rad) using an iQ SYBR™ Green Supermix Kit (Bio-Rad). The abundance of each gene product was calculated by relative quantification, with values for the target genes normalized with β-actin mRNA. Thermal cycling conditions for the PCR were 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, then a melting curve analysis from 65 °C to 95 °C, every 0.2 °C. The primer pairs for cytokines, GLUT4, and β-actin are shown in Table 4. Troglitazone was used as a reference compound. The test compound dissolved in DMSO was added to the differentiation and maintenance media (final DMSO concn was 0.1%). Values represent means ± SEM ($n = 3$). Significantly different from the control group, $^{**}p < 0.01$.[9]

Table 4. Primer sequences for mouse adiponectin, PPARγ2, IL-6, GLUT4, and β-actin

Adiponectin[10]	Sense	5′-AAGGACAAGGCCGTTCTCT-3′
	Antisense	5′-TATGGGTAGTTGCAGTCAGTTGG-3′
PPARγ2[11]	Sense	5′-GGTGAAACTCTGGGAGATTC-3′
	Antisense	5′-CAACCATTGGGTCAGCTCTTG-3′
IL-6[12]	Sense	5′-AACGATGATGCACTTGCAGA-3′
	Antisense	5′-GAGCATTGGAAATTGGGGTA-3′
GLUT4[13]	Sense	5′-CCTGAGAGCCCCAGATACCTCTAC-3′
	Antisense	5′-GTCGTCCAGCTCGTTCTACTAAG-3′
β-Actin[12]	Sense	5′-ATGGGTCAGAAGGACTCCTACG-3′
	Antisense	5′-AGTGGTACGACCAGAGGCATAC-3′

Table 5. Effects of hydrangenol (**1**) and troglitazone on 2-deoxyglucose uptake in 3T3-L1 cells

	2-Deoxyglucose uptake (%) Concentration (μM)					
	0	1	3	10	30	100
Hydrangenol (**1**)	100 ± 3	117 ± 2	160 ± 10**	221 ± 9**	245 ± 8**	302 ± 12**
Troglitazone	100 ± 4	—	388 ± 24**	—	—	—

3T3-L1 cells (5.0×10^4 cells/well) in DMEM supplemented with 10% FCS were seeded into a 48-well multiplate. After the initial differentiation, the medium was replaced with the maintenance medium. After 4 d (on day 8), the cells were incubated in the medium without serum for 4 h and then washed with Krebs–Ringer phosphate (KRP) buffer. After 20-min incubation in KRP buffer containing insulin (100 nM), 2-deoxy-D-glucose (final concn 0.1 mM) and 2-deoxy-D-(2,6-^{3}H)-glucose (1 μCi/mL) were added to the wells. After 10 min, the cells were washed with ice-cold PBS to terminate the reaction, and then a 1 M NaOH solution (200 μL/well) was added to each well and incubated for 2 h at 37 °C to dissolve the cells. After neutralization with 1 M HCl, an aliquot was transferred to a vial and the radioactivity was measured using a liquid scintillation counter (Backman LS6500). Nonspecific uptake was determined in the presence of 20 μM cytochalasin B and was subtracted from the total value. Troglitazone was used as a reference compound. The test compound dissolved in DMSO was added to the differentiation and maintenance media (final DMSO concn was 0.1%). Values represent means ± SEM ($n = 4$). Significantly different from the control group, $^{**}p < 0.01$.[9]

Table 6. Effects of hydrangenol (**1**) and troglitazone on blood glucose, TG, and FFA in KK-A^y mice

	Dose (mg/kg, po)	*n*	Blood glucose levels (mg/dL)			Blood TG levels (mg/dL)		
			0 w	1 w	2 w	0 w	1 w	2 w
Normal (C57 BL/6)	—	6	239.1 ± 11.2**	253.2 ± 12.8**	236.1 ± 9.3**	87.0 ± 3.7**	83.9 ± 7.0**	82.1 ± 8.8**
Control (vehicle)	—	14	343.9 ± 24.7	482.1 ± 10.7	491.3 ± 8.9	360.6 ± 18.0	480.0 ± 38.2	538.8 ± 34.7
Hydrangenol (**1**)	100	13	336.3 ± 20.0	389.1 ± 23.0**	422.7 ± 15.5*	332.1 ± 21.2	429.3 ± 24.0	483.9 ± 28.4
	200	13	336.0 ± 20.6	397.5 ± 26.9*	411.2 ± 25.4**	362.8 ± 17.9	413.8 ± 33.6	428.8 ± 38.6
Control (vehicle)	—	5	312.9 ± 12.8	443.2 ± 32.8	486.3 ± 15.6	329.2 ± 18.5	451.3 ± 30.7	592.4 ± 18.4
Troglitazone	100	5	307.5 ± 17.7	293.3 ± 13.3**	354.5 ± 43.7*	329.4 ± 36.4	303.4 ± 23.5**	464.6 ± 38.6*

	Dose (mg/kg, po)	*n*	Blood FFA levels (mEq/L)		
			0 w	1 w	2 w
Normal (C57 BL/6)	—	6	1.09 ± 0.05	0.82 ± 0.06	1.01 ± 0.10
Control (vehicle)	—	14	1.02 ± 0.07	1.03 ± 0.06	1.16 ± 0.08
Hydrangenol (**1**)	100	13	0.96 ± 0.08	0.91 ± 0.04	1.07 ± 0.07
	200	13	1.01 ± 0.08	0.81 ± 0.04**	0.89 ± 0.06*
Control (vehicle)	—	5	0.95 ± 0.04	0.85 ± 0.03	1.00 ± 0.06
Troglitazone	100	5	0.95 ± 0.02	0.62 ± 0.05**	0.80 ± 0.05*

Test sample suspended in 5% acacia solution and vehicle (5% acacia solution) were given orally to male KK-A^y mice (5 weeks old) once a day (17:00–18:00) for 2 weeks. Blood sample (ca. 0.2 mL) was collected just before the experiment (0 w) and at 7th (1 w) and 14th day (2 w) after the administration. Blood glucose, triglyceride (TG) and free fatty acid (FFA) levels of non-fasted KK-A^y mice were determined using commercial kits (Glucose CII-test Wako, Triglyceride E-test Wako and NEFA C-test Wako, Wako Pure Industries, Ltd.). C57BL/6 mice were used as non-diabetic mice (normal mice). Troglitazone was used as a reference compound. Values represent means ± SEM. Significantly different from $^*p < 0.05$, $^{**}p < 0.01$.[9]

compound, troglitazone, did not induce the expression of PPARγ2 mRNA and its inhibitory activity against the expression of IL-6 mRNA was weak. These results suggested that the mechanism of action by **1** was different from that of troglitazone, although the mechanism involved should be studied further. Furthermore, **1** (3–100 μM) concentration-dependently enhanced the uptake of 2-deoxyglucose in 3T3-L1 cells (Table 5).

Finally, effects of hydrangenol (**1**) on blood glucose, TG, and free fatty acid (FFA) levels in KK-A^y mice, as a model of type 2 diabetes, were examined. As shown in Table 6, compound **1** significantly lowered blood glucose levels 2 weeks after its administration at 200 mg/kg/d. In addition, compound **1** at a dose of 200 mg/kg/d decreased FFA levels, which is believed to be one of the factors inducing insulin resistance, similar to troglitazone.

In conclusion, hydrangenol (**1**), phyllodulcin (**5**), thunberginol A (**8**), and hydrangeaic acid (**11**) from the processed leaves of *H. macrophylla* var. *thunbergii* (Hydrangeae Dulcis Folium) promoted adipogenesis in 3T3-L1 cells. Hydrangenol (**1**), a principal constituent, significantly increased the amount of adiponectin released into the medium and levels of adiponectin, PPARγ2, and GLUT4 mRNA, while **1** decreased the expression of IL-6 mRNA. Furthermore, **1** significantly lowered blood glucose and FFA levels 2 weeks after its administration at a dose of 200 mg/kg/d. This is the first report that 3-phenyldihydroisocoumarins have an antidiabetic effect, and **1** is a promising seed compound with anti-diabetic activity.

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