

## 5,7-Dihydroxy-6-geranylflavanone improves insulin sensitivity through PPAR $\alpha$ / $\gamma$ dual activation

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**Abstract.** In the present study, we demonstrate that 5,7-dihydroxy-6-geranylflavanone (DGF) isolated from *Amorpha fruticosa* (*A. fruticosa*) is a novel peroxisome proliferator-activated receptor (PPAR) $\alpha$ / $\gamma$  dual agonist which may be used to improve insulin sensitivity. The extract from *A. fruticosa* increased the transcriptional activity of both PPAR $\alpha$  and PPAR $\gamma$  which was, in part, driven by the active ingredient DGF. Treatment with DGF markedly enhanced the adipogenesis of 3T3-L1 preadipocytes, which was comparable to the effect of the PPAR $\gamma$  agonist, troglitazone. In addition, DGF was found to enhance fatty acid oxidation and glucose utilization through the dual activation of PPAR $\alpha$ / $\gamma$ . In addition treatment with DGF led to an improvement in insulin sensitivity, resulting in enhanced glucose uptake in muscle cells. The findings of our study data suggest that DGF may be used as potential therapeutic agent in the treatment of type 2 diabetes and related metabolic disorders by enhancing glucose and lipid metabolism.

### Introduction

Type 2 diabetes mellitus (T2DM), which accounts for >90% of diabetes cases, is a common metabolic disease that is characterized by the resistance of target tissues to insulin stimulation (1). T2DM is usually associated with hyperglycemia, dyslipidemia, obesity, hypertension, fatty liver disease, atherosclerosis, certain types of cancer and cardiovascular diseases (2). The peptide hormone, insulin, lowers blood glucose levels by facilitating

glucose uptake, mainly into skeletal muscle and fat tissue, and by inhibiting endogenous glucose production in the liver; insulin resistance occurs when a normal dose of the hormone is incapable of eliciting these metabolic responses (3). Multiple defects in intracellular events, including an impairment of the insulin signaling axis, disrupt glucose metabolism and reduce glycogen synthesis, thus contributing to insulin resistance (4-6).

Relieving insulin resistance has been considered a promising approach to the treatment of T2DM (7). Thiazolidinediones (TZDs) and fibrates are some of the most commonly used medications in the treatment of T2DM, hyperlipidemia and insulin resistance. These drugs bind to and activate peroxisome proliferator-activated receptors (PPARs), which results in the upregulation of several genes involved in glucose and lipid metabolism (8). The PPAR family, belonging to the nuclear hormone receptor family, consists of 3 isoforms, PPAR $\alpha$ , PPAR $\beta$ / $\delta$  and PPAR $\gamma$  (9). PPAR $\gamma$  is mainly expressed in adipose tissue and vascular tissue/macrophages (10), and is present in muscle and  $\beta$  cells at low levels (11); it affects genes involved in lipid synthesis and storage, and glucose homeostasis. It is the functioning receptor for TZDs, including rosiglitazone and pioglitazone (8). PPAR $\alpha$  is predominantly expressed in the liver (12) and stimulates lipid consumption by enhancing the expression of fatty acid oxidation genes, resulting in the amelioration of hyperlipidemia (13). PPAR $\alpha$  agonists, including fenofibrate have been shown to exert potent effects by reducing plasma triglyceride levels (14). The distinct metabolic effects of PPAR $\alpha$  and PPAR $\gamma$  agonists on insulin sensitivity and lipid metabolism has encouraged the development of novel drugs which target both PPAR $\alpha$  and PPAR $\gamma$  (15-17). It has been proposed that the dual activation of PPAR $\alpha$  and PPAR $\gamma$  may guarantee more desirable effects with limited adverse effects (15-17). A number of PPAR $\alpha$ / $\gamma$  dual agonists have been identified and tested in obese and insulin-resistant individuals (18,19); however, the majority of these drugs have been shown to have unexpected side-effects, including heart failure, renal failure, urinary cancer and anemia (18,19). Therefore, the development of novel PPAR $\alpha$ / $\gamma$  dual agonists with few adverse effects is urgently required.

*Amorpha fruticosa* (*A. fruticosa*) (Leguminosae) has been applied traditionally in the treatment of hypertension, hematomas and contusions in China, Japan and Korea. Previous studies on this plant have reported a number of phenolic compounds,

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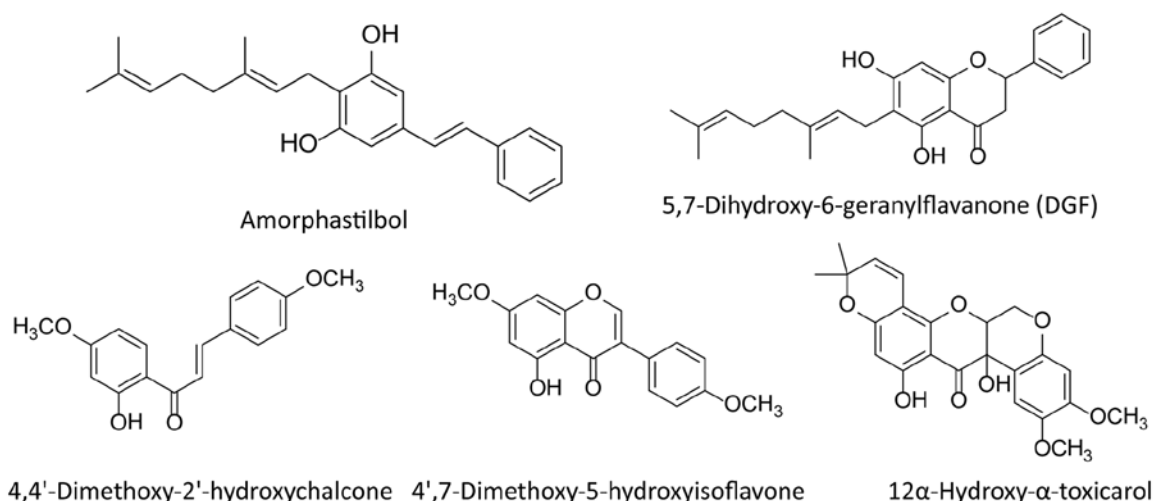


Figure 1. Chemical structures of the ingredients from *Amorpha fruticosa* (*A. fruticosa*).

including stilbenes, flavonoids and rotenoids. In addition, their pharmacological activities, such as antimicrobial, antitumor and tumor necrosis factor (TNF)- $\alpha$  inhibitory activities have been investigated (20,21). In our previous studies, we demonstrated the therapeutic potential of amorphastilbol isolated from *A. fruticosa* in metabolic disorders through the dual activation of PPAR $\alpha/\gamma$  in 3T3-L1 cell systems and animal models (22,23). In the present study, we identified another active ingredient, 5,7-dihydroxy-6-geranylflavanone (DGF; chemical structure shown in Fig. 1), isolated from *A. fruticosa* which promotes the dual activation of PPAR $\alpha/\gamma$  and explored its pharmacological properties.

## Materials and methods

**Cell culture and chemicals.** The CV-1 kidney cells, 3T3-L1 preadipocyte mouse embryonic fibroblasts and C2C12 mouse myoblasts were obtained from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen). The HepG2 hepatocellular carcinoma cells were purchased from ATCC and cultured in minimum essential medium with Earle's balanced salts (MEM/EBSS), supplemented with 10% FBS, 1% penicillin/streptomycin, 1X non-essential amino acid and 1 mM sodium pyruvate (both from Welgene, Daegu, Korea) at 37°C with 5% CO<sub>2</sub> in air. Troglitazone (TRO; PPAR $\gamma$  agonist) and WY14643 (PPAR $\alpha$  agonist) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and GW501516 (PPAR $\delta$  agonist) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Extraction, isolation and identification of DGF from *A. fruticosa*.** Fruits of *A. fruticosa* were collected in Gangwon Province, Korea in December 2012. The plant was authenticated and deposited at the KIST Gangneung Institute Herbarium, Gangneung, Korea. The fruit of *A. fruticosa* (300 g) was extracted 3 times with ethanol and evaporated under a vacuum at 40°C. The extract was reconstituted with 0.5 liter of water and re-extracted with 0.5 liter of ethyl acetate. A total of 5 pure compounds,

amorphastilbol, DGF, 4,4'-dimethoxy-2'-hydroxychalcone, 4',7-dimethoxy-5-hydroxyisoflavone and 12 $\alpha$ -hydroxy- $\alpha$ -toxicarol, were isolated by preparative high-performance liquid chromatography (DeltaPrep 150 ml System, SunFire Silica 150x10 mm Column, Waters, MA, USA) and identified from this extract, the chemical structures of which are shown in Fig. 1.

**Cell-based transactivation assay.** The CV-1 cells were seeded in 24-well plates and cultured for 24 h prior to transfection. After 24 h, the medium was changed to 10% charcoal dextran-treated FBS-DMEM; 4 h following the change in medium, a DNA mixture containing a 3X multimerized PPRE-luciferase reporter plasmid (0.3  $\mu$ g), pcDNA3-hPPAR (30 ng) and an internal control plasmid, pRL-SV-40 (5 ng), was transfected into the cells using the TransFast™ transfection reagent (Promega, Madison, WI, USA) (23). For RXR $\alpha$  reporter gene analysis, RXR $\alpha$  plasmid (30 ng) and RXRE-luciferase reporter plasmid (0.3  $\mu$ g) were transfected into the CV-1 cells. Following 24 h of transfection, the cells were treated with 10  $\mu$ M TRO or the indicated concentrations of plant extracts (1, 3, 10 and 30  $\mu$ g/ml) or DGF (1, 3, 10 and 30  $\mu$ M) and incubated for an additional 24 h. The luciferase activities of the cell lysates were measured using the Dual-Luciferase® reporter assay system according to the manufacturer's instructions (Promega). The relative luciferase activity was normalized to the corresponding *Renilla* luciferase activity to determine the transfection efficiency.

**Adipocyte differentiation.** Adipocyte differentiation was induced by treating the cells with differentiation medium (DM) containing hormonal cocktail (10  $\mu$ g/ml insulin, 1  $\mu$ M dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine) with 10% FBS in DMEM for 48 h before the cells were switched to a maintenance medium with 10% FBS and 10  $\mu$ g/ml insulin. After an additional 48 h, the medium was replaced with 10% FBS-DMEM. Thereafter, the medium was changed every 2 days. The test drugs were administered at the initiation of differentiation and at every medium change for 8 days. The lipid accumulation in the cells was detected by Oil Red O staining.

Table I. Primer sequences used for RT-PCR and qPCR.

| Gene name       | Oligonucleotide sequence (5'→3')                             | Accession no. | Gene expression analysis |
|-----------------|--|---------------|--------------------------|
| mC/EBP $\alpha$ | F: AGGTGCTGGAGTTGACCAGT<br>R: CAGCCTAGAGATCCAGCGAC           | NM_007678     | qPCR/RT-PCR              |
| mPPAR $\gamma$  | F: CAAGAATACCAAAGTGCGATCAA<br>R: GAGCTGGGTCTTTTCAGAATAATAAG  | NM_011146     | qPCR                     |
| mPPAR $\gamma$  | F: TTCGCTGATGCACTGCCTAT<br>R: GCCAACAGCTTCTCCTTCTC           | NM_011146     | RT-PCR                   |
| maP2            | F: AGTGAAAACCTTCGATGATTACATGAA<br>R: GCCTGCCACTTTCCTTGTG     | NM_024406     | qPCR                     |
| maP2            | F: ATGTGTGATGCCTTTGTGGGA<br>R: TGCCCTTTCATAAACTCTTGT         | NM_024406     | RT-PCR                   |
| mGLUT4          | F: AGAGTCTAAAGCGCCT<br>R: CCGAGACCAACGTGAA                   | NM_009204     | qPCR/RT-PCR              |
| mAdiponectin    | F: AGCCTGGAGAAGCCGCTTAT<br>R: TTGCAGTAGAACTTGCCAGTGC         | NM_009605     | qPCR/RT-PCR              |
| mResistin       | F: TCAACTCCCTGTTTCCAAATGC<br>R: TCTTCACGAATGTCCCACGA         | NM_022984     | qPCR/RT-PCR              |
| mGAPDH          | F: TTGTTGCCATCAACGACCCC<br>R: GCCGTTGAATTGCCGTGAG            | NM_008084     | qPCR                     |
| mGAPDH          | F: ACCACAGTCCATGCCATCAC<br>R: TCCACCACCCTGTTGCTGTA           | NM_008084     | RT-PCR                   |
| hACO2           | F: GCGGACATGGCTACTCAAAGC<br>R: GCAGTGCACCTTAGCAGCCTG         | NM_003500.1   | RT-PCR                   |
| hCPT1 $\alpha$  | F: AGACGGTGGAACAGAGGCTGAAG<br>R: TGAGACCAAACAAAGTGATGATGTCAG | NM_001876.1   | RT-PCR                   |
| hGAPDH          | F: ACCACAGTCCATGCCATCAC<br>R: TCCACCACCCTGTTGCTGTA           | NM_002046     | RT-PCR                   |

**Ligand binding assay.** The LanthaScreen™ TR-FRET PPAR competitive binding assay (Invitrogen) was performed according to the manufacturer's instructions (24). In brief, a mixture of 5 ng glutathione S-transferase fused to the PPAR ligand-binding domain (GST-PPAR-LBD), 5 nM Tb-GST antibody, 5 nM Fluormone Pan-PPAR Green, and serial dilutions of DGF ( $\leq 100 \mu\text{M}$ ) was added to the wells of black 384-well plates to a total volume of 18 ml. Following 2 h of incubation in the dark, the FRET signal was measured by excitation at 340 nm and emission at 520 nm using the Infinite® M1000 microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

**Measurement of triglyceride content.** Triglyceride contents were measured using the triglyceride determination kit (Sigma-Aldrich). Following treatment, the differentiated 3T3-L1 adipocytes were rinsed twice with PBS, scraped in 200  $\mu\text{l}$  of saline solution (150 mM NaCl, 2 mM EDTA, 50 mM sodium phosphate, pH 7.4), sonicated to homogenize the cell suspension and the total triglyceride content was then measured. The total triglyceride content was determined by measuring the increase in absorbance at 540 nm using a microplate reader (PowerWave XS; BioTek Instruments Inc., Winooski, VT, USA) according to the manufacturer's instructions. The results were expressed as milligrams of triglyceride per milligram of cellular protein. The residual cell lysate was centrifuged at

12,000 rpm to remove the fatty layer, and was then assayed for the measurement of the protein content using a BCA protein assay kit (Sigma-Aldrich).

**Measurement of glycerol-3-phosphate dehydrogenase (GPDH) activity.** GPDH activity was measured using a GPDH activity assay kit (MK426; Takara Bio Inc., Shiga, Japan). Following treatment, the differentiated 3T3-L1 adipocytes were rinsed twice with PBS, scraped into 200  $\mu\text{l}$  enzyme extraction buffer (provided with the kit) and sonicated. GPDH activity was determined according to the decrease in NADH activity by measuring the decrease in the absorbance at 340 nm using a microplate reader, according to the manufacturer's instructions.

**Gene expression analysis.** Total RNA was isolated from the 3T3-L1 adipocytes or HepG2 cells transfected with pcDNA3-hPPAR $\alpha$  plasmid using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA concentration of each sample was determined using a spectrophotometer (Nanodrop 2000; Thermo Fischer Scientific, Inc., Wilmington, DE, USA) at 260 nm; the integrity of each RNA sample was evaluated using the Agilent 2100 BioAnalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). cDNA synthesis was performed using 1  $\mu\text{g}$  of total RNA in 20  $\mu\text{l}$  with random primers and SuperScript II reverse

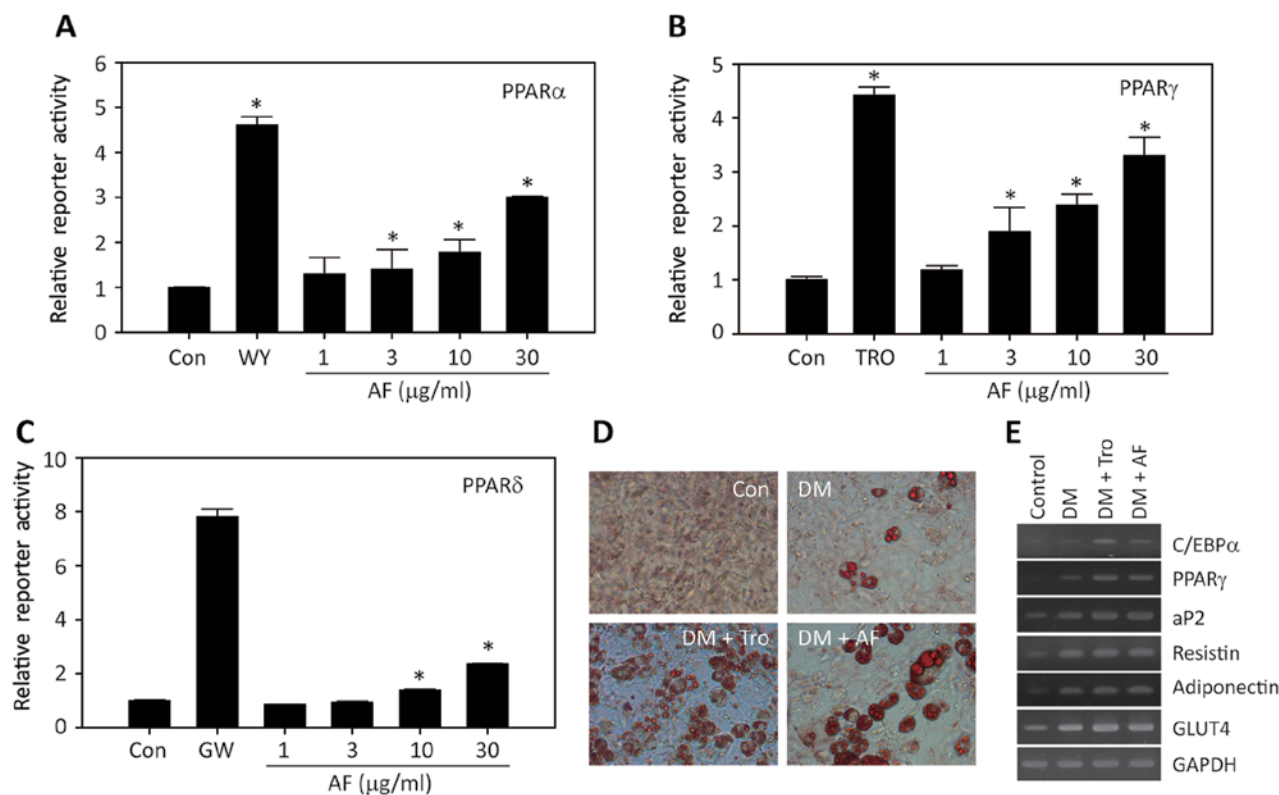


Figure 2. *Amorphia fruticosa* (AF) extract selectively activates peroxisome proliferator-activated receptor (PPAR) $\alpha$  and PPAR $\gamma$  *in vitro*. CV-1 cells were transiently co-transfected with human (A) PPAR $\alpha$ , (B) PPAR $\gamma$  or (C) PPAR $\delta$  expression vectors, the PPRE-luciferase reporter construct, and the pRL-SV40 vector. The cells were then treated with 10  $\mu$ M WY14643 (WY), 10  $\mu$ M troglitazone (TRO), 1  $\mu$ M GW501516 (GW), or various concentrations of AF (1,3,10 or 30  $\mu$ g/ml) for 24 h. The harvested cells were analyzed by reporter assay as described in the Materials and methods. Each bar represents the mean  $\pm$  SD of duplicates. \* $P$ <0.05 vs. control (untreated cells). (D) 3T3-L1 cells were differentiated using a hormonal cocktail in the absence or presence of TRO or AF for 8 days; the lipids were then stained using Oil Red O. (E) Relative mRNA expression of each gene from the TRO- or AF-treated 3T3-L1 cells was determined by RT-PCR and confirmed by agarose gel electrophoresis with ethidium bromide staining.

transcriptase. Quantitative PCR (q-PCR) was performed with SYBR-Green fluorescent dye using the 7500 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). Data analyses were performed using the 7500 system SDS software version 1.3.1 (Applied Biosystems Life Technologies). In some cases, conventional PCR was performed using the GeneAmp PCR system 9700 (Applied Biosystems Life Technologies), and the integrity of the PCR product was verified by agarose gel electrophoresis and ethidium bromide staining. The sequences of the primers used in this study are listed in Table I.

**Myotube formation and western blot analysis.** The C2C12 myoblasts were cultured in DMEM until reaching 90% confluence. The cells were differentiated into myotubes with DMEM containing 2% horse serum for 4 days, and then incubated for 16 h in DMEM containing 2% BSA and 10% FBS in the absence or presence of 0.75 mM palmitate to induce insulin resistance. Subsequently, the DGF-treated cells were stimulated with 100 nM insulin for 10 min. Following stimulation, the cells were washed twice with PBS and harvested. For western blot analysis, antibodies to Akt (cs9272; 1:1,000), phospho-Ser473 Akt (cs4060; 1:1,000), insulin receptor (IR)  $\beta$  (cs3020; 1:1,000), phospho-Tyr1135/1136 insulin growth factor (IGF)-1R $\beta$ /Tyr1150/1151 IR $\beta$  (cs3024; 1:1,000) were used (Cell Signaling Technology, Beverly, MA, USA).

**2-NBDG glucose uptake assay.** The myotubes, which were obtained from the above-mentioned procedures, were stimulated with 100 nM insulin for 1 h. Following insulin stimulation, the myotubes were incubated with 50  $\mu$ M 2-NBDG (Invitrogen) for 15 min and then washed with PBS 3 times to remove free 2-NBDG. The fluorescence intensity of the cells containing 2-NBDG was measured using the Infinite M1000 microplate reader (Tecan Group Ltd.) with excitation at 485 nm and emission at 535 nm.

**Statistical analysis.** Data are expressed as the means  $\pm$  SD. Differences between the mean values in the 2 groups were analyzed using one-way analysis of variance (ANOVA). A value of  $P$ <0.05 was considered to indicate a statistically significant difference.

## Results

**Dual activation of PPAR $\alpha/\gamma$  transcriptional activity by *A. fruticosa*.** To explore the pharmacological properties of *A. fruticosa*, we examined the effects of *A. fruticosa* on the transcriptional activation of PPARs. As shown in Fig. 2A and B, treatment with the extract of *A. fruticosa* led to an increase in PPAR $\alpha$ - and PPAR $\gamma$ -reporter gene activities in a dose-dependent manner; however, it had a minimal effect on the transcriptional activation of PPAR $\delta$  (Fig. 2C). In addition, treatment with *A. fruticosa*

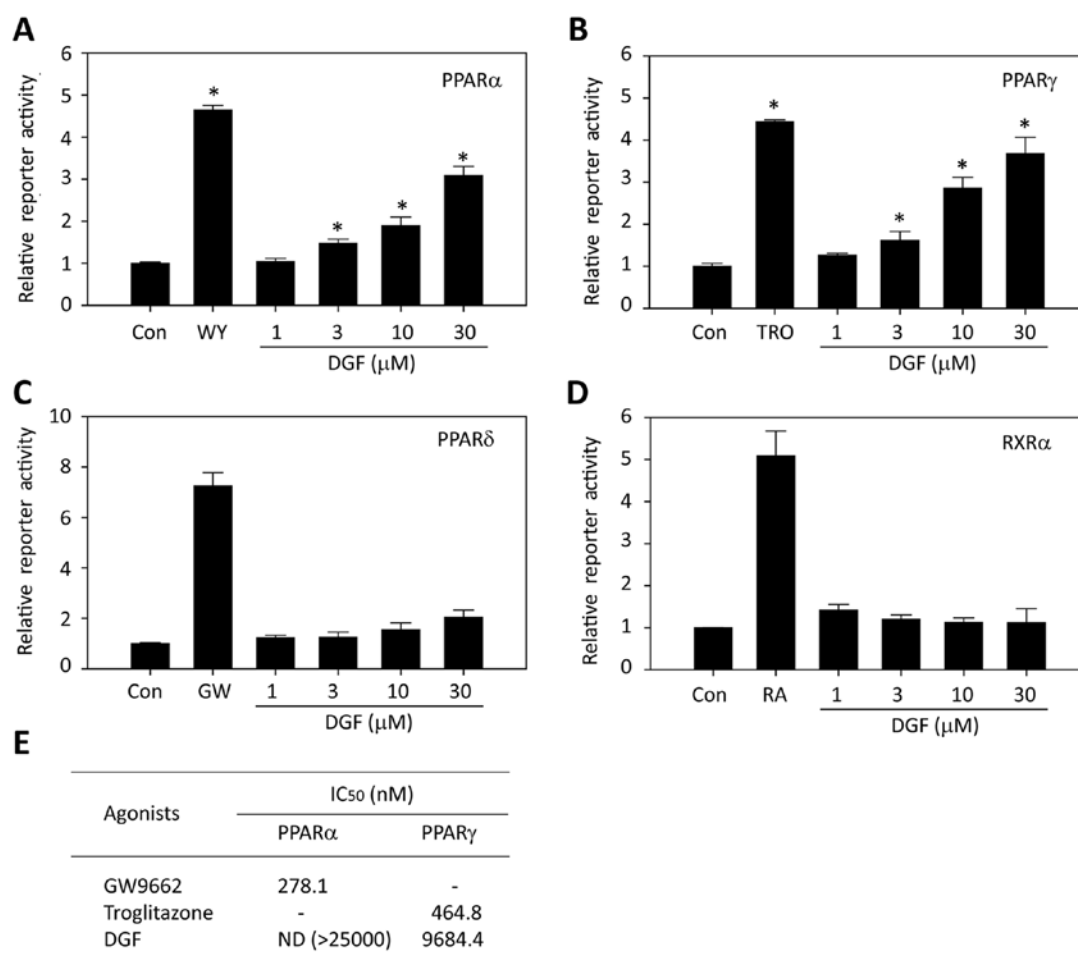


Figure 3. 5,7-Dihydroxy-6-geranyl flavanone (DGF) from *Amorpha fruticosa* (*A. fruticosa*) selectively activates peroxisome proliferator-activated receptor (PPAR) $\alpha$  and PPAR $\gamma$  *in vitro*. Human (A) PPAR $\alpha$ , (B) PPAR $\gamma$ , or (C) PPAR $\delta$  or (D) RXR $\alpha$  expression vector, PPRE-luciferase reporter construct, and pRL-SV40 vector were transiently co-transfected into the CV-1 cells. The cells were then treated with 10  $\mu$ M WY14643 (WY), 10  $\mu$ M troglitazone (TRO), 1  $\mu$ M GW501516 (GW), or various concentrations of DGF (1, 3, 10 or 20  $\mu$ M) for 24 h. A reporter assay was performed as described in the Materials and methods. Each bar represents the mean  $\pm$  SD of duplicates. \*P<0.05 vs. control. (E) Binding affinities of DGF to PPAR $\alpha$  and PPAR $\gamma$  were analyzed using the LanthaScreen™ TR-FRET PPAR competitive binding assay, as described in the Materials and methods.

Table II. Effects of the compounds from *A. fruticosa* on PPAR $\alpha$ / $\gamma$  transcriptional activity.

| Compounds                                 | Transactivation (fold) |               |
|---|------------------------|---------------|
|   | PPAR $\alpha$          | PPAR $\gamma$ |
| DGF                                       | 2.97                   | 3.68          |
| Amorphastilbol                            | 3.94                   | 3.61          |
| 4,4'-Dimethoxy-2'-hydroxychalcone         | 1.38                   | 1.27          |
| 4',7-Dimethoxy-5-hydroxyisoflavone        | 1.65                   | 2.38          |
| 12 $\alpha$ -Hydroxy- $\alpha$ -toxicarol | 2.01                   | 2.85          |
| WY14643                                   | 4.71                   | -             |
| TRO                                       | -                      | 4.4           |

Transcriptional activities of peroxisome proliferator-activated receptor (PPAR)- $\alpha$  and PPAR $\gamma$  in the presence of each compounds (30  $\mu$ M) were analyzed, and the data are expressed as the fold increase in gene expression compared with the control. WY14643 (10  $\mu$ M) and troglitazone (TRO) (10  $\mu$ M) were used as positive controls of PPAR $\alpha$  and PPAR $\gamma$ , respectively. DGF, 5,7-dihydroxy-6-geranyl flavanone; *A. fruticosa*, *Amorpha fruticosa*.

extract markedly enhanced the adipocyte differentiation of the 3T3-L1 cells, as shown by Oil Red O staining (Fig. 2D), which was accompanied by an increase in the expression of adipocyte marker genes, including CCAAT-enhancer-binding protein (*C/EBP*) $\alpha$ , PPAR $\gamma$ , adipocyte protein 2 (*aP2*), resistin, adiponectin, and glucose transporter type 4 (*GLUT4*) (Fig. 2E). These results strongly indicate the presence of a novel PPAR $\alpha$ / $\gamma$  agonist in the fruit extract of *A. fruticosa*.

*DGF is a novel PPAR $\alpha$ / $\gamma$  dual activator.* As the PPAR $\alpha$ / $\gamma$ -reporter gene activities and adipocyte differentiation were markedly enhanced by the extract of *A. fruticosa*, we wished to identify the active ingredients of *A. fruticosa*. Thus, we isolated and identified 5 compounds from *A. fruticosa* and evaluated their activities on PPAR $\alpha$ / $\gamma$  transcriptional activation. As shown in Table II, although amorphastilbol exhibited the most potent promoting effect on PPAR $\alpha$ / $\gamma$  transactivation, DGF also had a similar effect. The other compounds, 4,4'-dimethoxy-2'-hydroxychalcone, 4',7-dimethoxy-5-hydroxyisoflavone and 12 $\alpha$ -hydroxy- $\alpha$ -toxicarol, exerted weaker effects than those of amorphastilbol and DGF. Since the pharmacological properties of amorphastilbol have already been reported (22),



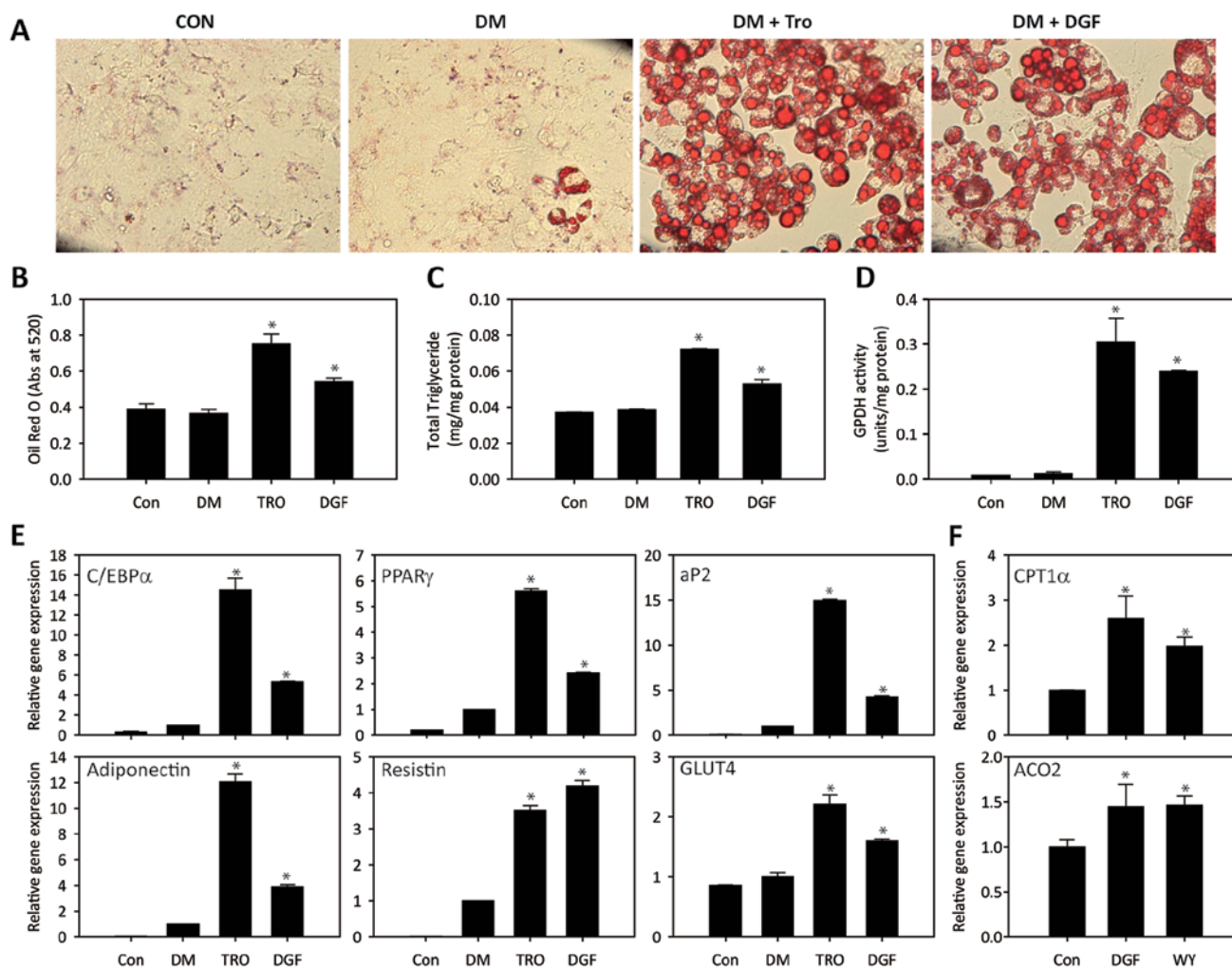


Figure 4. 5,7-Dihydroxy-6-geranyl flavanone (DGF) upregulates specific genes involved in both adipocyte differentiation and fatty acid oxidation. (A) Lipid accumulation in differentiated 3T3-L1 cells treated with troglitazone (TRO) or DGF (10  $\mu$ M) shown by Oil Red O staining. DM, differentiation medium. (B) The quantification of the lipid accumulation was based on the OD values measured at 520 nm of destained Oil Red O from the adipocytes. (C) Triglyceride contents were measured using a triglyceride determination kit. (D) Glycerol-3-phosphate dehydrogenase (GPDH) activity was measured on day 8 after differentiation. \* $P$ <0.05 vs. DM control. (E) The expression of adipocyte-enriched genes in these cells was analyzed by qPCR ( $n=3$  experiments) and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each bar represents the mean  $\pm$  SD. \* $P$ <0.05 vs. DM control. (F) HepG2 cells transfected with human peroxisome proliferator-activated receptor (PPAR) $\alpha$  were incubated with WY or DGF for 48 h. The relative gene expression was analyzed by qPCR and normalized to GAPDH expression. Each bar represents the mean  $\pm$  SD of triplicate experiments. \* $P$ <0.05 vs. control (untreated cells).

in this study, we focused on the properties of DGF. As shown in Fig. 3A and B, treatment with DGF led to an increase in both PPAR $\alpha$ - and PPAR $\gamma$ -reporter gene activities in a dose-dependent manner. However, DGF exerted a minimal effect on PPAR $\delta$  transcription (Fig. 3C) and no detectable effect on retinoid X receptor (RXR $\alpha$ ) was observed (Fig. 3D). The DGF binding affinities to PPAR $\alpha$  and PPAR $\gamma$  were weaker than those of the positive controls, GW501516 and TRO (Fig. 3E), which is in agreement with the weak ability of DGF to activate the transcription of PPAR $\alpha/\gamma$  in comparison to that of the positive control (Fig. 3A and B).

*DGF upregulates specific genes involved in both adipocyte differentiation and fatty acid oxidation.* To evaluate the potential effects of DGF on adipogenesis, 3T3-L1 preadipocytes were differentiated for 8 days in the presence of DGF. As shown in Fig. 4A and B, treatment with DGF induced a marked increase in lipid accumulation in the differentiated adipocytes,

which was comparable to the effects of TRO. To further characterize the adipogenic potential of DGF, the cellular triglyceride content and GPDH enzyme activity were measured. The triglyceride content in the differentiated adipocytes was also increased by DGF treatment up to 1.5-fold (Fig. 4C); this observation was in accordance with the result that DGF induced lipid droplet accumulation in adipocytes, as shown in Fig. 4A. In addition, GPDH activity was significantly enhanced by DGF treatment (Fig. 4D). Taken together, these results strongly support the notion that DGF promotes the adipocyte differentiation of 3T3-L1 cells. As adipogenesis is governed by the increased expression of various transcription factors and adipocyte-specific genes (25), we examined the effects DGF on the expression of adipogenic transcription factors and marker genes in the differentiated adipocytes. Following the induction of adipocyte differentiation in the presence of DGF, the mRNA levels of *C/EBP $\alpha$* , *PPAR $\gamma$* , *aP2*, *adiponectin* and *resistin* were measured by qPCR. In the DGF-treated adipocytes, the mRNA

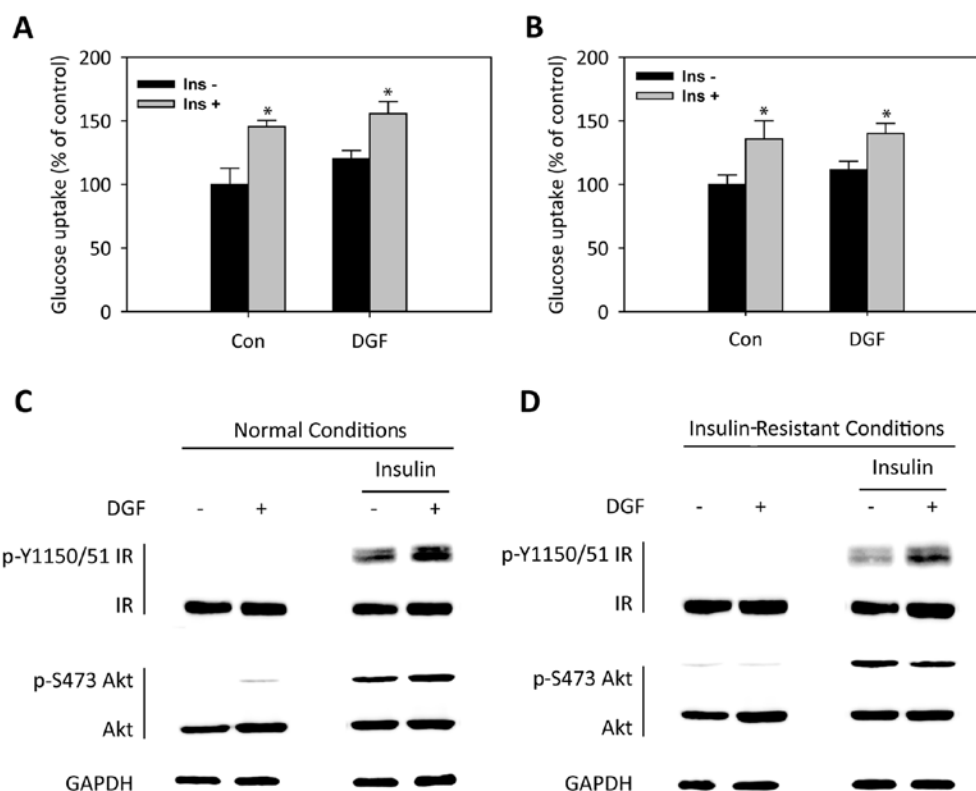


Figure 5. 5,7-Dihydroxy-6-geranyl flavanone (DGF) enhances insulin sensitivity under both normal and insulin-resistant conditions. (A and B) Basal and insulin-stimulated glucose uptake in DGF-treated C2C12 myotubes under both normal and insulin-resistant conditions. Each bar represents the mean  $\pm$  SD of triplicate experiments. \* $P < 0.05$  vs. cells without any treatment. (C and D) Level of phosphorylation of insulin receptor (IR) and Akt in DGF-treated C2C12 myotubes under both normal and insulin-resistant conditions. Protein levels were determined by western blot analysis.

levels of these genes were markedly increased compared to their levels in the absence of DGF (Fig. 4E). In addition, treatment with DGF induced an increase in the mRNA level of *GLUT4*, which is responsible for insulin-mediated glucose uptake (26), indicating that the insulin sensitivity of adipose tissue may be enhanced by DGF treatment. Subsequently, we examined the effects of DGF on fatty acid oxidation to confirm its PPAR $\alpha$  agonistic effect. In the HepG2 cells transfected with the PPAR $\alpha$  overexpression vector, the expression levels of two different PPAR $\alpha$  target genes involved in  $\beta$ -oxidation, carnitine palmitoyltransferase 1 $\alpha$  (*CPT1 $\alpha$* ) and acyl-CoA oxidase 2 (*ACO2*), were increased by DGF treatment (Fig. 4F), supporting the hypothesis that DGF promotes fatty acid oxidation through the activation of PPAR $\alpha$ .

**DGF improves insulin sensitivity.** To further ascertain the positive effects of DGF on insulin sensitivity, we first examined glucose uptake in C2C12 myotubes under normal conditions. As shown in Fig. 5A, glucose uptake in C2C12 myotubes in the absence or presence of insulin was significantly enhanced by DGF treatment under normal conditions. Correspondingly, treatment with DGF also enhanced the insulin-induced phosphorylation of IR and Akt under normal conditions (Fig. 5C). We then examined the effects of DGF on glucose uptake under palmitate-induced insulin-resistant conditions. DGF enhanced glucose uptake under insulin-resistant conditions as well (Fig. 5B). The effects of DGF on insulin sensitivity under insulin-resistant conditions were further confirmed by the elevated phosphorylation levels of these downstream proteins

in insulin signaling (Fig. 5D). All these results suggest that the enhancement of insulin sensitivity by DGF is mediated by the insulin-induced activation of the IR-Akt signaling axis and enhanced glucose uptake.

## Discussion

T2DM and related metabolic diseases pose a serious health concern in modern societies. We have recently reported that amorphastilbol isolated from *A. fruticosa* exerts beneficial effects on glucose and lipid metabolism by selectively activating both PPAR $\alpha$  and PPAR $\gamma$ , thus ameliorating metabolic disorders without being associated with any severe adverse reactions that have been observed for other PPAR agonists, such as weight gain and hepatomegaly (22). In this study, we demonstrated that DGF, as another novel PPAR $\alpha/\gamma$  dual agonist isolated from *A. fruticosa*, promoted adipogenesis through the upregulation of adipocyte specific marker gene expression, and improved insulin sensitivity. However, the binding affinities of DGF to both PPAR $\alpha$  ( $>25 \mu\text{M}$ ) and PPAR $\gamma$  ( $9.7 \mu\text{M}$ ) were much weaker than those of amorphastilbol; the binding affinities of amorphastilbol to both PPAR $\alpha$  and PPAR $\gamma$  were reported as  $1.5 \mu\text{M}$  and  $0.84 \mu\text{M}$ , respectively (22). Although its binding affinities to PPAR $\alpha/\gamma$  were much weaker than those of amorphastilbol, DGF significantly increased their transcriptional activities, which resulted in the upregulation of adipocyte specific marker gene expression and the improvement of insulin sensitivity, indicating that DGF is one of the active ingredients of *A. fruticosa* with anti-diabetic properties.

It has been demonstrated that rosiglitazone, a full agonist of PPAR $\gamma$ , is associated with an increased risk of heart attacks, which potentially limits its appeal and further clinical use, in spite of its potent improvement of glucose metabolism (27). However, recently, FDA demonstrated that there is no direct evidence for the cardiovascular risk of rosiglitazone after reviewing the results of clinical trials (28). In addition, pioglitazone, another PPAR $\gamma$  agonist, is less frequently associated with increased cardiovascular risk (29). All these facts imply that PPAR $\gamma$  activity itself may not be directly associated with this cardiovascular risk. Therefore, the development of novel partial PPAR agonists that are structurally unrelated to TZDs, particularly rosiglitazone, remains appealing. From this point of view, it is noteworthy that amorphastilbol and DGF isolated from *A. fruticosa* are partial dual agonists of PPAR $\alpha/\gamma$  and that their chemical structures are unique from those of TZDs. In addition, amorphastilbol and DGF do not affect hERG K<sup>+</sup> channel activity, which is associated with cardiovascular toxicity (data not shown). Taken together, these data support, in part, the relative safe use of amorphastilbol and DGF with regard to cardiovascular risk.

Insulin resistance occurs when a normal level of insulin is incapable of eliciting its metabolic responses (3) and results from multiple intracellular defects, including an impairment of the insulin signaling axis (4-6). Our data demonstrated that DGF ameliorated insulin resistance by activating the insulin signaling axis, resulting in the enhancement of glucose uptake (Fig. 5A and B).

In conclusion, in this study, we demonstrate that DGF exerts beneficial effects on glucose and lipid metabolism, thus ameliorating metabolic disorders by selectively activating both PPAR $\alpha$  and PPAR $\gamma$ . These results strongly suggest that DGF is one of active components of *A. fruticosa* with anti-diabetic and anti-metabolic properties. Therefore, our data provide strongly support the further development of *A. fruticosa* and its component DGF as anti-metabolic agents for the treatment of glucose and lipid abnormalities, as well as insulin resistance.

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