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Kojyl cinnamate ester derivatives promote adiponectin production during adipogenesis in human adipose tissue-derived mesenchymal stem cells

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ABSTRACT

The subcutaneous fat tissue mass gradually decreases with age, and its regulation is a strategy to develop anti-aging compounds to ameliorate the photo-aging of human skin. The adipogenesis of human adipose tissue-mesenchymal stem cells (hAT-MSCs) can be used as a model to discover novel anti-aging compounds. *Cinnamomum cassia* methanol extracts were identified as adipogenesis-promoting agents by natural product library screening. Cinnamates, the major chemical components of *Cinnamomum cassia* extracts, promoted adipogenesis in hAT-MSCs. We synthesized kojyl cinnamate ester derivatives to improve the pharmacological activity of cinnamates. Structure–activity studies of kojyl cinnamate derivatives showed that both the α,β-unsaturated carbonyl ester group and the kojic acid moiety play core roles in promoting adiponectin production during adipogenesis in hAT-MSCs. We conclude that kojyl cinnamate ester derivatives provide novel pharmacophores that can regulate adipogenesis in hAT-MSCs.

Subcutaneous fat tissue gradually thins with age.[1] This is associated with the increased risk of skin injury, a deteriorated skin function to regulate body temperature, and impaired dermal elasticity.[2,3] Human mesenchymal stem cells (hMSCs) exist in virtually all human mesenchymal tissues and can differentiate into adipocytes.[4] Therefore, chemical compounds that promote adipocyte differentiation in MSCs resident in subcutaneous fat tissue may counteract the aging-related functional decreases. Human adipose tissue-mesenchymal stem cells (hAT-MSCs) can be used as a model system to study adipocyte differentiation in fat tissue.[4] In murine pre-adipocytes like 3T3-L1 cells, treatment with an adipogenic cocktail consisting of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) (the IDX condition), can induce nearly all pre-adipocytes to acquire differentiated adipocyte phenotypes.[5,6] However, the IDX condition cannot induce the adipocyte differentiation of all the hMSCs tested in two dimensional cell cultures.[6,7] Approximately ten percent of hMSCs in cell culture have an adipocyte phenotype. When sulfonylurea-type antidiabetic drugs like glibenclamide and peroxisome proliferator-activated receptor γ (PPARγ) agonists like troglitazone are added to hMSCs cultured in the IDX condition, the fraction of differentiated adipocytes is increased. Chemical compounds with the potential to promote adipocyte differentiation in hAT-MSCs can be evaluated by testing them in combination with the IDX adipogenic cocktail in cell culture.[6,8] To discover novel pharmacophores capable of regulating the thickness of subcutaneous fat tissue, we have screened natural product libraries using hAT-MSCs as an adipocyte differentiation model. Adiponectin can be measured to quantitatively evaluate the level of adipocyte differentiation in hAT-MSCs.[9] A preliminary screening showed that ethanol extracts of *Cinnamomum cassia* (Ramulus Cinnamomi and Cortex Cinnamomi) promoted adipocyte differentiation (Fig. 1A).[10] The major chemical components of *Cinnamomum cassia* include cinnamaldehyde, cinnamic acid, and eugenol (Fig. 1B).[11] After determining the highest non-cytotoxic concentrations for cinnamaldehyde, cinnamic acid, and eugenol on hAT-MSCs (Fig. 1C–E), we evaluated the effects of the major chemical components in *Cinnamomum cassia* on adipocyte differentiation. The results (Fig. 1F) showed that supplementation with

Abbreviations: hAT-MSCs, human adipose tissue-derived mesenchymal stem cells; PPARγ, peroxisome proliferator-activated receptor gamma.

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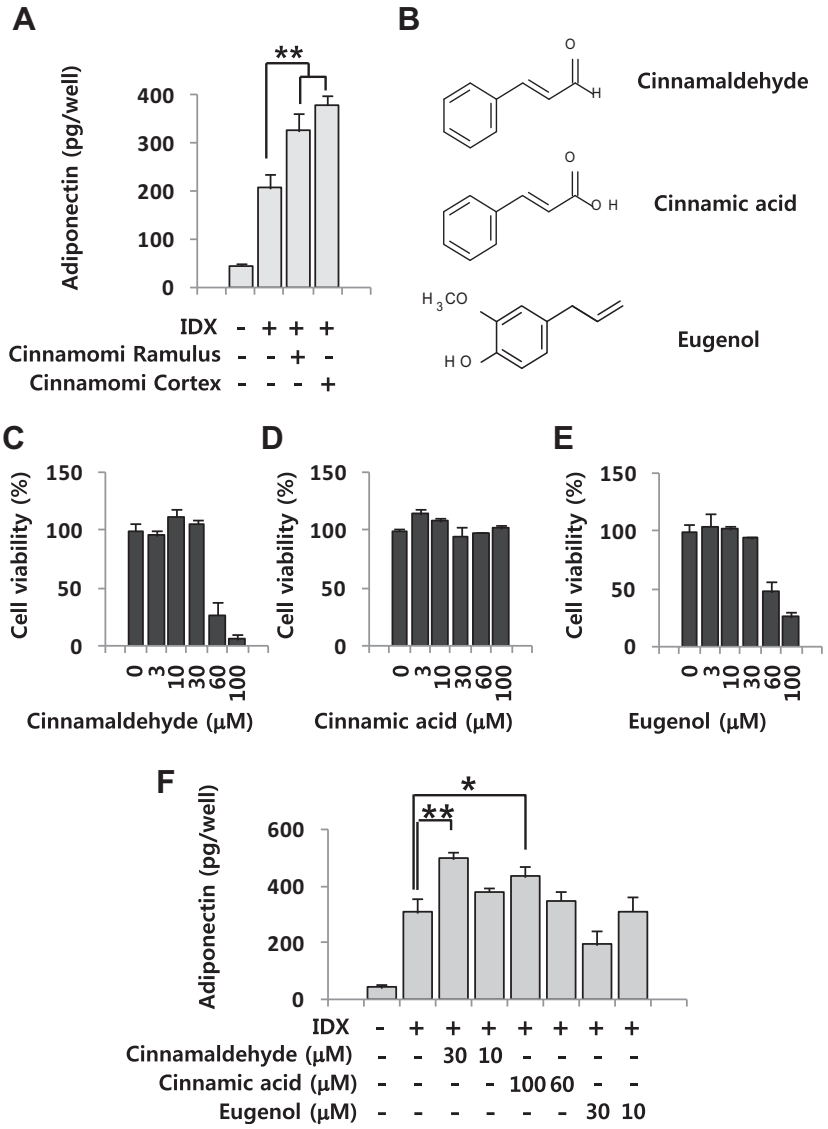
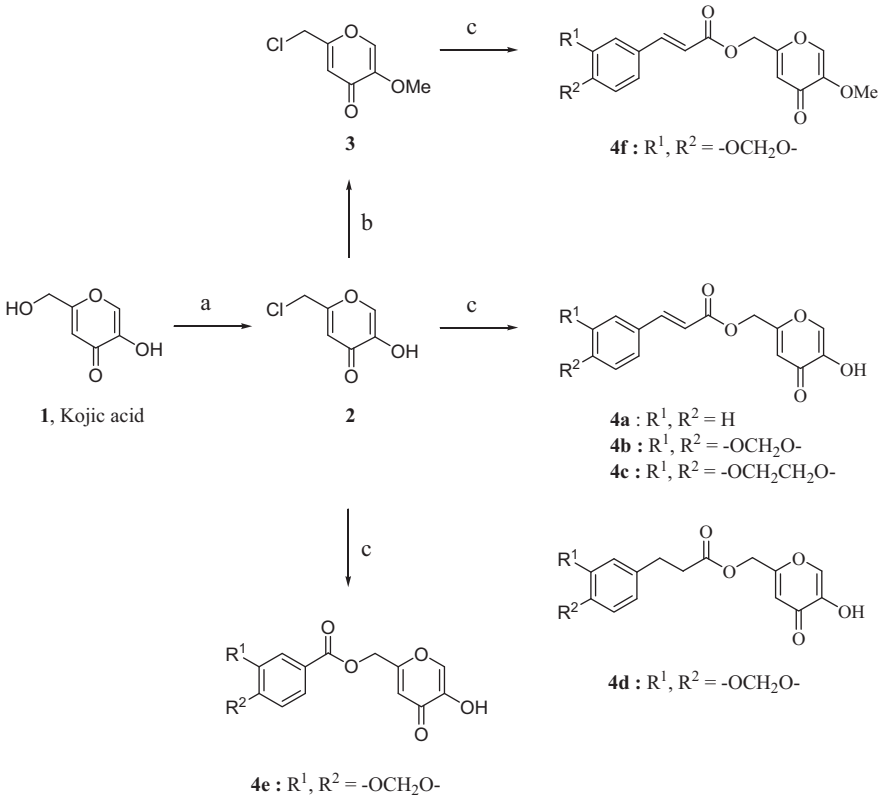


Figure 1. Effects of *Cinnamomum cassia* extracts on adipogenesis in hAT-MSCs. hAT-MSCs were grown under IDX conditions and/or co-treated with 30 μg/ml of ethanol extracts of Cinnamomi Ramulus and Cinnamomi Cortex (A). After the induction of adipogenesis, the media were replaced every two or three days. On the 12th day in culture, cell culture supernatants were harvested. ELISA was performed to measure the levels of adiponectin accumulated in the supernatants during the 48 h after the last medium exchange. Cytotoxicity of the major chemical components, cinnamaldehdye, cinnamic acid, and eugenol, on hAT-MSCs was determined using 4-3-[4-lodophenyl]-2-4(4-nitrophenyl)-2*H*-5-tetrazolio-1,3-benzene disulfonate (WST, Roche Molecular Biochemical, Indianapolis, IN, USA). The absorbance of the samples (A_{450}) was determined, and absolute optical density expressed as a percentage of the control value (B–E). The effects of cinnamaldehdye, cinnamic acid, and eugenol on adiponectin production in hAT-MSCs during adipogenesis were evaluated (F). Values represent mean ± SD ($n = 3$). $^{*}P \leqslant 0.05$ and $^{**}P \leqslant 0.01$.

cinnamaldehyde (30 μM) and cinnamic acid (100 μM) in the IDX condition significantly increased adiponectin production (by 70% and 46%, respectively), compared with that in the IDX control. In contrast, eugenol did not promote adipogenesis in hAT-MSCs (Fig. 1F). Therefore, cinnamaldehyde and cinnamic acid may contribute to the effect of the *Cinnamomum cassia* methanol extract in promoting adipogenesis in hAT-MSCs.

Previously, we synthesized the 3,4-methylenedioxy cinnamate derivative of kojic acid (**4b**, Seletinoid G, Scheme 1) as a new anti-aging compound.[12] Kojic acid is produced from carbohydrate sources in an aerobic process by a variety of microorganisms.[13] It exerts various biological effects-such as an inhibition of tyrosinase, chelation of metal ions, free radical scavenging, and prevention of photo-damage due to its γ-pyranone structure containing an enolic hydroxyl group.[14] Compound **4b** was designed based on the assumption that the γ-pyranone ring of kojic acid would mimic the carboxylic acid moiety in retinoid structures. Because retinoic acid derivatives have in vivo anti-aging activity in human skin, we intuitively expected that compound **4b** may counteract the functional regression of aged human skin. In human clinical studies, we demonstrated that compound **4b** increased procollagen synthesis and decreased matrix metalloproteinase-1 (MMP-1) in photo-aged human skin in vivo.[12] Because compound **4b** also contains a cinnamate moiety, we evaluated whether, like cinnamaldehyde and cinnamic acid, it could affect adipogenesis in hAT-MSCs. In a preliminary screen, we found 100 μM of **4b** promoted adiponectin production in hAT-MSCs (data not shown). Therefore, to study the structure activity relationship of kojyl cinnamate derivatives of **4b** in promoting adiponectin biosynthetic activity in hAT-MSCs, we synthesized additional cinnamate derivatives (**4a** and **4c**) and modified compounds (**4d**–**4f**).[15] The synthetic pathways are shown in Scheme 1. Cinnamate derivatives (**4a**–**4c**), a benzoate derivative (**4e**), and a hydrocinnamate derivative (**4d**) were synthesized by the condensation of kojyl chloride **2**



Scheme 1. Reaction and conditions: (a) $SOCl_2$, DMF, rt; (b) dimethylsulfate, K_2CO_3, acetone, reflux; (c) potassium salts of acids, DMF, 100–120 °C.

with the potassium salts of acids.[15] The 5-methoxy-protected compound (**4f**) was synthesized by the condensation of 5-methoxy kojyl chloride **(3)** with the potassium salt of 3,4-methylenedioxy cinnamic acid.[15] Compared with the IDX condition, the addition of **4a**, **4b**, or **4c** to the IDX adipogenic cocktail promoted adiponectin production in hAT-MSCs by 280%, 420%, or 370%, respectively. Kojyl cinnamate ester derivatives **4a**, **4b**, and **4c**, showed more potent activities than cinnamic acid (Table 1 and Fig. 2). Treatment with the benzoate derivative **4e** increased adiponectin production by 111%, making it almost as potent as cinnamic acid. A hydrocinnamate derivative **4d** was inactive. The inactive compound **4d** has the reduced form of the α,β-unsaturated double bond next to the carbonyl ester of the active compound **4b**, suggesting that the α,β-unsaturated carbonyl ester structure plays an important role in promoting adiponectin production by the hAT-MSCs. In addition, **4e**, the compound lacking the α,β-unsaturated double bond moiety adjacent to the carbonyl ester, was less potent than compounds with this moiety **4a**, **4b** and **4c**. This supports the core role of the α,β-unsaturated carbonyl ester structure in promoting adipogenesis in hAT-MSCs. Importantly, **4f**, in which the kojic acid moiety of compound **4b** is 5-methoxy substituted, had no effect on adipogenesis (Table 1). Because a chemical modification of the kojic acid moiety in **4b** resulted in the loss of pharmacological activity, the kojic acid moiety of kojyl cinnamate ester derivatives played an essential role in the promotion of adipogenesis in hAT-MSCs.

Next, we tested the concentration-dependent effect of **4a**, **4b**, and **4c** (Fig. 3A), and compared their potencies with those of glibenclamide and troglitazone in promoting adiponectin production during hAT-MSC adipogenesis (Fig. 3B). In dose-response analyses for glibenclamide or troglitazone, adiponectin in culture supernatants reached maximal levels at higher drug concentrations. From the maximal level in the dose-response curves, the effective concentration 50 (EC_{50}) values for glibenclamide and troglitazone were 3.49 and 0.32 μM (Fig. 3A). In parallel experiments, the EC_{50}-values for **4a**, **4b**, and **4c** were 60.5, 40.3, and 45.5 μM, respectively. The increase in adiponectin levels in culture supernatants during adipogenesis in hAT-MSCs by the addition of cinnamaldehyde and cinnamic acid were far below the half-maximal adiponectin level in the dose-response curve (Table 1). Taken together, both the α,β-unsaturated carbonyl ester structure and the kojic acid moiety play roles in the pharmacological effects of kojyl cinnamate ester derivatives in promoting adiponectin production during adipogenesis in hAT-MSCs.

The kojyl ester compound of cinnamaldehyde, **4b**, was initially identified as an anti-aging compound because it increased dermal collagen levels in photo-aged human skin.[12] Although it is clinically effective, the pharmacological mechanism of action of the compound **4b** has not been fully explained. Adiponectin is known to stimulate the production of extracellular matrix components such as type I collagen and hyaluronic acid in dermal fibroblasts.[16] Because **4b** significantly promoted adipocyte differentiation in hAT-MSCs (Fig. 3), it may increase adipocyte differentiation in tissue-resident hMSCs to affect the mass of subcutaneous fat tissue in human skin. Therefore, these results suggest that the pharmacological mechanisms of the **4b** compound associated with the increased ECM production in human skin are indirectly mediated by paracrine effects mediated by subcutaneous fat tissue-derived adiponectin on dermal fibroblasts in human skin.

Additionally, the up-regulation of adiponectin production is also associated with an increase in insulin sensitivity.[6–8] In this regard, kojyl cinnamate ester derivatives provide novel pharmacophores for developing anti-diabetic drugs. However, the adipogenesis model using hMSCs, one example of a phenotypic cell-based assay for measuring insulin sensitivity, does not provide direct information about the molecular targets. As both sulfonylurea-type

Table 1
Effects of cinnamate-kojic acid ester derivatives on adiponectin production during adipogenesis in hAT-MSCs

Compound	Adiponectin (pg/ml) Mean ± SD, n = 3	Cell viability** (CV%, mean ± SD)	
		CV50 (μM)	CV90 (μM)
Vehicle control	32 ± 11		
Glibenclamide 30 μM	30 ± 5	93 ± 11	36 ± 14
Troglitazone 10 μM	24 ± 13	56 ± 11	24 ± 2
Cinnamic acid 60 μM	26 ± 17	202 ± 46	79 ± 2
Kojic acid 400 μM	34 ± 3	1345 ± 292	504 ± 32
4a 60 μM	29 ± 4	151 ± 17	61 ± 21
4b 60 μM	27 ± 17	181 ± 10	82 ± 5
4c 60 μM	32 ± 8	161 ± 10	72 ± 18
4d 60 μM	29 ± 10	197 ± 23	87 ± 29
4e 60 μM	35 ± 3	192 ± 29	95 ± 22
4f 60 μM	26 ± 15	181 ± 27	85 ± 32
IDX control	302 ± 50		
IDX + glibenclamide 30 μM	1832 ± 110#		
IDX + troglitazone 10 μM	2005 ± 193#		
IDX + cinnamic acid 60 μM	501 ± 62*		
IDX + kojic acid 400 μM	312 ± 103		
IDX + **4a** 60 μM	1068 ± 176#		
IDX + **4b** 60 μM	1435 ± 113#		
IDX + **4c** 60 μM	1300 ± 140#		
IDX + **4d** 60 μM	364 ± 16		
IDX + **4e** 60 μM	614 ± 77*		
IDX + **4f** 60 μM	463 ± 36*		

# Denotes p <0.01 for statistical comparison between the IDX control and the IDX + compound.
* Denotes p <0.05.
** Cell viability (CV) was determined using the WST method in hAT-MSCs as described in Figure 1. CV50 and CV90 are the concentration of 50% and 90% of cells are viable compared to control, respectively.

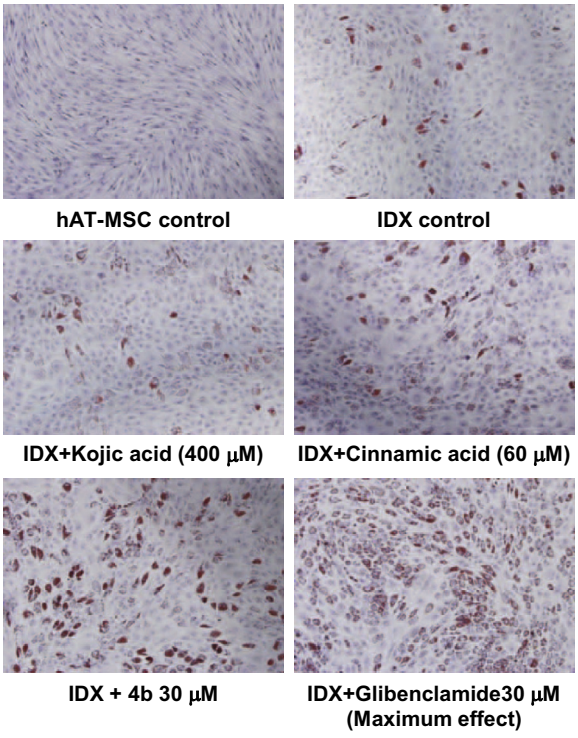


Figure 2. Phenotypic changes induced in hAT-MSCs by the kojyl cinnamate ester derivative **4b**. Twelve days after adipogenic stimulation with IDX, lipid droplets in adipocytes were stained with Oil Red O (ORO). The differentiated adipocytes in hAT-MSC cultures were photographed using an Olympus IX71 inverted phase-microscope.

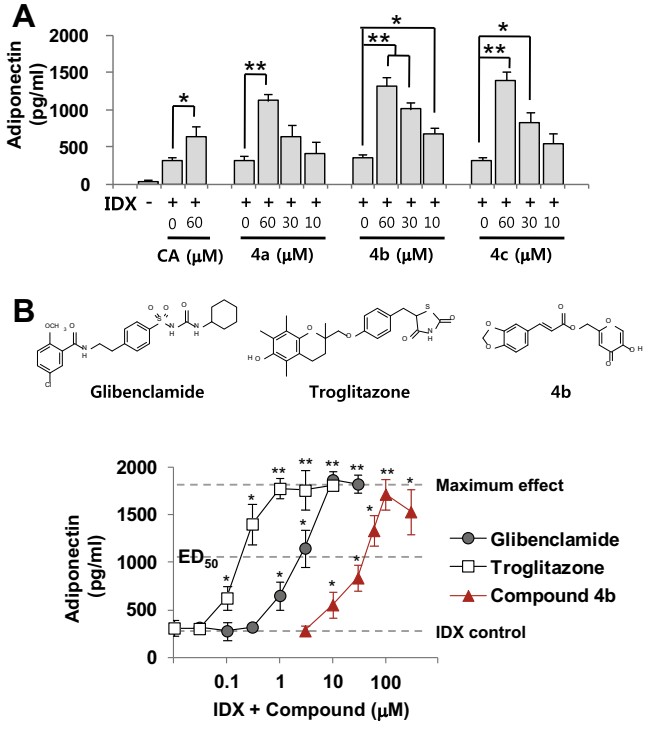


Figure 3. Concentration-dependent effects of kojyl cinnamate ester derivatives on adiponectin production during adipogenesis in hAT-MSCs. After the induction of adipogenesis, the media were replaced every two or three days. On the 12th day in culture, cell culture supernatants were harvested. ELISA was performed to measure the levels of adiponectin accumulated during the 48 h after the last medium change (A). CA denotes cinnamic acid. Glibenclamide and troglitazone were used as positive controls to plot the concentration dependence curve for **4b** (B). Values represent mean ± SD (n = 3). $^{*}P \leqslant 0.05$ and $^{**}P \leqslant 0.01$.

antidiabetic drugs and PPARγ agonists promote adipogenesis in hMSCs,[6–8] it is possible that kojyl cinnamate ester derivatives can regulate the same molecular targets. When we evaluated whether kojyl cinnamate ester derivatives transactivate PPARγ,[6] no significant effect on PPARγ activity was detected (data not shown).

Therefore, kojyl cinnamate ester derivatives may affect other molecular targets to regulate adipogenesis. Further studies examining hAT-MSCs as a model will aim to synthesize novel compounds with an improved activity to stimulate adiponectin production during adipogenesis, and to identify the direct molecular targets of kojyl cinnamate ester derivatives.

In conclusion, kojyl cinnamate ester derivatives promoted adipogenesis in hAT-MSCs and increased adiponectin production. Both the α,β-unsaturated carbonyl ester structure and the kojic acid moiety are core structural features important in the pharmacological activity. These results suggest that the clinically proven anti-aging activity of kojyl cinnamate ester derivatives in human skin may be mediated by up-regulation of adiponectin synthesis in hAT-MSCs residing in subcutaneous fat tissue. In addition, kojyl cinnamate ester compounds may exert anti-diabetic effects by increasing insulin sensitivity, as shown using hAT-MSCs in an adipogenesis model. Therefore, kojyl cinnamate ester derivatives provide novel pharmacophores that may play anti-aging roles in photo-aged human skin as well as anti-diabetic roles by improving insulin sensitivity.

Acknowledgments

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9. hAT-MSCs were purchased from Lonza, Inc. (Walkersville, MD, USA). These cells were grown in low glucose (1 g/L) DMEM containing 10% fetal bovine serum (FBS) and supplemented with penicillin–streptomycin and Glutamax™ (Invitrogen, Carlsbad, CA). To induce adipocyte differentiation, the growth medium was replaced by DMEM containing a high concentration of glucose (4.5 g/L) and supplemented with 10% FBS, Glutamax™, 10 μg/mL insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). Ethanol extracts of *Cinnamomum cassia* were obtained from Korea Plant Extract Bank (http://extract.pdrc.re.kr/). Cinnamaldehyde, cinnamic acid, and eugenol were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Adipocyte differentiation was assessed using an oil red O (ORO) stain as an indicator of intracellular lipid accumulation. After hBM-MSCs had differentiated to adipocytes, cells were rinsed twice with phosphate-buffered saline (PBS), fixed with 10% formalin in PBS (pH 7.4) for 1 h, and then washed with 60% isopropanol, before being allowed to dry completely. hBM-MSCs were stained with 0.2% ORO reagent for 10 min at room temperature, and then washed with H_2O four times. Following a 10 min elution of each hBM-MSCs sample with 100% isopropanol, absorbance was measured at 500 nm using a spectrophotometer. To visualize the nucleus, hBM-MSCs were counterstained with hematoxylin reagent for 2 min and then washed twice with H_2O. The level of adipocyte differentiation was observed and counted using an inverted phase-microscope. For quantitative determination of adiponectin in cell culture supernatants, Quantikine™ immunoassay kits were used (R&D Systems, Minneapolis, MN, US.
10. The methanol extracts for 200 medicinal plants traditionally used in China and Korea were obtained from the Plant Extract Bank of the Korea Research Institute of Bioscience and Biotechnology (http://extract.kribb.re.kr/extract/f.htm). Ten μg/ml of a methanol extract was treated in the adipogenesis-induced hAT-MSCs for a preliminary screening to discover candidate plant extracts to promote adiponectin production.
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15. *The data of selected compounds:* Synthesis of compound **4b**: To a stirred solution of kojyl chloride **2** (4.80 g, 30.0 mmol) in DMF (100 mL) under N_2 was added potassium salt of 3,4-methylenedioxycinnamic acid (6.88 g, 30.0 mmol) with 3,4-methylenedioxycinnamic acid (4.32 g, 22.5 mmol). The reaction mixture was stirred for 1 h at 110–120 °C, after which DMF was evaporated in vacuo. The residue was extracted with ethyl acetate (500 mL), washed with water. The organic layer was dried with anhydrous $MgSO_4$ and concentrated to give a crude product. The resultant was purified by crystallization from ethyl acetate–hexane to give **4b** (7.40 g) in 78% yields. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.29 (s, 1H), 8.12 (s, 1H), 7.68 (d, 1H, J = 16.2 Hz), 7.46 (s, 1H), 7.25 (d, 1H, J = 8.1 Hz), 6.98 (d, 1H, J = 8.1 Hz), 6.64 (d, 1H, J = 16.2 Hz), 6.52 (s, 1H), 6.08 (s, 2H), 5.07 (s, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.5, 165.6, 161.6, 149.5, 148.0, 146.0, 145.7, 139.8, 128.2, 125.3, 114.6, 112.5, 108.4, 106.7, 101.6, 61.2. FABMS, m/e 317 $[M+H]^+$ Cho, J.-C.; Rho, H. S.; Baek, H. S.; Ahn, S. M.; Woo, B. Y.; Hong, Y. D.; Cheon, J. W.; Heo, J. M.; Shin, S. S.; Park, Y.-H.; Suh, K.-D. *Bioorg. Med. Chem. Lett.* **2004**, *2012*, 22.
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