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AMP-activated protein kinase (AMPK) activators from *Myristica fragrans* (nutmeg) and their anti-obesity effect

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ABSTRACT

AMP-activated protein kinase (AMPK) is a potential therapeutic target for the treatment of metabolic syndrome including obesity and type-2 diabetes. As part of an ongoing search for new AMPK activators from plants, this study found that the total extract of *Myristica fragrans* (nutmeg) activated the AMPK enzyme in differentiated C2C12 cells. As active constituents, seven 2,5-bis-aryl-3,4-dimethyltetrahydrofuran lignans, tetrahydrofuroguaiacin B (**1**), saucernetindiol (**2**), verrucosin (**3**), nectandrin B (**4**), nectandrin A (**5**), fragransin C_1 (**6**), and galbacin (**7**) were isolated from this extract. Among the isolates, compounds **1**, **4**, and **5** at 5 μM produced strong AMPK stimulation in differentiated C2C12 cells. In addition, the preventive effect of a tetrahydrofuran mixture (THF) on weight gain in a diet-induced animal model was further examined. These results suggest that nutmeg and its active constituents can be used not only for the development of agents to treat obesity and possibly type-2 diabetes but may also be beneficial for other metabolic disorders.

Metabolic disorders, including obesity and type-2 diabetes, are becoming an epidemic in the developed and developing countries. Today, more than 1.1 billion adults worldwide are overweight, and 312 million of them obese. Unfortunately, the increase in the prevalence of obesity is closely linked to the upsurge in type-2 diabetes.[1] Because the Western lifestyle, which involves decreasing physical activity and over-consumption of high energy foods, is a major contributing factor in obesity, exercise is beneficial for overcoming obesity by increasing the total energy expenditure.[2] Recently, many studies suggested that AMP-activated protein kinase (AMPK) activators mimic or potentiate the exercise-related effects.[3] AMPK, as a heterotrimeric complex, is activated when the AMP concentration is increased by the depletion of ATP in metabolic stress, and by upstream kinases, such as LKB1 and CaMKK.[4] Activation of the AMPK enzyme switches off the ATP-consuming anabolic pathways, such as fatty acid synthesis, and switches on the ATP-generating catabolic pathways by fatty acid oxidation to supply energy to the cell. Therefore, the selective activation of AMPK is considered a potential drug target for the prevention and treatment of obesity and diabetes by regulating the whole-body glucose and lipid homeostasis.[5]

Myristica fragrans Houtt. (Myristicaceae) is an aromatic evergreen tree cultivated in South Africa, India, and other tropical countries. After nutmeg, which refers to the dried kernels of this plant, was imported into Europe at the 12th century, it has long been used indigenously as a spice in many Western foods.[6] Nutmeg is also prescribed for medicinal purposes in Asia to treat many diseases, such as rheumatism, muscle spasm, decreased appetite, and diarrhea.[7] As part of an ongoing screening program to search for new AMPK activators from natural plants, the total extract of *M. fragrans* (nutmeg) was found to activate the AMPK enzyme in differentiated C2C12 cells.

The EtOH extract of *M. fragrans* was subjected to an HP-20 column (10 × 60 cm), eluted with a gradient of EtOH in H_2O (60%, 80%, 90%, and 100%, each 3 L), and finally washed with acetone (2 L) to give five fractions. A bioassay of the five fractions on the AMPK activity revealed the 80% ethanol-eluted fraction to be most active. This active fraction was further subjected to a succession of chromatographic procedures, including silica gel chromatography, RP-C18, and preparative HPLC, to yield seven tetrahydrofuran-type lignans.[8,9] The chemical structures of the isolated compounds were determined by based on physicochemical and spectroscopic data analyses (IR, UV, $[\alpha]_D$, 1D- and 2D-NMR, and MS data), as well as a comparison with those reported in the literature. Compound **1** was obtained as a colorless oil with the molecular formula, $C_{20}H_{24}O_5$, as determined by high resolution mass spectroscopy [HREIMS *m*/*z* 344.1624 (calcd for $C_{20}H_{24}O_5$, 344.1614)]. The ^1H and ^{13}C NMR spectra of compound **1** revealed the presence of 12 protons and 10 carbon atoms.[9] This suggested the symmetric

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nature of the molecular structure of compound **1** with each signal in the ^{1}H and ^{13}C NMR spectra corresponding to two identical sets of protons and carbon atoms, respectively. Furthermore, the NMR spectral data of compound **1** suggested the presence of a 3,4-dimethyl-2,5-disubstituted tetrahydrofuran moiety.[10] The coupling constant (6.0 Hz) of the doublet at δ_H 0.61 for 3-methyl and 4-methyl suggested that these two methyl groups are alpha and in a *cis*-configuration with the adjacent protons, H-2 and H-5, respectively. A comparison of the spectroscopic data and physiochemical values between compound **1** and the published data[11] indicated the structure of compound **1** to be tetrahydrofuroguaiacin B. Compounds **2** and **3** were also purified as colorless oils, and determined to be saucernetidiol[12] and verrucosin[13] by ^{1}H and ^{13}C NMR spectroscopic data analyses, and a comparison with the published data. The ^{1}H and ^{13}C NMR spectra of compound **4** also showed the presence of only twelve protons and ten carbon atoms. The molecular formula, which was confirmed to be $C_{20}H_{24}O_5$ by HREIMS [*m*/*z* 344.1610 (calcd for $C_{20}H_{24}O_5$, 344.1624)], also suggested the symmetric structure of compound **4**. However, the two methyl groups at C-3 and C-4 were shifted down field [δ_H 1.05 (6H, d, J = 6.6 Hz)] and the two protons at C-2 and C-5 were shifted up field [δ_H 4.53 (2H, d, J = 6.6 Hz)] compared to those of compound **1**. These observations indicated that H-2 and H-5 have a *trans*-configuration with the adjacent protons of H-3 and H-4, respectively. From the spectroscopic data and physiochemical values between compound **4** and the published data,[14] compound **4** was determined to be nectandrin B. Compound **5** was isolated as a colorless oil with the molecular formula, $C_{21}H_{26}O_5$, by the high resolution mass spectrum [HREIMS *m*/*z* 358.1715 (calcd for $C_{21}H_{26}O_5$ 358.1728)]. A comparison of the ^{1}H and ^{13}C NMR spectra of compound **5** with those of compound **4** indicated the presence of an additional methoxy group in compound **5** [δ_H 3.82 (3H, s); δ_C 56.2]. Compound **5** was identified as nectandrin A by a comparison of the ^{1}H and ^{13}C NMR spectral data with those published in the literature.[11] The other known compounds were characterized as fragransin C_1 (**6**) and galbacin (**7**) by a comparison of their physicochemical values, ^{1}H and ^{13}C NMR spectra, and the MS data with those published in the literature.[11,13,14] Among these, compounds **1**, **2**, and **7** were isolated from this plant for the first time (Fig. 1).

As AMPK has recently attracted attention as the next generation target for total metabolic control, it was suggested that AMPK activators can be used to treat type-2 diabetes and obesity.[4,5] A part of an ongoing screening program for new AMPK activators from natural plants using cell-based assays,[15,16] seven 2,5-bis-aryl-3,4-dimethyltetrahydrofuran lignans were isolated from *M. fragrans*. The effect of the individual tetrahydrofuran-type lignans on AMPKα and its downstream target ACCs (ACC1 and ACC2) in cultured skeletal cells were examined. Western blot analysis indicated that the phosphorylation levels of AMPKα Thr^{172} and its intracellular substrate, ACCs Ser^{79}, were increased after the cells were treated with these compounds. Among them, compounds **1**, **4**, and **5** showed the most potential activation, exhibiting stimulation at a final concentration of 5 μM (Fig. 2). A detailed examination of the chemical and biological properties of the isolates indicated that the tetrahydrofuran lignans with symmetric structures (compounds **1**, **4**, and **5**) had stronger stimulation effects on the AMPK activity than the asymmetrical ones (compounds **2**, **3**, and **6**). Furthermore, the chemical structure of the isolates showed that all compounds had 4′- and 4″-OH and/or 3′- and 3″-OCH_3 in the two aromatic rings (compounds **1**–**6**), with the exception of compound **7**, which was replaced by two methylenedioxy moieties in the structure.

As the consumption of high levels of dietary fat is believed to be a major factor in promoting obesity in human and animals, the

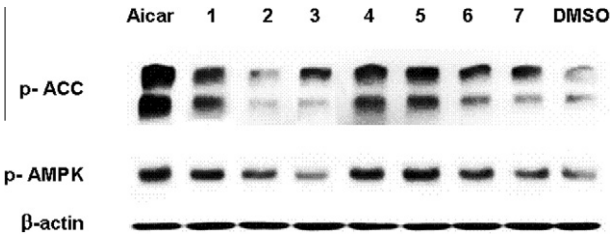

Figure 2. Stimulatory effects of the isolated tetrahydrofuran-type lignans **1**–**7** at 5 μM on the AMPK activity. The Western blotting data after AICAR as the positive control (1 mM) and differentiated C2C12 cells treated with the isolates **1**–**7** (5 μM) were analyzed using ImageJ software (National Institute of Health).

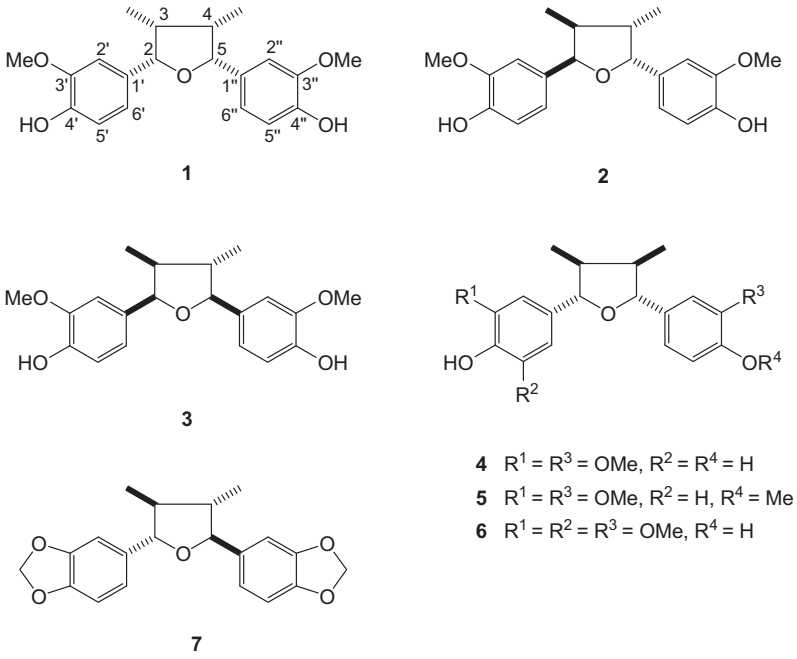

Figure 1. Structures of isolates **1**–**7** from *Myristica fragrans*.

C57BL/6 animal model was used to examine the protective effect of THF against weight gain in high-fat diet (HFD)-induced mice.[17] Figure 3A shows the phenotypic characterizations of the HFD-induced C57BL/6 mice treated with or without THF and non-diet (ND) mice. All groups of mice started with similar body weights (20.86 ± 1.10 g, $n = 30$), but the average body weight (31.41 ± 3.04 g, $n = 10$) of the HFD-induced mice after six weeks was increased by 10.55 g more than those of the ND mice. The groups administered THF showed significantly lower body weight (28.72 ± 3.23 g, $n = 10$) than the HFD-treated group without any difference in food intake between the two groups (Table 1). After the HFD and HFD + THF diets for six weeks, the epididymis fat content was compared. The epididymis fat content of the THF-fed mice (1.24 ± 0.40 g) was approximately 30% lower than those of the HFD-treated mice (1.74 ± 0.53 g) (Fig. 3B and Table 1). The physiological changes in the C57BL/6 mice treated with or without THF and ND mice for six weeks were investigated further. The blood chemistry revealed a decrease in the plasma glucose level in the THF-fed mice (199.10 ± 29.89 mg/dL) compared to those of the HFD-treated mice (232.7 ± 23.39 mg/dL). The levels of TG and LDL, 79.70 ± 9.88 and 25.90 ± 2.47 mg/dL, respectively, in the blood collected from the THF-treated mice were significantly lower than those of the HFD-treated mice; 88.40 ± 14.83 and 32.20 ± 3.97 mg/dL, respectively. This was also accompanied by lower concentrations of cholesterol; 153.46 ± 22.06 and 170.91 ± 15.35 mg/dL for THF-treated mice and HFD-treated mice, respectively (Table 1). The decrease (68.06 ± 56.29 mg/dL) in the level of glutamate pyruvate transaminase (GPT) in the THF-treated mice compared to those in HFD-treated mice (81.84 ± 67.63 mg/dL) shows that the administration of THF in the HFD-induced C57BL/6 mice offers some protection against the development and progression of liver damage induced by HFD in C57BL/6 mice.[18]

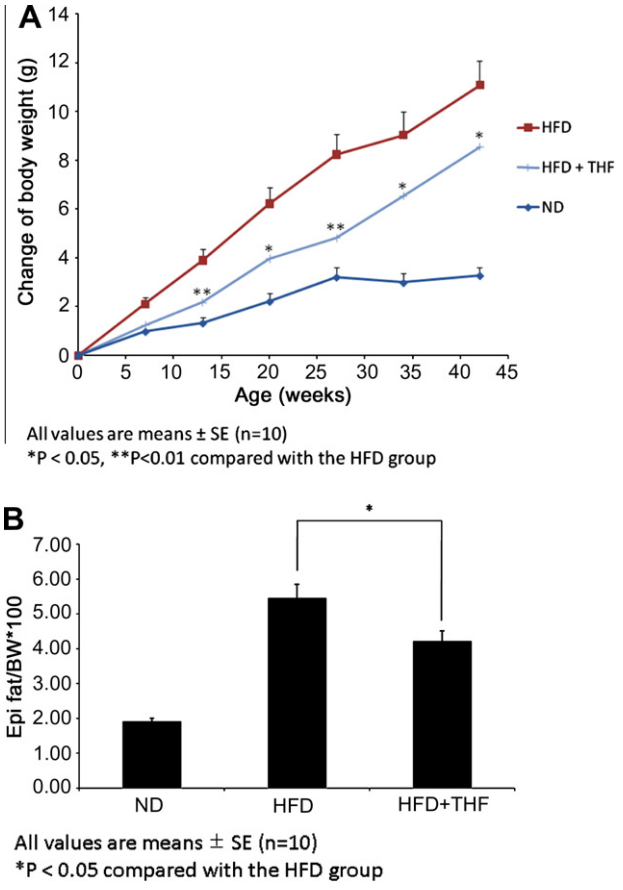

Figure 3. Changes in body weight (A) and relative fat mass (B) in the C57BL/6 mice after oral administration for six weeks with normal diet mice (ND), high-fat diet mice treated with the vehicle (HFD) and tetrahydrofuran mixture (HFD + THF, 200 mg/kg/day), respectively.

Table 1
Characterization of the ND mice as control with a normal diet, and HFD mice treated with or without THF for six weeks

Blood chemistry	ND[h] ($n = 10$)	HFD[h] ($n = 10$)	HFD + THF[h] ($n = 10$)
GPT[a] (mg/dL)	48.14 ± 15.22	81.84 ± 67.63	68.06 ± 56.29
BUN[b] (mg/dL)	25.93 ± 3.09	23.89 ± 1.77	22.74 ± 1.89
GLUC[c] (mg/dL)	159.33 ± 28.44	232.7 ± 23.39*	199.10 ± 29.89**
CHOL[d] (mg/dL)	115.73 ± 17.16	170.91 ± 15.35*	153.46 ± 22.06
HDLC[e] (mg/dL)	90.64 ± 14.52	125.48 ± 5.40*	125.98 ± 12.39
LDLC[f] (mg/dL)	22.78 ± 2.54	32.20 ± 3.97*	25.90 ± 2.47**
TRIG[g] (mg/dL)	99 ± 15.64	88.40 ± 14.83	79.70 ± 9.88
Kidney weight (g)	0.29 ± 0.03	0.31 ± 0.02	0.29 ± 0.02***
Liver weight (g)	1.04 ± 0.10	1.01 ± 0.08	0.95 ± 0.12***
Epididymis fat (g)	0.46 ± 0.06	1.74 ± 0.53	1.24 ± 0.40**
Food intake (g/day)	2.71 ± 0.08	2.24 ± 0.05	2.20 ± 0.07***

[a] Glutamate pyruvate transaminase.
[b] Blood urea nitrogen.
[c] Glucose.
[d] Cholesterol.
[e] High-density lipoprotein cholesterol.
[f] Low-density lipoprotein cholesterol.
[g] Triglyceride.
[h] Data were expressed as means ± SD.
* P <0.0001 versus ND.
** P <0.05 versus HFD.
*** P >0.05 versus HFD.

M. fragrans has been used traditionally as a spice and for various medicinal purposes, such as stomachic, carminative, tonic, aphrodisiac, and a nervous stimulant. These results showed that final body weights and weight gain in the THF-treated mice were significantly lower than those of the HFD-induced obesity mice. THF prevented not only the increase in body weight and adipose tissue mass but also the increases in glucose and LDL levels in the THF-treated group compared to those of the HFD group. These effects may be due partly to whole-body energy regulation through the phosphorylation of ACCs by the AMPK activators in this extract.[19] Therefore, the enriched extract with tetrahydrofuran-type lignans from *M. fragrans* can be used not only for the development of agents to treat obesity and possibly type-2 diabetes but may also be a new class of AMPK activators for treating metabolic disorders.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.05.067.

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8. *Bioassay-guided isolation of active compounds*: The dried semen of *Myristica fragrans* (nutmeg) was purchased at a folk medicine market in Gwangju city, Republic of Korea. The sample was identified by Professor YH Moon at Chosun University, and a specimen (No. 0010) was deposited at the Department of Pharmacy, Chosun University, Republic of Korea. The seed (nutmeg) of *M. fragrans* (3 kg) was extracted with 30% EtOH at room temperature for 7 days. The 30% ethanol-soluble extract was filtered, and placed directly onto a Diaion HP-20 column (10 × 60 cm), eluted with H_2O/EtOH (40:60, 20:80, 10:90, 0:100, each 3 L), and finally washed with acetone (2 L) to give five fractions. A bioassay of the five fractions on AMPK revealed the 80% ethanol-eluted fraction to be most active. This was further chromatographed over silica gel (6 × 60 cm; 63–200 μm particle size) using a gradient of *n*-hexane/acetone (from 6:1 to 0:1), to yield five fractions (F.1–F.5) according to their TLC profiles. Compound **4** (370 mg) was purified from a part of fraction 2 by chromatography on a reversed phase ODS-A column (5.0 × 60 cm, 150 μm particle size) eluted with MeOH/H_2O (1.5:1–2:1, each 3 L). Further chromatography of fraction 1 on a reversed phase ODS-A column (5.0 × 60 cm, 150 μm particle size) eluted with MeOH/H_2O (2:1, 3:1 to 5: 1, each 2.5 L) yielded compounds **5** (12 mg) and **7** (8.5 mg). Purification of fraction 3 by semi-preparative Gilson HPLC systems [using RS Tech OptimaPak C18 column (10 × 250 mm, 10 μm particle size); mobile phase MeOH/H_2O (60:40); flow rate 2 mL/min; UV-detections at 205 and 280 nm] resulted in the isolation of compounds **1** (28.9 mg, t_R 33.4 min), **6** (12.7 mg, t_R 37 min), **2** (6.7 mg, t_R 39.5 min), and **3** (7.8 mg, t_R 43.5 min).
9. *Physicochemical and spectroscopic data of compounds* **1–7**: *Tetrahydrofuroguaiacin B* (**1**): colorless oil; HREIMS *m*/*z* 344.1624 (calcd for $C_{20}H_{24}O_5$, 344.1614); ^1H and ^{13}C NMR, see Tables S1 and S2 (Supplementary data). *Saucernetindiol* (**2**): colorless oil; $[\alpha]_D^{25}$ +10.0 (*c* 1.38, $CHCl_3$); HREIMS *m*/*z* 344.1636 (calcd for $C_{20}H_{24}O_5$ 344.1626); ^1H and ^{13}C NMR, see Supplementary Tables S1 and S2. Verrucosin (**3**): colorless oil; $[\alpha]_D^{25}$ +14.8 (*c* 1.38, $CHCl_3$); HREIMS *m*/*z* 344.1676 (calcd for $C_{20}H_{24}O_5$ 344.1622); ^1H and ^{13}C NMR, see Supplementary Tables S1 and S2. *Nectandrin B* (**4**): colorless oil; $[\alpha]_D^{25}$ −0 (*c* 0.3, MeOH); HREIMS *m*/*z* 344.1610 (calcd for $C_{20}H_{24}O_5$, 344.1624); ^1H and ^{13}C NMR, see Supplementary Tables S1 and S2. *Nectandrin A* (**5**): colorless oil; $[\alpha]_D^{25}$ +11.5 (*c* 1.38, $CHCl_3$); HREIMS *m*/*z* 358.1715 (calcd for $C_{21}H_{26}O_5$ 358.1728); ^1H and ^{13}C NMR, see Supplementary Tables S1 and S2. *Fragransin* C_1 (**6**): colorless oil; $[\alpha]_D^{25}$ +3.5 (*c* 0.4, MeOH); HREIMS *m*/*z* 377.1715 (calcd for $C_{21}H_{26}O_6$ 374.1728); ^1H and ^{13}C NMR, see Supplementary Tables S1 and S2. *Galbacin* (**7**): colorless oil; HREIMS *m*/*z* 340.1311 (calcd for $C_{20}H_{20}O_5$ 340.1322); ^1H and ^{13}C NMR, see Supplementary Tables S1 and S2.
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15. *Cell culture*. Mouse C2C12 skeletal myoblasts were maintained in DMEM supplemented with 10% fetal bovine serum in an atmosphere containing 95% air and 5% CO_2 at 37 °C. To prepare for each assay, the cells were seeded in 12-well plates, 10^5 cells/well in 2 mL growth medium. Differentiation of C2C12 myoblasts was induced by replacing the growth medium with DMEM containing 5% horse serum when the cells reached confluence. The medium was changed every 48 h until the formation of myotubes was observed. The cells were used in the experiments at 4–5 days after differentiation.
16. *AMPK assay by Western blot analysis*: C2C12 myotubes were incubated with the appropriate concentration of the compounds for 30 min and then lysed in EBC lysis buffer [50 mM Tris–HCl (pH 7.6), 120 mM NaCl, 1 mM EDTA (pH 8.0), 0.5% NP-40, and 50 mM sodium fluoride]. The cell debris was removed by centrifugation at 12,000 rpm for 15 min, at 4 °C. The protein concentrations in the cell lysates were determined using a Bio-rad protein assay kit. Approximately 30 μg proteins of the total cell extracts were subjected to western blot analysis using anti-phosphospecific AMPKα Thr^{172}, anti-phosphospecific ACC Ser^{79}. β-Actin protein levels were used as a control for equal protein loading. The immunoreactive antigen was then recognized using a horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Lee, Y. S.; Kim, W. S.; Kim, K. H.; Yoon, M. J.; Cho, H. J.; Shen, Y.; Ye, J. M.; Lee, C. H.; Oh, W. K.; Kim, C. T.; Cordula, H. B.; Gosby, A.; Kraegen, E. W.; James, D. E.; Kim, J. B. *Diabetes* **2006**, *55*, 2256).
17. *Animal experiments*: Thirty eight-week-old male C57BL/6 mice were housed in plastic cages in a temperature-controlled (22 ± 1 °C) room and maintained on a reverse 12 h light/dark cycle. The mice were divided randomly into three groups: normal diet group (ND), high-fat diet group (HFD) with a daily gavage of the vehicle as control; and HFD + 200 mg/kg of tetrahydrofuran mixture-fed group (HFD + THF). The HFD (DIO series diets, Research Diets Inc., USA) was based on a modified Western diet and contained 21% (w/w) lard and 0.15% (w/w) cholesterol (Moon, H. S.; Lee, H. G.; Seo, J. H.; Chung, C. S.; Kim, T. G.; Choi, Y. J.; Cho, C. S. *J. Nutr. Biochem.* **2009**, *20*, 187). The tetrahydrofuran mixture was administered for 6 weeks, and the weight gain was measured every week. The food intake was measured for three consecutive days per week by subtracting the food jar pre- and post-weights for 6 weeks, and the mean values were used as the daily food intake. At the necropsy, both sides of the inguinal and epididymal adipose tissues were removed and weighed, and the relative adipose tissue weight to body weight was calculated. All animal experiments were approved by the Institutional Animal Care and Use Committee, and were performed in accordance with the institutional guidelines at AmorePacific Corporation, Korea.
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