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Suppression of Adipogenesis and Obesity in High-Fat Induced Mouse Model by Hydroxylated Polymethoxyflavones

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ABSTRACT: This study demonstrated that hydroxylated polymethoxyflavones (HPMFs) effectively and dose-dependently suppressed accumulation of lipid droplets in adipocytes by approximately 51−55%. Western blot analysis revealed that HPMFs markedly down-regulated adipogenesis-related transcription factors peroxisome proliferator-activated receptor (PPAR) γ and sterol regulatory element-binding protein (SREBP)-1c as well as downstream target fatty acid binding protein 2 (aP2), fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC). In addition, HPMFs also activated adenosine monophosphate-activated protein kinase (AMPK) signaling in 3T3-L1 adipocytes. In the early phase of adipogenesis, HPMF-treated preadipocytes displayed a delayed cell cycle entry into G_2/M phase at 24 h (35.5% for DMI group and 4.8% for 20 μg/mL HPMFs-treated group) after initiation of adipogenesis. Furthermore, administration of HPMFs (0.25 and 1%) decreased high-fat diet (HFD) induced weight gain (15.3 ± 3.9 g for HFD group, 10.3 ± 0.3 g and 7.9 ± 0.7 g for 0.25 and 1% HPMFs groups, respectively) and relative perigonadal, retroperitoneal, mesenteric fat weight in C57BL/6 mice. Administration of HPMFs reduced serum levels of aspartate aminotransferase (GOT), alanine aminotransferase (GPT), triglycerides (TG), and total cholesterol (T-cho). The results suggested that HPMFs may have a potential benefit in preventing obesity.

KEYWORDS: *hydroxylated polymethoxyflavones (HPMFs), high-fat diet (HFD), 3T3-L1, obesity, adenosine monophosphate-activated protein kinase (AMPK)*

INTRODUCTION

The major phytochemicals in citrus peel include polymethoxylated flavonoids, terpenoids, such as limonene and linalool, and other volatile oils.[1] Among these, polymethoxyflavones, particularly in the peel of sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulate*), have been demonstrated to have various biological activities including anticarcinogenic, anti-inflammatory, and antitumor activities.[1,2] Our previous studies reported that dietary administration of HPMFs significantly decreased aberrant crypt foci (ACF) and tumor formation in azoxymethane (AOM)-treated mice.[3] One of major ingredient in HPMFs, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, has been also shown to induce apoptosis in human leukemia cells.[4] However, the effects of HPMFs on adipogenesis are not fully understood.

Obesity is a serious health problem and a highly prevalent condition related to metabolic diseases in the world. Numerous studies indicate the high risk of obesity in the development of cardiovascular disease, type 2 diabetes, hypertension, fatty liver disease, cancer, and increased mortality.[5] As caloric intake increases, adipocytes store energy in the form of triglycerides (TGs) that result in enhanced adipogenesis, increased adipose tissue mass, and consequently obesity.[6] In addition to fat storage, adipose tissue is a major endocrine and metabolic organ secreting adipocytokines, cytokines, growth factors, and hormones involved in host immunity, energy homeostasis, systemic insulin sensitivity, and tissue regeneration. In obesity, dysfunction of adipose tissue contributes to abnormal cytokine and hormone production in adipocyte and results in metabolic syndrome.[6] Adipocyte differentiation is a pivotal biological process during adipogenesis in adipose tissue that is characterized by alteration of cellular properties. Differentiation of preadipocytes undergoes morphological and biochemical transition from growth arrest of confluent preadipocytes, reentry to the cell cycle for an additional two rounds of division, term mitotic clonal expansion (MCE), and terminal differentiation to mature adipocytes followed by changes in genetic programs for lipid synthesis, storage, and utilization.[7] This process is controlled by a set of transcription factors such as CCAAT/enhancer-binding protein (C/EBP)s, peroxisome proliferator-activated receptor gamma (PPARγ), and sterol regulatory element-binding protein (SREBP-1c), and cellular signaling cascade involved in cell cycle and insulin-dependent signaling pathways in the early phase of adipogenesis that are known to play critical roles.[8] In addition, activation of PPARγ is sufficient to promote adipose differentiation in fibroblastic cells.[9] Besides C/EBPs and PPARγ, insulin and insulin-like growth factor-1

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Table 1. Composition of Experimental Diets

	ND	HFD	HFD + 0.25% HPMFs	HFD + 1% HPMFs
macronutrient composition				
protein % of energy	20.0	14.0	14.0	14.0
carbohydrate % of energy	65.0	46.0	46.0	46.0
fat % of energy	15.0	40.0	40.0	40.0
ingredient, g/kg				
lard		129.0	129.0	129.0
soybean oil		12.9	12.9	12.9
cholesterol		5.0	5.0	5.0
HPMFs			2.5	10.0
energy content, kJ/g	35.8	50.7	50.7	50.7

(IGF-1) signaling are also involved in regulation of adipocyte differentiation.[10] Adenosine monophosphate-activated protein kinase (AMPK) acts as a nutrient sensor and central regulator of cellular energy homeostasis. Studies indicate that activation of AMPK leads to inhibition of adipocyte differentiation and decreased adipogenesis.[11,12] Phosphorylation of metabolic enzyme acetyl-CoA carboxylase (ACC) 1 and HMG-CoA reductase (HMGCR) by AMPK promotes fatty acid oxidation and reduces cholesterol synthesis.[13,14]

It is believed that the combination of decreased caloric intake and increased physical exercise is effective for obesity prevention[15] but most often difficult to achieve. Some antiobesity drugs have been used to treat overweight or obese patients but with limited efficacy and concern for side effects and safety.[16] Nowadays, researchers have become increasingly interested in searching for natural products from dietary and edible plants that possess antiobesity activity. A large body of literature shows that many phytochemicals and natural products are used to treat obesity as metabolic stimulaters, appetite suppressants, and starch blockers and by regulation of glucose and lipid metabolism as well as targeting adipocyte differentiation.[17]

In this study, we investigated the inhibitory effects of HPMFs on differentiation of 3T3-L1 preadipocytes and high-fat diet-induced obese mice. We have demonstrated that HPMFs inhibited adipogenesis with no effect on the viability of the differentiating preadipocytes. In these model systems, HPMFs demonstrated multiple antiadipogenetic properties through suppressing cell cycle progression and expression of pro-adipogenic transcription factors, PPARγ and SREBP-1c, and modulation of AMPK signaling.

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY, USA). Fetal calf serum (FCS) was purchased from HyClone Laboratories (Logan, UT, USA). Insulin, 3-isobutylmethylxanthine (IBMX), dexamethasone (DEX), and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The rabbit FAS, PPARγ, and P-AMPKβ (Ser108) polyclonal antibodies and rabbit ap2, P-AMPKα (Thr172), AMPKα, AMPKβ, and ACC monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The rabbit SREBP-1c polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The mouse β-actin monoclonal antibody was purchased from Sigma Chemical Co. Cholesterol used in the animal diet was purchased from Hi-Media Laboratories (Mumbai, India). HPMFs were prepared according to the method of Li et al.[18] The composition and content of hydroxylated PMFs and PMFs were described before.[3]

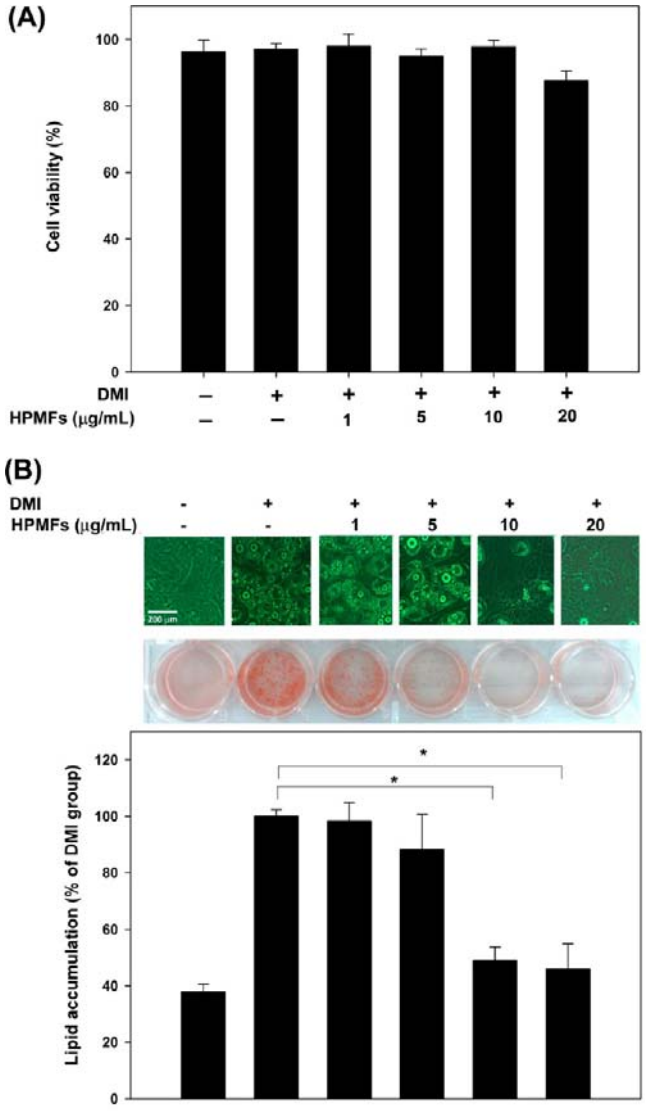

Figure 1. HPMFs inhibit differentiation and adipogenesis of 3T3-L1 preadipocytes: (A) cell viability determined by the trypan blue assay as described; (B) differentiation of 3T3-L1 preadipocytes stained with Oil Red O and photographed (upper and middle) (magnification, ×400). Scale bars, 200 μm. 3T3-L1 preadipocytes were incubated with DMI (DMEM with IBMX, DEX, and insulin) for 2 days and then replaced with DMEM containing insulin with or without HPMFs (1, 5, 10, and 20 μg/mL), respectively, for 8 days. Lipid content was extracted from Oil Red O stained cells by 2-propanol and quantified by spectrophotometric analysis at 520 nm. Data are presented as the mean ± SE, and each experiment was independently performed three times with similar results. Statistical analysis was done by Student's t test. (*) $P < 0.001$, statistically significant differences from DMI-treated group.

Cell Culture and Adipocyte Differentiation. Mouse 3T3-L1 preadipocytes purchased from the American Type Culture Collection (Rockville, MD, USA) were grown in DMEM supplemented with 2 mM glutamine (GIBCO BRL), 1% penicillin/streptomycin (10000 units of penicillin/mL and 10 mg streptomycin/mL), and 10% FCS at 37 °C under a humidified 5% CO_2 atmosphere. Differentiation of 3T3-L1 preadipocytes was as described before.[19] Briefly, cells were seeded into a 24-well (2×10^4/mL) or 10 cm dish with DMEM containing 10% FBS to full confluence. Two days after confluence (defined as

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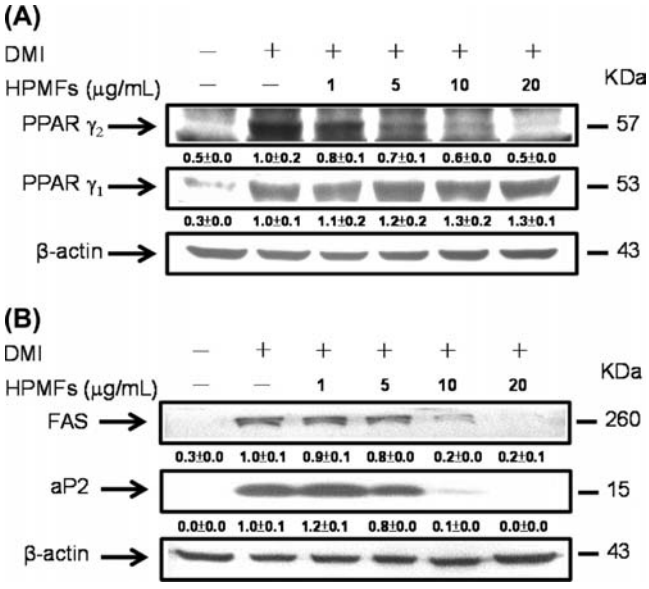

Figure 2. HPMFs reduce protein levels of PPARγ and its downstream targets, FAS and aP2, in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were treated as described previously. After 8 days of treatment, total protein was extracted, and the protein levels of (A) PPARγ, (B) FAS, and (C) aP2 were determined by Western blot analysis. β-Actin was used as a loading control. This experiment was repeated three times with similar results. The values under each lane indicate the relative density of the band normalized to β-actin and present as the mean ± SE.

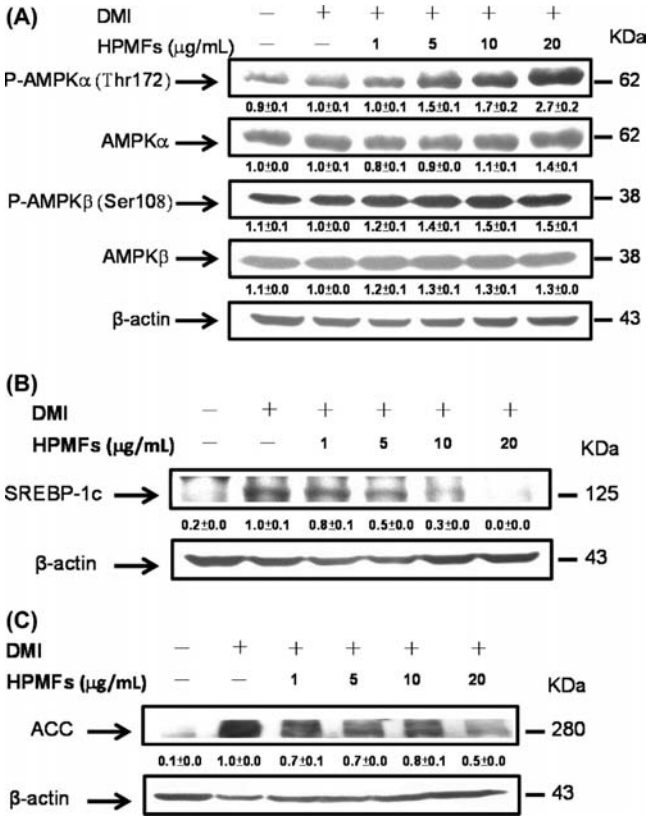

Figure 3. HPMFs inhibit the differentiation of 3T3-L1 preadipocytes by regulation of AMPKs and PI3K/Akt signaling. 3T3-L1 preadipocytes were treated as described previously. (A) Phospho-APMKα (Thr172), total AMPKα, phospho-AMPKβ (Ser108), total AMPKβ, (B) SREBP-1c, and (C) ACC were examined by Western blot analysis with specific antibodies. Each experiment was repeated three times with similar results. The values under each lane indicate the relative density of the band normalized to β-actin and are presented as the mean ± SE.

day 0), cells were incubated in differentiation medium (DMI) containing 1.7 μM insulin, 0.5 mM IBMX, and 12.7 μM DEX in DMEM containing 10% FBS for 2 days. The medium was then replaced by DMEM containing 10% FBS and insulin (1.7 μM) with or without HPMFs, which was replaced every 2 days. The final concentrations of dimethyl sulfoxide (DMSO) in the culture medium were <0.05%. The cells were harvested after 8 days (at day 10) for Oil Red O staining, trypan blue exclusion assay, and protein extraction.

Cytotoxicity Assay. The effect of HPMFs on cell viability of 3T3-L1 adipocyte was analyzed by trypan blue exclusion assay. Differentiation of 3T3-L1 preadipocytes and HPMF treatment were described above. At the end of treatment (day 10), the cells were harvested, and cytotoxicity was determined by trypan blue exclusion and microscopy examination.

Oil Red O Staining. At the end of differentiation, cells were washed twice with phosphate-buffered saline (PBS), fixed with 10% formalin for 60 min, and stained with 0.5% Oil Red O in isopropanol for 1 h at room temperature. Excess Oil Red O dye was washed twice with distilled water and then dried. The stained lipid droplets within cells were visualized by light microscope and photographed with a digital camera at 100× magnification. To quantify lipid accumulation, the stained lipid droplets were dissolved in isopropanol, and the absorbance was measured at 520 nm.

Western Blotting. The total proteins of differentiated 3T3-L1 adipocytes were extracted via addition of gold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at 10000g for 30 min at 4 °C. The total proteins were measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min and were subjected to 10% SDS–polyacrylamide minigels at a constant current of 20 mA. Electrophoresis was then carried out on SDS–polyacrylamide gels. Proteins on the gel were electrotransferred onto an immobile membrane (PVDF; Millipore Corp., Bedford, MA, USA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution containing 20 mM Tris-HCl and then immunoblotted with primary antibodies and β-actin. The blots were rinsed three times with PBST buffer (0.2% Tween 20 in 1× PBS buffer) for 10 min each. Then blots were incubated with 1:5000 dilution of the horseradish peroxidase (HRP)-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA, USA) and then washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK).

Cell Cycle Analysis. Postconfluent 3T3-L1 preadipocytes were cultured in DMI medium with or without HPMFs for 18 and 24 h. The cells were then harvested, washed with PBS, resuspended in 200 μL of PBS, and fixed in 800 μL of iced 100% ethanol at −20 °C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at 37 °C for 30 min. Next, cells were stained with PI (50 μg/mL) for 30 min. Fluorescence emitted from the PI–DNA complex was quantified after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA, USA). The quantitation of cell cycle distribution was performed with ModFit LT for Mac 3.0 software (Becton Dickinson).

Animal Experiments. Male C57BL/6J mice at 5 weeks of age were purchased from the BioLASCO Experimental Animal Center (Taiwan Co., Ltd., Taipei, Taiwan) and housed in a controlled

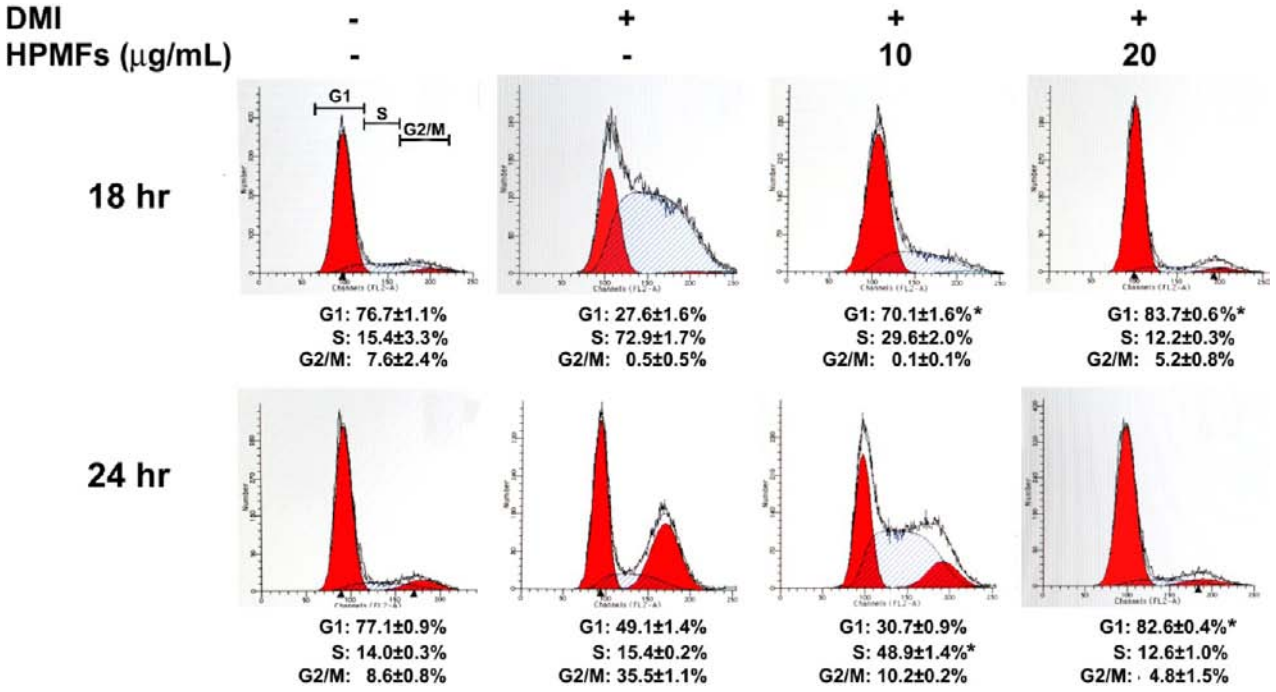

Figure 4. Effects of HPMFs on cell cycle during mitotic clonal expansion (MCE) process of adipogenesis. 3T3-L1 preadipocytes were exposed to DMI in the presence or absence of HPMFs. The cells were harvested at 18 and 24 h after initiating differentiation and stained with PI. The labeled cells were analyzed using FACScan benchtop cytometer, and percentages of cells in the G0/G1, S, and G2/M phases were calculated using ModFit LT software. The data shown here are from a typical experiment repeated three times, and values represent the mean ± SE. Statistical analysis was done by Student's *t* test. (∗) $P < 0.001$, statistically significant differences from DMI-treated group.

atmosphere (25 ± 1 °C at 50% relative humidity) with a 12 h light/12 h dark cycle. After 1 week of acclimation, animals were randomly distributed into four groups of 10 animals each as follows: normal diet (ND, 15% energy as fat), high-fat diet (HFD; 40% energy as fat consists of 13% lard, 1.3% soybean oil, and 0.5% cholesterol in the ND), and HFD supplemented with 0.25 or 1% HPMFs (2.5 or 10 g HPMFs/kg diet), respectively, for 10 weeks. The experimental diets were modified from the Purina 5001 diet (LabDiet, PMI Nutrition International), and the composition is listed in Table 1. Animals had free access to food and water at all times. Food cups were replenished with fresh diet daily. The diet intake of animals was monitored every day, and the body weight was recorded weekly. All animal experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU). At the end of the study, all animals were fasted overnight and sacrificed by CO_2 asphyxiation. Blood samples were collected from the heart for biochemical analysis. Liver, spleen, kidney, and fat pads (perigonadal, retroperitoneal, and mesenteric fat) were immediately removed, weighed, and photographed.

Biochemical Analysis. Plasma samples were separated by centrifugation at 1000*g* for 15 min. Plasma levels of GOT, GPT, TG, and T-chol were measured using a commercial assay kit. Briefly, serum was spotted onto respective Fujifilm Dri-Chem slides (Fujifilm, Kanagawa, Japan), and each biochemical indicator was determined using a blood biochemistry analyzer (Fujifilm Dri-Chem 3500s; Fujifilm) according to the manufacturer's instructions.

Histopathological Examinations. A portion of the median lobe of liver was dissected and fixed in 10% buffered formalin for at least 24 h, dehydrated with a sequence of ethanol solutions, and processed for embedding in paraffin. Sections of 5−6 μm in thickness were cut, deparaffinized, rehydrated, stained with hematoxylin and eosin (H&E), and subjected to photomicroscopic observation.

Statistical Analysis. Data are presented as means ± SE for the indicated number of independently performed experiments. Comparisons of statistical significance between groups were made by one-way Student's *t* test or one-way analysis of variance (ANOVA). A *P* value <0.05 was considered statistically significant.

RESULTS

Effect of HPMFs on Lipid Accumulation in 3T3-L1 Adipocytes. The composition and content of hydroxylated PMFs and PMFs have been described in our previous study.[3] To further investigate the effect of HPMFs on adipocyte differentiation, 3T3-L1 preadipocytes were treated with differentiated medium and insulin with or without HPMFs. As shown in Figure 1A, HPMFs showed no significant cytotoxicity in 3T3-L1 cells. Ten days after the initiation of differentiation, lipid accumulation was measured by Oil Red O staining. Figure 1B shows the result of Oil Red O staining under a microscope. HPMFs potently reduced lipid accumulated in 3T3-L1 adipocytes in a dose-dependent manner.

HPMFs Inhibited PPARγ, FAS, and aP2 Expression in 3T3-L1 Adipocytes. Several studies have shown that the activation of C/EBPα and PPARγ leads to terminal differentiation through their subsequent transactivation of adipocyte-specific genes such as fatty acid synthase and fatty acid binding protein 4, and C/EBPs are master transcription factors in the regulation of adipogenesis in adipocytes.[9,20] Hence, we next investigated the expression of PPARγ, FAS, and aP2 to characterize the effect of HPMFs on 3T3-L1 adipocyte differentiation. As shown in Figure 2, HPMF treatment resulted in dramatic dose-dependent reduction in the levels of PPARγ2, FAS, and aP2, but not PPARγ1 in differentiated adipocytes.

HPMFs Activated AMPK Signaling in 3T3-L1 Adipocytes. Recent studies suggest that AMPK is involved in the regulation of glucose and lipid metabolism and acts as a regulator for adipocyte differentiation.[11,12] To clarify the inhibitory effect of HPMFs on suppression of 3T3-L1 adipocyte differentiation, we examined whether HPMFs can modulate AMPK signaling. As shown in Figure 3A, HPMFs dramatically increased phosphorylated AMPKα, a catalytic subunit. Moreover,

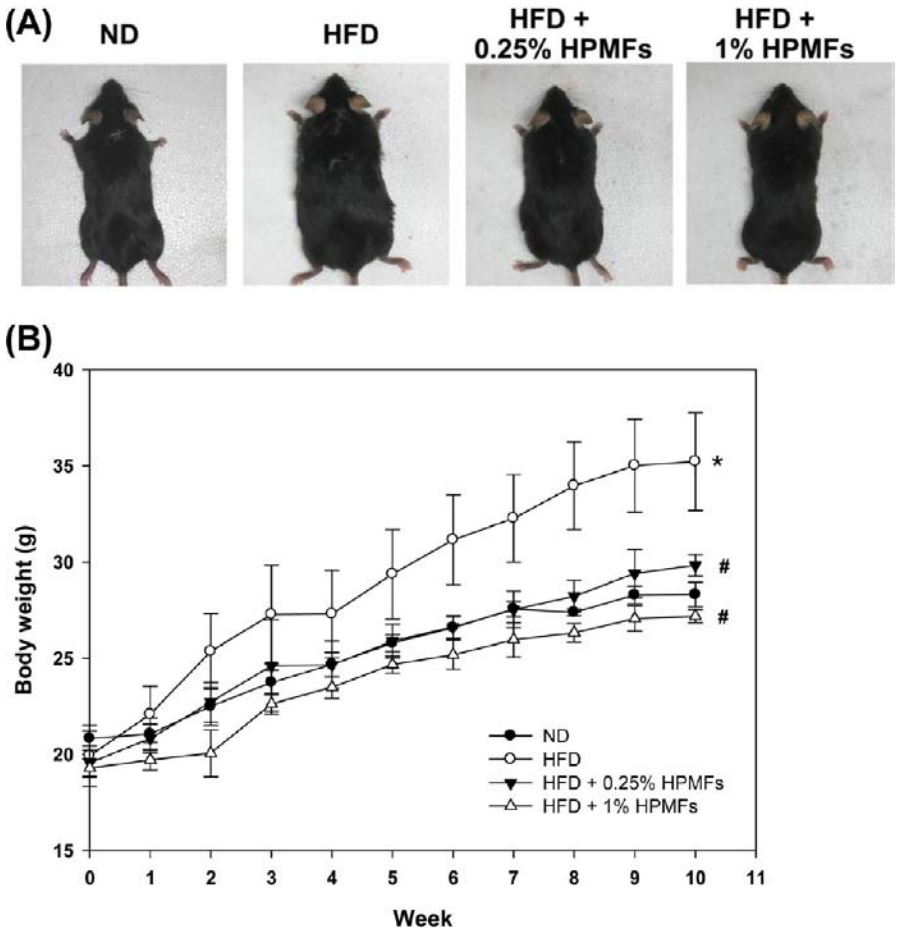

Figure 5. Effects of supplement with HPMFs on body weight in HFD fed C57BL/6 mice. Mice (n = 10) were fed HFD supplement with or without HPMFs (0.25 and 1%) for 10 weeks. ND group was administered control diet as vehicle. At the end of experiment, all mice of each group were killed by CO_2 asphyxiation. (A) Representative photographs of each group are shown at the end of week 10. (B) Body weight was monitored weekly, and the average body weight of each group was expressed as the mean ± SE. Statistical analysis was done by Student's t test. (∗) $P < 0.05$, compared with ND group; (#) $P < 0.05$, compared with HFD group. ND, normal diet; HFD, high-fat diet; HPMFs, hydroxylated polymethoxyflavones.

Table 2. Effects of HPMFs on Body Weight Gain and Food Intake in Mice Fed HFD[a]

	ND	HFD	HFD + 0.25% HPMFs	HFD + 1% HPMFs
initial wt (g)	20.3 ± 0.9	19.9 ± 1.5	19.2 ± 0.9	19.3 ± 0.4
final wt (g)	28.3 ± 0.6	35.1 ± 3.1*	29.8 ± 0.6##	27.2 ± 0.3###
wt gain (g)	7.5 ± 0.9	15.3 ± 3.9*	10.3 ± 0.3#	7.9 ± 0.7##
food intake (g/mouse/day)	4.9 ± 0.1	4.3 ± 0.2*	4.6 ± 0.2	4.1 ± 0.5

[a]Mice were fed diet for 10 weeks as described under Materials and Methods, and the body weights were monitored twice weekly. The average body weight of each group is expressed as the mean ± SE (n = 10 per group), and statistical analysis was done by Student's t test. ND, normal diet; HFD, high-fat diet; HPMFs, hydroxylated polymethoxyflavones. *, $P < 0.01$, compared with ND group. #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$, compared with HFD group.

phosphorylation of AMPKβ, a regulatory subunit, was also elevated in HPMF-treated cells. Previous studies demonstrated that AMPK activation inhibited ACC activity directly by phosphorylation and inhibited ACC expression indirectly via the suppression of SREBP-1c.[21] Therefore, we also determined whether SREBP-1c and ACC are important targets for HPMFs subsequent to AMPK activation. As shown in Figure 3B,C, SREBP-1c and ACC were markedly decreased by HPMF treatment. Taken together, these results suggested that HPMFs suppressed 3T3-L1 adipocye differentiation through both inhibition of PPAR and activation of AMPK signaling.

HPMFs Blocked Cell Cycle Progression in 3T3-L1 Adipocytes. To further confirm the effect of HPMFs on cell mitosis after adipogenic induction, the effect of HPMFs on cell cycle was analyzed by FACScan cytometry. Histograms of flow cytometric data showed that HPMF-treated cells displayed a delayed cell cycle progression at both 18 and 24 h after induction of differentiation with DMI (Figure 4). Approximately 72.9% of differentiating 3T3-L1 cells were in the S phase of the cell cycle after 18 h of DMI treatment, whereas only 12.2% of HPMF-treated cells were in the S phase, and >83% of HPMF-treated cells were in the G1 phase of the cell cycle. This in turn resulted in 35.5% of DMI-treated control cells found in the G2/M phase, whereas only 4.8% of HPMFs-treated cells were found to be in the G_2/M phase after 24 h of treatment. Thus, our results demonstrated that HPMFs impaired induction of

Table 3. Effects of HPMFs on Relative Organ Weights in Mice Fed HFD[a]

	ND	HFD	HFD + 0.25% HPMFs	HFD + 1% HPMFs
liver (%)	4.33 ± 0.79	4.78 ± 0.38	4.86 ± 0.27	4.54 ± 0.85
kidney (%)	1.70 ± 0.08	1.74 ± 0.21	1.58 ± 0.13	1.59 ± 0.19
spleen (%)	0.24 ± 0.02	0.23 ± 0.04	0.23 ± 0.02	0.26 ± 0.04

[a]Mice were fed HFD supplemented with or without HPMFs (0.25 and 1%) for 10 weeks. Mice of each group were killed by decapitation at the end of week 10; the liver, spleen, and kidney were removed, photographed, weighed, and recorded. Data are presented as the mean ± SE (n = 10 per group). The relative organ weight is expressed as a percentage of body weight (liver weight/body weight × 100). ND, normal diet; HFD, high-fat diet; HPMFs, hydroxylated polymethoxyflavones.

early adipogenic transcription factors and cell cycle programs in the early phase of adipogenesis of 3T3-L1 cells.

HPMFs Prevented HFD-Induced Obesity. We next determined the effects of HPMFs on metabolic abnormality in HFD-fed mice. Mice fed the HFD for 10 weeks had significantly higher body weights than animals fed the normal diet. Furthermore, dietary supplementation of HFD with HPMFs at 0.25 and 1% to mice significantly reduced both final body weight and body weight gain after 10 weeks of feeding compared with the values for HFD mice (Figure 5 and Table 2). There were no statistical differences in food intake among the three different diet groups (Table 2). The relative organ weights of liver, kidney, and spleen showed no significant difference between HFD and HFD supplement with HPMF groups (Table 3). Compared to those of HFD-fed mice, dietary HPMF-treated mice significantly and dramatically reduced the relative perigonadal, retroperitoneal, and mesenteric weight in a dose-dependent manner (Figure 6).

HPMFs Dramatically Decreased Signs of Liver Pathology. We also examined the effect of HPMFs on the signs of liver pathology in HFD mice. Photomicrographs of liver samples stained with H&E are shown in Figure 7. Livers from the ND group were normal, and livers from the control group fed a HFD for 10 weeks exhibited an increased number of fatty

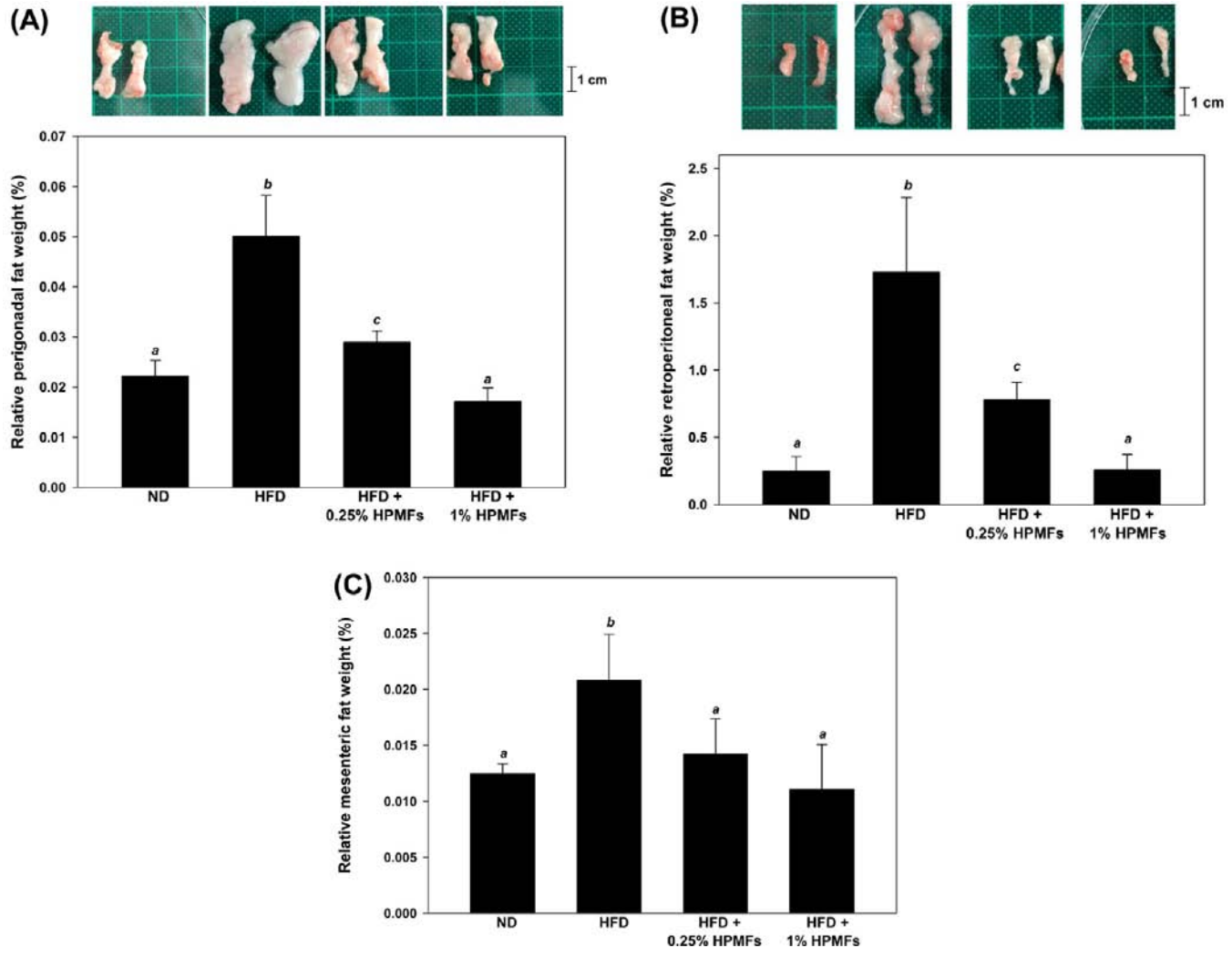

Figure 6. Effect of HPMF supplement on relative adipose tissue weights in HFD-fed C57BL/6 mice. Mice were fed HFD diet supplement with or without HPMFs (0.25 and 1%) for 10 weeks. The adipose tissues were removed immediately, photographed, weighed, and recorded. The relative (A) perigonadal, (B) retroperitoneal, and (C) mesenteric fat weight was expressed as a percentage of body weight (adipose tissue weight/body weight × 100). Data are expressed as the mean ± SE, and statistical difference was analyzed using one-way ANOVA. ND, normal diet; HFD, high-fat diet; HPMFs, hydroxylated polymethoxyflavones.

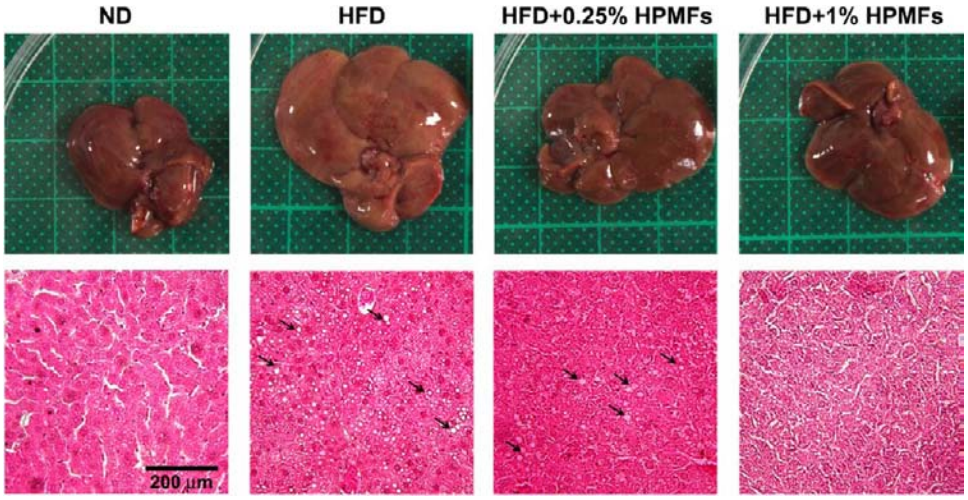

Figure 7. HPMFs attenuate liver pathogenesis in HFD-fed C57BL6 mice. Liver morphology was photographed (upper), and changes in liver histological from each group were stained with H&E (lower) (magnification, ×400) at the end of week 10. Lipid deposition is indicated as arrows. ND, normal diet; HFD, high-fat diet; HPMFs, hydroxylated polymethoxyflavones.

Table 4. Effects of HPMFs on Serum Biochemical Parameters in Mice Fed HFD[a]

	ND	HFD	HFD + 0.25% HPMFs	HFD + 1% HPMFs
GOT (U/L)	72.25 ± 9.00	79.25 ± 60.42	73.50 ± 12.04	49.00 ± 9.13
GPT (U/L)	18.60 ± 2.70	35.60 ± 6.73**	27.20 ± 3.96#	25.60 ± 3.56#
TG (mg/dL)	82.00 ± 26.58	122.25 ± 20.27*	106.00 ± 6.48	81.75 ± 16.17#
T-cho (mg/dL)	74.25 ± 9.07	166.50 ± 24.31**	125.75 ± 10.44#	117.50 ± 6.24##

[a]Data are presented as the mean ± SE (n = 10 per group), and statistical analysis was done by Student's t test. (*) $P < 0.01$, and (**) $P < 0.001$, compared with ND group. (#) $P < 0.05$, and (##) $P < 0.05$, compared with HFD group. ND, normal diet; HFD, high-fat diet; HPMFs, hydroxylated polymethoxyflavones.

droplets. However, dietary supplement of the HFD with HPMFs markedly decreased the number of fatty droplets in a dose-dependent manner. TG and T-cho levels were also significantly reduced following HPMF (1%) treatment (Table 4)

DISCUSSION

Numerous studies have documented that adipocytes may act as potential targets for antiobesity strategy, and adipogenesis regulation is considered to be mediated by many plant extracts with less detrimental effects.[17] In a recent study, citrus flavonoids and nobiletin significantly suppressed the differentiation of 3T3-L1 preadipocytes into adipocytes, thus improving obesity and insulin resistance in high-fat diet-induced obese mice.[22,23] In the present study, the chemical compositions of HPMFs used include nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, tangeretin, 5-hydroxy-6,7,3′,4-tetramethoxyflavone, 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, 5-hydroxy-6,7,4′-trimethoxyflavone, and 5-hydroxy-6,7,8,4′-tetramethoxyflavone.[3] For the first time, we found that HPMFs markedly suppressed lipid accumulation in 3T3-L1 cells through modulation of multiple molecular pathways and improved HFD-induced obesity in mice. Although nobiletin is a major PMF in citrus peel and has been shown to enhance lipolysis and suppress differentiation in 3T3-L1 adipocytes through activation of cAMP-responsive element binding protein (CREB) and down-regulation of PPARγ, respectively,[24,25] and to improve insulin resistance in HFD induced obese mice,[22] but the content of nobiletin in our HPMF sample is relatively minor compared with other 5-hydroxylated PMFs and PMFs (only 10.41 ± 0.14 mg/g HPMFs).[3] Thus, we suggest that the antiobesity effect of HPMFs might contribute to higher levels of 5-hydroxylated PMFs listed above.

PPARγ has been suggested as a master regulator of adipogenesis and is necessary for adipocyte differentiation.[26] It is possible that the suppressive effect of HPMFs on adipocyte differentiation is due to the down-regulation of PPARγ2 levels. The latter speculation was supported by the observation of the decrease in the levels of FAS and aP2, the downstream target genes of PPARγ, in 3T3-L1 adipocytes (Figure 2). Emerging studies suggest that AMPK is a master metabolic regulator responsible for modulating cellular metabolism and a promising target for metabolic disorder such as obesity and type 2 diabetes.[27] AMPK is involved in the modulation of adipose tissue metabolism through mediation of glucose uptake, β-oxidation of fatty acid, lipolysis, and adipokine secretion.[28] AMPK is also found to inhibit adipocyte differentiation and directly phosphorylate downstream target ACC at Ser 79 that inhibits the enzymatic activity and subsequent decreases in fatty acid synthesis.[11] Here we also explore the effects of HPMFs on AMPK signaling. In the results of HPMF treatment, our findings showed that HPMFs significantly induced phosphorylation of AMPKα and AMPKβ as well as suppressed the levels of SREBP-1c and ACC (Figure 3), which contributed to decreased lipid accumulation in differentiated 3T3-L1 adipocytes. This information indicates that HPMFs may activate AMPK through modulation of upstream signaling, but further verification is needed. Moreover, mitotic clonal expansion is a prerequisite for differentiation of 3T3-L1 preadipocytes into adipocyte.[29] Indeed, our cell cycle analysis result reveals this phenomenon with delayed cell cycle progression of differentiating 3T3-L1 cells by HPMF treatment after 24 h of DMI treatment (Figure 4).

In addition, we used a HFD induced obesity mouse model in this investigation.[30] The mice in obesity groups have higher body, liver, and kidney weights and higher serum triglyceride and GPT levels compared to normal control group (Tables 2−4). Consistent with our in vitro results, the administration of HPMFs over 10 weeks to mice fed the HFD decreased the body weight and fat pad weights of the mice with no change in food intake (Figures 5 and 6). We also analyzed the effects of HPMFs on the development of fatty liver, which is strongly associated with obesity.[31] In HFD mice, the livers were enlarged and yellowish, indicating liver steatosis, whereas the livers of mice administered HPMFs remained healthy looking. In histological analysis, the livers of HFD-induced obesity mice exhibited an accumulation of numerous fat droplets, a typical sign of fatty liver. However, an interesting finding was that HMPFs markedly suppressed lipid accumulation, and fewer signs of pathology were evident (Figure 7).

In conclusion, we demonstrated that HPMFs significantly suppressed 3T3-L1 adipocyte differentiation and lipid accumulation by down-regulating the adipocyte-specific transcriptional regulators PPARγ and SREBP-1c and modulating multiple signaling pathways including activation of AMPK signaling. Furthermore, HMPFs also attenuated the mitotic clonal expansion process of adipocyte differentiation. Finally, the administration of HPMFs to mice with HFD-induced obesity reduced body weight gain, fat pad weight, and accumulation of fatty droplets in the liver. More work is warranted to fully elucidate the mechanism underlying the suppression of adipocyte differentiation and HFD-induced obesity by HPMFs. These results provided the molecular mechanism of HPMFs on suppression of adipocyte differentiation. On the basis of these findings, we conclude that HPMFs have a great potential as a novel agent for the prevention and treatment of obesity.

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Notes

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ABBREVIATIONS USED

HPMFs, hydroxylated polymethoxyflavones; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; C/EBP, CCAAT/enhancer binding protein; FAS, fatty acid synthase; HMGCR, HMG-CoA reductase; IGF-1, insulin-like growth factor 1; PPAR, peroxisome proliferator-activated receptor

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