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Anti-obesity effects of Asian dayflower, *Commelina communis*, in mice with high-fat diet-induced obesity and in 3T3-L1 cells

Shiori Nagai [a], *Eri Wakai* [a], *Makio Shibano* [b], *Ko Fujimori* [a,*]

[a] *Laboratory of Biodefense and Regulation, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan*

[b] *Laboratory of Pharmacognosy, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan*

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ABSTRACT

Asian dayflower, *Commelina communis* tea (CCT), decreased the body weight gain and reduced the mass of visceral and subcutaneous adipose tissue in the high-fat diet (HFD)-fed mice. The serum glucose and triacylglycerol levels were lowered in CCT-administered HFD-fed mice. Moreover, CCT slightly improved their insulin sensitivity. To elucidate the molecular mechanism of anti-adipogenic effect of *C. communis*, we focused on the flavonoids that have various physiological functions. The major flavonoids from *C. communis* were purified by HPLC, and their structures determined by NMR and mass spectrometry. Glucoluteolin (luteolin-7-O-glucoside), a flavonoid of *C. communis*, reduced the intracellular triacylglycerol level in 3T3-L1 cells. The transcription level of glucose transporter 4 (GLUT4) and glucose uptake were decreased by glucoluteolin. These results indicate that CCT reduced the body weight gain and slightly improved the insulin sensitivity in HFD-fed mice. Glucoluteolin repressed the accumulation of intracellular lipids by suppressing GLUT4-mediated glucose uptake into adipocytes.

1. Introduction

Obesity is a global health problem and its prevalence is rapidly increasing (Finucane et al., 2011). Obesity is caused by an increase in the adipose tissue mass and is associated with the development of numerous pathological disorders (Attie & Scherer, 2009; Cornier et al., 2008; Pi-Sunyer, 2009). Adipocytes play critical roles in maintaining energy balance. These cells store lipids as an energy source and release fatty acids in

* *Corresponding author.* Laboratory of Biodefense and Regulation, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan. Tel.: +81 72 690 1215; fax: +81 72 690 1215.
E-mail address: fujimori@gly.oups.ac.jp (K. Fujimori).

Abbreviations: C/EBP, CCAAT/enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; GLUT, glucose transporter; CCT, *C. communis* tea; WAT, white adipose tissue; HFD, high-fat diet; LFD, low-fat diet; qPCR, quantitative PCR; TBP, TATA-binding protein; NEFA, non-esterified fatty acid; ITT, insulin tolerance test; MS, mass spectrometry; UHPLC, ultra-HPLC; DMEM, Dulbecco's modified Eagle's medium; 2-NBDG, 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose; vWAT, visceral WAT; sWAT, subcutaneous WAT; aP2, fatty acid binding protein 4; LPL, lipoprotein lipase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; ATGL, adipocyte triglyceride lipase; HSL, hormone sensitive lipase; MGL, monoglyceride lipase; TNFα, tumour necrosis factor α; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1

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response to various nutritional signals and/or energy insufficiency (Spiegelman & Flier, 2001). Adipocytes are also known as endocrine cells that secrete a number of adipocytokines (Antuna-Puente, Feve, Fellahi, & Bastard, 2008; Attie & Scherer, 2009; Galic, Oakhill, & Steinberg, 2010; Rasouli & Kern, 2008). Adipogenesis is controlled via a complex process including changes in hormone sensitivity and gene expression. A number of proteins, including transcription factors involved in the regulation of adipogenesis, have been identified (Lefterova & Lazar, 2009; Rosen, 2005; Rosen, Walkey, Puigserver, & Spiegelman, 2000). Among them, CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) are key adipogenesis-regulating transcription factors (Lefterova & Lazar, 2009; Rosen, 2005; Rosen et al., 2000). These transcription factors regulate the expression of a number of adipogenesis-related genes to control adipogenesis (adipocyte differentiation). Thus, for the prevention of obesity and development of anti-obesity foods and beverage, elucidation of the regulatory mechanism of obesity (adipogenesis) is important.

Natural compounds from plants and vegetables with anti-obesity effects have been investigated. Flavonoids, which are well-known natural polyphenolic compounds, are found in fruits, vegetables, and beverages (Di Carlo, Mascolo, Izzo, & Capasso, 1999). The compounds have various biological activities such as anti-cancer (Adhami, Syed, Khan, & Mukhtar, 2012; Nishiumi et al., 2011), antioxidant (Pandey & Rizvi, 2009), anti-inflammatory (Rathee et al., 2009), and anti-obesity (Meydani & Hasan, 2010; Wang et al., 2014) effects.

Commelina communis, known as the Asian dayflower, is widely distributed throughout the world. *C. communis* has been traditionally used in Chinese medicine (Bing et al., 2009; Shibano, Kakutani, Taniguchi, Yasuda, & Baba, 2008; Shih et al., 2002; Youn, Park, & Cho, 2004; Zhang et al., 2013), for it has a variety of biological activities, such as anti-inflammatory, anti-bacterial, anti-hyperglycaemic, and antioxidant effects. Moreover, *C. communis* contains a number of chemical constituents, such as flavonoids, alkaloids, polysaccharides, terpenes, and sterols. Two of these constituents, 1-deoxynojirimycin (DNJ) and (2R, 3R, 4R, 5R)-2,5-bis(hydroxymethyl)-3,4-dihydroxypyrrolidine (DMDP), inhibit the activity of α-glucosidase (Shibano et al., 2008; Youn et al., 2004), the enzyme that digests starches and oligosaccharides in the small intestines.

Insulin activates glucose transport into cells via glucose transporters (GLUTs), activation of which is the rate-limiting step during glucose uptake (Muretta & Mastick, 2009). GLUT4 is dominantly expressed in adipose tissues and muscle, where it plays a central role in glucose homeostasis in their tissues (Huang & Czech, 2007). GLUT4 is translocated to the plasma membrane from the cytosol when insulin binds to the insulin receptor. Most of the glucose incorporated into cells is used to synthesise ATP. However, excess incorporation of glucose into cells increases their intracellular lipid levels, which can increase the prevalence of diabetes and obesity. Thus, the control of glucose uptake into cells is important for regulation of the intracellular lipid level.

In the present study, we investigated the anti-obesity effects of *C. communis* tea (CCT) in high-fat diet (HFD)-fed mice, and found that CCT decreased the body weight gain by reducing the visceral and subcutaneous white adipose tissue (WAT) mass and slightly improved the insulin sensitivity in HFD-fed mice. Moreover, glucoluteolin (luteolin-7-O-glucoside), which is an abundant flavonoid in *C. communis*, repressed the accumulation of intracellular lipids by repressing GLUT4-mediated glucose uptake into mouse adipocytic 3T3-L1 cells. Thus, CCT decreased the nutrient-mediated body weight gain seen in HFD-fed mice and could be useful for the development of anti-obesity and anti-diabetic foods and beverage.

2. Materials and methods

2.1. *Preparation of C. communis tea (CCT)*

C. communis tea (CCT) leaves were obtained from Aobana Shokkenn (Osaka, Japan) and JA KUSATSU (Shiga, Japan). The tea leaves (1 g) were extracted in 500 mL of hot water at 80 °C for 15 min. The resultant hot water-extracts of *C. communis* were designated as CCT, cooled to room temperature, and used in experiments within 2 days.

2.2. *Animal study*

Mice (C57BL/6J, 5-week-old males) were purchased from Japan SLC (Shizuoka, Japan) and maintained in a 12-h light/12-h dark photoperiod in a humidity- and temperature-controlled room (55% at 24 °C). The mice were divided into 4 groups [N = 8–10 mice for each HFD group and N = 4 mice for each low-fat diet (LFD) group] of similar average body weights. They were provided CCT or water, respectively, to drink and each group was fed either the LFD (FR-2, 4.8% fat; Funabashi Farm, Chiba, Japan) or the HFD (35% fat; Research Diet, New Brunswick, NJ, USA). These 4 groups were designated as CCT/LFD, CCT/HFD, water/LFD, and water/HFD groups, respectively. Water or CCT and food were available *ad libitum*. Body weight was measured weekly for 8 weeks. The animal study was approved by the Animal Committee of Osaka University of Pharmaceutical Sciences, and animals were handled in accordance with the principles and guidelines established by the committee.

2.3. *Quantification of mRNA level by quantitative PCR (qPCR)*

Total RNA was extracted by the use of TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) according to the instructions prescribed by the manufacturer. When RNA was prepared from tissues, they were disrupted in TriPure Isolation Reagent by using a Bead beater-type homogeniser μT-01 (TAITEC, Saitama, Japan). After removal of the cell debris and lipids by centrifugation, the supernatant was then utilised to prepare RNA.

First-strand cDNAs were synthesised with total RNA (1 μg), random hexamer (Takara-Bio, Kyoto, Japan), and ReverTra Ace Reverse Transcriptase (Toyobo, Osaka, Japan) at 42 °C for 60 min after denaturation at 72 °C for 3 min, and this was followed by heat-inactivation of the reverse transcriptase at 99 °C for 5 min. The cDNAs were further utilised for quantitative PCR (qPCR) analysis.

Transcription levels of the desired genes were measured by using a LightCycler System (Roche Diagnostics) with

THUNDERBIRD SYBR qPCR Mix (Toyobo) or a StepOnePlus System (Thermo Fisher Scientific, Waltham, MA, USA) with *Power* SYBR Green PCR Master Mix (Thermo Fisher Scientific) and gene-specific primer sets (Table S1). The mRNA levels of the desired genes were normalised to the level of the TATA-binding protein (TBP).

2.4. *Serum biochemical markers*

Serum insulin levels were measured by using a Mouse Insulin ELISA KIT (TMB; Shibayagi, Gunma, Japan). Serum triacylglycerol, non-esterified fatty acid (NEFA), total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels were determined by using the L-Type TG M test, NEFA-C, Cholesterol M, L-Type LDL-C, and L-Type HDL-C Kits, respectively (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions.

2.5. *Insulin tolerance test (ITT)*

Mice were fasted for 16 h prior to the ITT. Insulin (0.75 mU/g of body weight; HUMULIN®; Eli Lilly, Indianapolis, IN, USA) was intraperitoneally injected. Blood was collected from a tail vein and glucose was immediately measured by using a MEDISAFE MINI Blood Glucose Monitoring System (Terumo, Tokyo, Japan). The blood glucose levels were measured at 0, 15, 30, 60, 90 and 120 min after injection of insulin.

2.6. *Extraction and isolation of constituents of* C. communis

The aerial parts (400 g) of *C. communis* were dried and refluxed 3 times with 70%(v/v) ethanol (3 L) for 3 h. The solution was then concentrated under reduced pressure to give an extract (75.5 g), which was subsequently chromatographed on a Diaion HP-20 column (Mitsubishi Chemical, Tokyo, Japan). After the column has been washed with water, the adsorbed material was eluted with methanol (2 L). The eluted fraction was next concentrated *in vacuo* to give a flavonoid fraction (51.7 g). This fraction was chromatographed on a silica gel (400 g) column (5.3 i.d. × 56 cm) with a dichloromethane-methanol mobile phase, and all fractions were subsequently subjected to HPLC separation (Develosil ODS-5 10 i.d. × 250 mm, Nomura Chemical, Aichi, Japan) or Nucleosil C-18 AB 10 i.d. × 250 mm (Macherey-Nagel, Düren, Germany) with elution with 1%(v/v) acetic acid : acetonitrile = 88:12 or 85:15 (v/v).

2.7. *Spectral identification of constituents of* C. communis *by NMR and mass spectrometry (MS)*

Four flavonoid compounds, i.e., orientin, isoorientin, isorhamnetin 3-O-rutinoside, and glucoluteolin, were identified by comparison of their spectral (^{1}H-NMR, ^{13}C-NMR, and MS) data with the reported values (Kato & Morita, 1990; Park, Kim, & Kim, 2000; Sakakibara, Difeo, Nakatani, Timmermann, & Mabry, 1976). ^{1}H- and ^{13}C-NMR spectra were recorded on a VNMRS-400 spectrometer (Agilent Technologies; Santa Clara, CA, USA), operating at 400 MHz for proton and 100 MHz for carbon, with tetramethylsilane as an internal standard. Fast atom bombardment-mass spectrometry (FAB-MS) spectra were obtained with an MS700V mass spectrometer (JEOL, Tokyo, Japan). Spectrum data of the flavonoids were shown in Supplementary materials.

The purity of each of these 4 flavonoid compounds was checked by ultra-HPLC (UHPLC, Nexera; Shimadzu, Kyoto, Japan) using a diode-array detector. A Cosmosil 2.5C_{18}-MS-II column (100 × 2.0 mm i.d.; 2.5 μm; Nacalai Tesque, Kyoto, Japan) was utilised for UHPLC analysis with a mobile phase [1%(v/v) acetic acid] and acetonitrile at a ratio of 85:15 (v/v). The flow rate was 0.4 mL/min, column oven temperature was 40 °C, and the detection wavelength was at 400–200 nm.

2.8. *Cell culture*

Mouse adipocytic 3T3-L1 cells (Human Science Research Resources Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10%(v/v) foetal calf serum (Sigma) and antibiotics (Nacalai Tesque), and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Adipocyte differentiation of 3T3-L1 cells was initiated by incubation for 2 days in DMEM with insulin (10 μg/ml; Sigma), 1 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (Nacalai Tesque). On day 2, the medium was replaced with DMEM containing insulin (10 μg/ml) alone and then changed every 2 days. Oil Red O staining of the intracellular lipids was carried out as described previously (Fujimori, Yano, & Ueno, 2012).

2.9. *Cell toxicity assay*

Cell toxicity was assessed as described previously (Fujimori & Shibano, 2013). In brief, 3T3-L1 cells were seeded in 96-well plates and allowed to attach overnight at 37 °C. The cells were incubated for 6 days in DMEM containing various concentrations (0–50 μM) of each of the purified flavonoids: orientin, isoorientin, isorhamnetin 3-O-rutinoside, and glucoluteolin. Cell toxicity was measured with Cell Count Reagent SF (Nacalai Tesque) according to the instructions indicated by manufacturer.

2.10. *Measurement of intracellular triacylglycerol level*

3T3-L1 cells were allowed to differentiate into adipocytes for 6 days in DMEM with or without each of the purified flavonoids (50 μM). Intracellular triacylglycerol levels were measured by using a WAKO LabAssay Triglyceride Kit (Wako Pure Chemical). Protein concentrations were determined with Pierce BCA Protein Assay Reagent (Thermo Fisher Scientific), with BSA used as the standard.

2.11. *Glycerol release assay*

3T3-L1 cells were allowed to differentiate into adipocytes for 6 days in DMEM in the presence or absence of glucoluteolin. On day 5, the medium was changed to phenol red-free DMEM (Sigma) containing insulin with or without glucoluteolin (50 μM). On day 6, the culture medium was collected and used to measure the glycerol released into the medium by using Free Glycerol Assay Reagent (Cayman Chemical, Ann Arbor, MI, USA) according to the method prescribed by the manufacturer.

2.12. 2-Deoxyglucose uptake assay

3T3-L1 cells were allowed to differentiate for 6 days into adipocytes in 96-well plates in DMEM with or without glucoluteolin. The medium was removed, and the cells were washed 3 times with PBS(-), followed by incubation for 30 min at 37 °C in PBS(-) containing 150 μg/ml 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG; Cayman Chemical) for 30 min. The buffer was then discarded, and the cells were washed 3 times with PBS(-), after which the fluorescence was observed in PBS(-) under a fluorescent microscope (CKX-41FL, Olympus, Tokyo, Japan). Fluorescence was measured by using an Enspire 2300 Multimode Plate Reader (Perkin-Elmer, Waltham, MA, USA). The 2-NBDG uptake level was expressed as the degree of elevation compared with that of the vehicle-treated cells.

2.13. Statistical analysis

Two groups were compared by using Student's *t*-test. One-way analysis of variance and Tukey's *post-hoc* test were used to compare more than 2 groups with comparable variances. $p < 0.05$ was considered significant.

3. Results

3.1. Decrease in body weight gain by administration of CCT to HFD-induced obese mice (CCT/HFD group)

C. communis (Fig. 1A) has a variety of bioactive properties as a Chinese medicine (Bing et al., 2009; Shibano et al., 2008; Shih et al., 2002; Youn et al., 2004; Zhang et al., 2013). To investigate the effects of *C. communis* on obesity in mice, we administered CCT (Fig. 1B) to mice on either the LFD or HFD. After 8 weeks, the body weight gain in the CCT/HFD group was significantly reduced as compared with that in the water/HFD group, whose levels were similar to those of the CCT/LFD and water/LFD groups (Fig. 1C and D). In addition, there was no significant difference in food intake between the groups (Fig. 1E). To investigate changes in fat weight, we isolated subcutaneous WAT (sWAT) and visceral WAT (vWAT) including epididymal and perirenal adipose tissues and weighed them. The fat tissue masses in vWAT and sWAT, but not that mass in the liver, were significantly lower in the CCT/HFD group than in the water/HFD group (Fig. 1F). These results indicate that CCT effectively suppressed HFD-induced body weight gain by reducing the WAT mass in the mice.

3.2. Effect of CCT on serum biochemical parameters in HFD-induced obese mice (CCT/HFD group)

The serum triacylglycerol level in the CCT/HFD group was lowered as compared with that in the water/HFD group (Table 1). However, triacylglycerol levels remained unchanged between the CCT/LFD and water/LFD groups (Table 1). Total cholesterol, HDL-, and LDL-cholesterol levels in the CCT/HFD group were almost the same as those of the water/HFD group (Table 1). Thus, CCT decreased the serum triacylglycerol levels in the HFD-fed mice.

3.3. Expression of adipogenesis-related genes in WAT of CCT-administered HFD-fed mice (CCT/HFD group)

We measured the mRNA levels of adipogenic, lipogenic, and lipolytic genes in the WAT of each HFD-fed group. The mRNA levels of the adipogenic PPARγ, C/EBPα, fatty acid-binding protein 4 (aP2), CD36, lipoprotein lipase (LPL), and GLUT4 genes were significantly decreased in the WAT and sWAT of the CCT/HFD group, as compared with each of those in the water/HFD group (Fig. 2A). In contrast, the transcription levels of the acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD) genes, which are involved in lipogenesis, were either unaltered or rather increased in the vWAT and sWAT of the CCT/HFD group, as compared with each of those in the water/HFD group (Fig. 2B). These results indicate that the expression of adipogenic genes was suppressed by CCT in the WAT of HFD-fed mice, suggesting its ability to suppress adipogenesis in adipose tissues.

Then, we measured the transcription level of the lipolytic genes including adipocyte triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL) in the WAT of the CCT/HFD and water/HFD groups. The mRNA level of the ATGL, HSL, and MGL genes in the CCT/HFD group was significantly reduced, as compared with each of the water/HFD group (Fig. 2C). In contrast, the serum NEFA level was the same in the CCT/HFD and water/HFD groups (Fig. 2D). These results indicate that lipolysis was not altered even after the mice had been administered CCT under the HFD, although the expression level of the lipolytic genes in WAT was repressed by CCT. Lipolytic properties such as the activity of HSL, a rate-limiting enzyme in lipolysis, should be further investigated.

Table 1 – Effect of CCT on serum metabolic parameters in HFD-fed mice.

	LFD		HFD	
	Water	CCT	Water	CCT
Triacylglycerol (mg/dl)	68.0 ± 17.9	58.7 ± 12.3	92.0 ± 16.3	56.0 ± 11.3*
Total cholesterol (mg/dl)	118.0 ± 2.0	113.0 ± 5.0	200.5 ± 5.9	188.8 ± 6.9
LDL cholesterol (mg/dl)	10.0 ± 1.2	14.0 ± 4.8	18.5 ± 1.8	18.0 ± 2.8
HDL cholesterol (mg/dl)	71.0 ± 1.9	60.0 ± 2.8	89.5 ± 2.5	88.4 ± 4.8

Metabolic parameters in 13-week-old mice (N = 4–10). Data are the means ± S.E.
* $p < 0.01$, vs. water/HFD group.

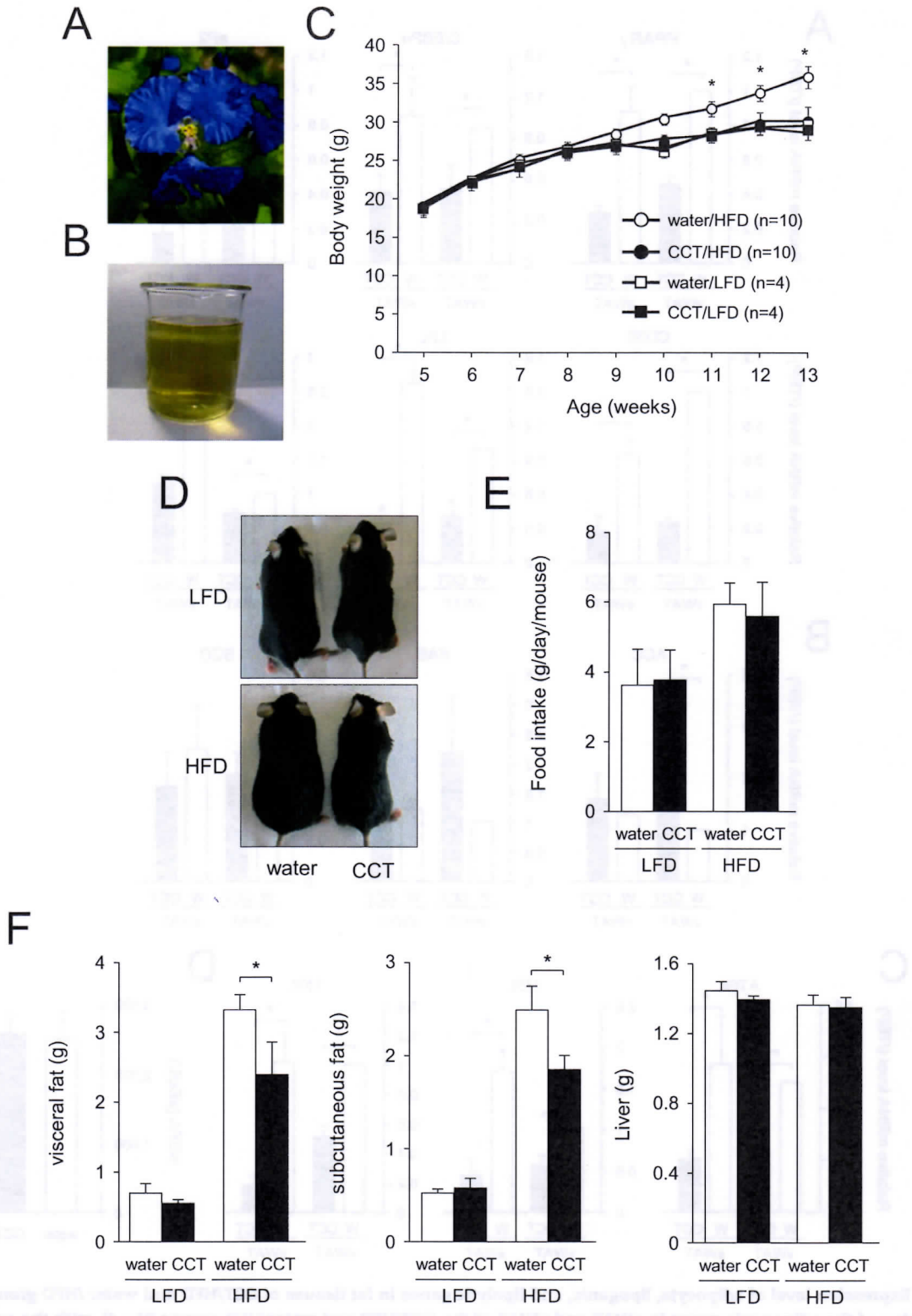

Fig. 1 – Decreased body weight gain in CCT/HFD group. (A) Photo of *C. communis*. (B) *C. communis* tea (CCT). (C) Body weight change in the CCT/HFD, water/HFD, CCT/LFD, and water/LFD groups (N = 4–10) for 8 weeks. Body weight was measured every week. Data are shown as the means ± S.E. *$p < 0.01$, as compared with those of the water/HFD group. (D) Representative photographs of mice in each group. (E) Daily food intake per gram body weight in 12-week-old mice. Data are shown as the means ± S.D. (N = 4–10). (F) Absolute weight of visceral and subcutaneous fats, and liver in each group. Data are shown as the means ± S.E. *$p < 0.01$, as indicated by the brackets.

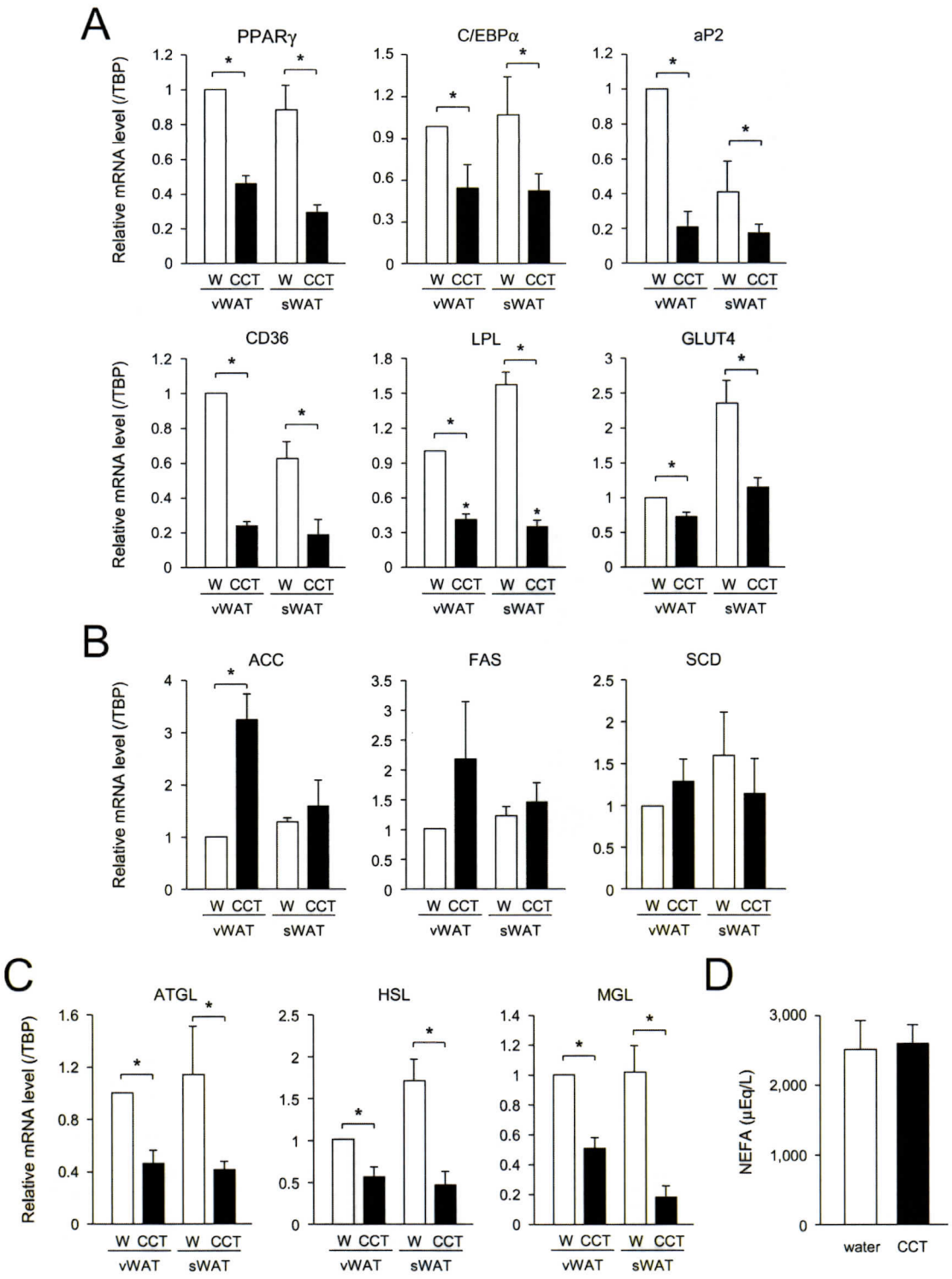

Fig. 2 – Expression level of adipocyte, lipogenic, and lipolytic genes in fat tissues of CCT/HFD and water /HFD groups. (A) Expression of the adipogenic genes in sWAT and vWAT of the CCT/HFD and water/HFD groups (N = 4), with the expression level in the vWAT of the water/HFD group taken as "1". Data are shown as the means ± S.D. *$p < 0.01$, as indicated by the brackets. W: water. (B) Transcription level of the lipogenic genes in sWAT and vWAT of the CCT/HFD and water/HFD groups (N = 4). Data are shown as the means ± S.D. *$p < 0.01$, as indicated by the brackets. W: water. (C) Expression level of the lipolytic genes in the sWAT and vWAT of the CCT/HFD and water/HFD groups (N = 4). Data are shown as the means ± S.D. *$p < 0.01$, as indicated by the brackets. (D) Measurement of serum NEFA level. Data are shown as the means ± S.E. (N = 4–5). W: water.

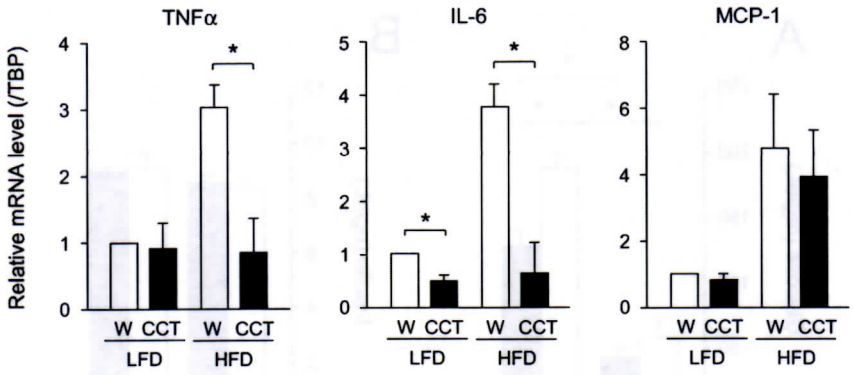

Fig. 3 – Expression level of macrophage-marker genes in vWAT of CCT/HFD and water /HFD groups. Expression levels of the macrophage-marker genes in the vWAT of the CCT/HFD and water/HFD groups (N = 4) are shown, with the expression level in the water/LFD group taken as "1". Data are presented as the means ± S.D. *$p < 0.01$, as indicated by the brackets. W: water.

3.4. *CCT-mediated change in expression of inflammation-related genes in HFD-induced obese mice (CCT/HFD group)*

Obesity is thought to be a state of chronic low-grade inflammation. When adipocytes are expanded and/or enlarged, infiltration of macrophage into adipose tissues is increased, and the secretion of pro-inflammatory cytokines such as tumour necrosis factor (TNF) α and interleukin-6 (IL-6), as well as that of chemokines such as monocyte chemoattractant protein-1 (MCP-1), is elevated. These factors are associated with the pathogenesis of insulin resistance and type 2 diabetes (Osborn & Olefsky, 2012; Suganami, Tanaka, & Ogawa, 2012; Tam, Covington, Ravussin, Redman, & Pennington, 2012).

When we measured the transcription level of these macrophage marker genes in the vWAT of the CCT/HFD and water/HFD groups (Fig. 3), the results showed that the mRNA level of the TNFα, IL-6, and MCP-1 genes was reduced in the vWAT of the CCT/HFD group, as compared with that of these genes in the water/HFD group, although their levels in the LFD groups were not changed. These results reveal that CCT suppressed the inflammation in the vWAT in the HFD-fed mice.

3.5. *Slight improvement of insulin sensitivity by CCT in HFD-induced obese mice (CCT/HFD group)*

To investigate the effect of CCT on insulin sensitivity, we conducted the ITT in LFD- or HFD-fed mice with or without administration of CCT. The serum glucose level in the CCT/HFD group was significantly reduced as compared with that in the water/HFD group (Fig. 4A), whereas serum insulin levels between these groups were similar, although their levels in the HFD-fed groups were slightly higher than those in the LFD-fed groups (Fig. 4B). Insulin sensitivity in the CCT/HFD group seemed to have been improved as compared with that in the water/HFD group, and it was almost the same as those in the CCT/LFD and water/LFD groups (Fig. 4C). Therefore, CCT slightly improved insulin sensitivity in the HFD-fed mice.

3.6. *Extraction, purification, structural identification, and cell toxicity of C. communis flavonoids*

Next, we elucidated the molecular mechanism of the anti-adipogenic effects of *C. communis* by using an *in vitro* cell-culture system. We focused on the flavonoids in *C. communis* because they are abundantly contained in *C. communis* and have a number of useful biological properties (Brown, Poudyal, & Panchal, 2015; Nijveldt et al., 2001; Wang et al., 2014). So, we purified them from *C. communis*. The aerial parts of *C. communis* were extracted in methanol, and the flavonoid constituents were purified as described in the Materials and Methods. Orientin, isoorientin, isorhamnetin 3-O-rutinoside, and glucoluteolin, which are the major flavonoids in *C. communis*, were isolated by preparative HPLC, and then checked by UHPLC for purity, which was shown to be more than at least 99% (Fig. 5A). Finally, we obtained each of the purified flavonoids from *C. communis* (400 g): orientin (178 mg, 0.045%), isoorientin (22 mg, 0.0055%), isorhamnetin-3-O-rutinoside (64 mg, 0.016%), and glucoluteolin (461 mg, 0.12%). Furthermore, we confirmed their chemical structures based on spectral analyses (^{1}H-NMR, ^{13}C-NMR, two-dimensional NMR, and MS; Fig. 5A, *inset*).

Next, we examined these flavonoids for possible cytotoxicity towards 3T3-L1 cells. The cells were cultured for 6 days in DMEM containing various concentrations of each of the purified flavonoids (0–50 μM), and then cell toxicity was measured. No significant cell toxicity was detected up to 50 μM of any of the purified flavonoids used in this study (Fig. 5B).

3.7. *Effects of flavonoids purified from C. communis on lipid accumulation in 3T3-L1 cells*

In furthering our study, we investigated the effects of orientin, isoorientin, isorhamnetin 3-O-rutinoside, and glucoluteolin on lipid accumulation in 3T3-L1 cells. The cells were allowed to differentiate into adipocytes for 6 days in the presence or absence of each purified flavonoid, and thereafter the intracellular lipids were stained with Oil Red O. The number of lipid droplets in the differentiated cells was elevated as

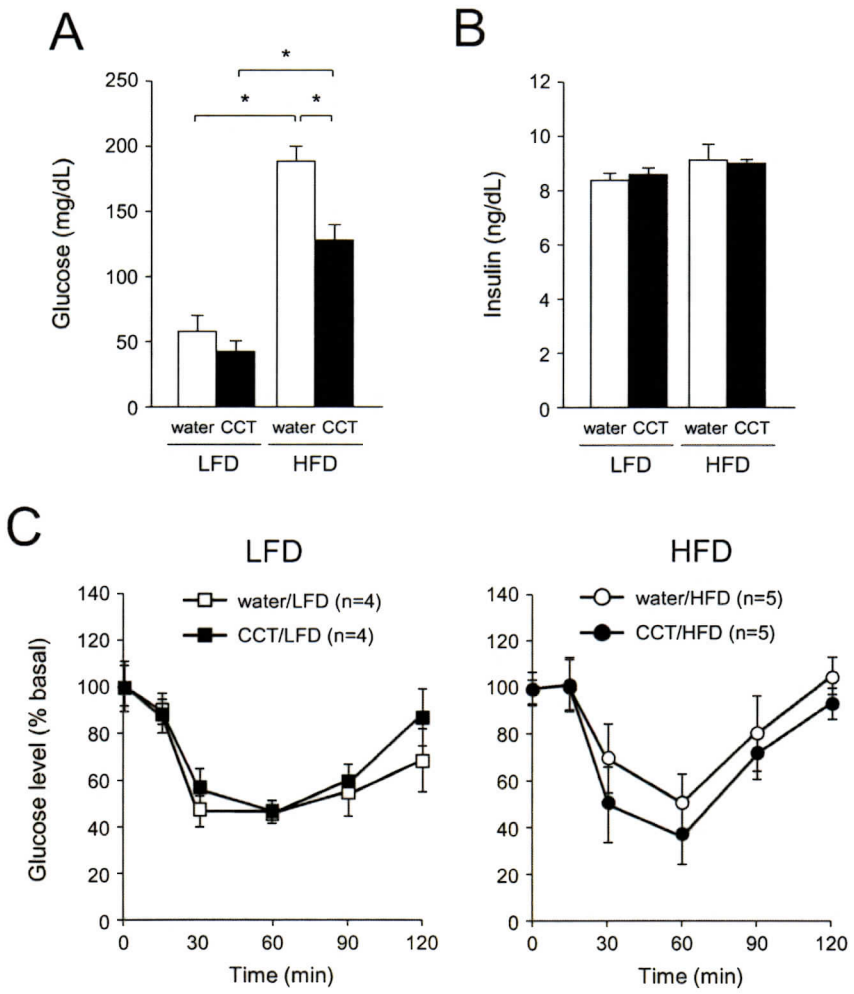

Fig. 4 – ITT. (A) Serum glucose level in each group. Mice were fasted overnight, after which their serum was prepared. Data are shown as the means ± S.E. (N = 4–10) *$p < 0.01$, as indicated by the brackets. (B) Serum insulin level in each group. Data are shown as means ± S.E. (N = 4–10) *$p < 0.01$, as indicated by the brackets. (C) ITT in each group. Blood glucose concentration was measured at indicated time points. Data are shown as the means ± S.E. (N = 4–5).

compared with that of those in the undifferentiated ones (Fig. 6A), whereas the number of intracellular lipid droplets was clearly decreased when orientin, isorhamnetin 3-O-rutinoside or glucoluteolin, but not isoorientin, were present (Fig. 6A).

Then, we measured the levels of intracellular triacylglycerol in the flavonoid-treated cells. 3T3-L1 cells were allowed to differentiate into adipocytes for 6 days in DMEM with or without each of the flavonoids. The intracellular triacylglycerol level in the differentiated cells was clearly enhanced as compared with that in the undifferentiated cells (Fig. 6B). When we caused the cells to differentiate into adipocytes in the presence of each of the flavonoids, the intracellular triacylglycerol level in orientin- or glucoluteolin-treated cells was decreased as compared with that in the differentiated cells (Fig. 6B). In contrast, isoorientin and isorhamnetin 3-O-rutinoside did not affect the accumulation of intracellular triacylglycerol (Fig. 6B). These results indicate that glucoluteolin has the most suppressive effect on the accumulation of intracellular lipids in 3T3-L1 cells. Thus, we next investigated the molecular mechanism of suppression of adipocyte differentiation by glucoluteolin.

3.8. *Change in expression level of adipogenesis-related genes by glucoluteolin in 3T3-L1 cells*

To determine the nature of this mechanism, we investigated changes in the expression level of the adipogenic genes in glucoluteolin-treated 3T3-L1 cells. The mRNA level of the adipogenic PPARγ, C/EBPα, aP2, and LPL genes was significantly elevated during adipocyte differentiation in the absence of the flavonoid, but reduced in its presence (Fig. 7A).

Next, we investigated the mRNA level of the lipogenic genes in the glucoluteolin-treated 3T3-L1 cells. When the cells were allowed to differentiate into adipocytes for 6 days in DMEM with or without glucoluteolin, the transcription level of the lipogenic genes such as ACC, FAS, and SCD was enhanced during adipocyte differentiation of 3T3-L1 cells in the absence of

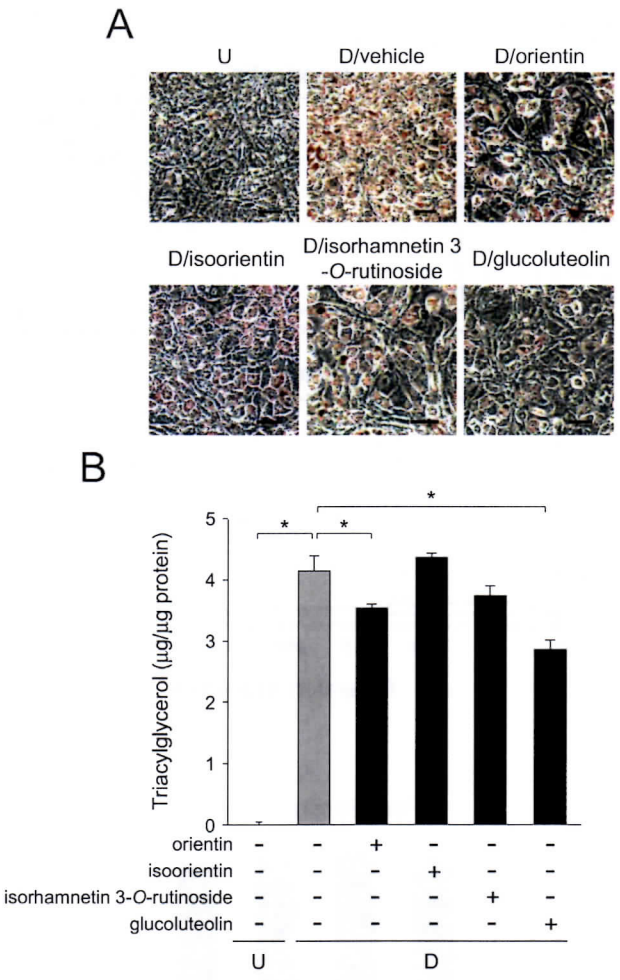

Fig. 6 – Suppression of accumulation of intracellular lipids by C. *communis* flavonoids. (A) Oil Red O staining of the intracellular lipids in 3T3-L1 cells. The cells (undifferentiated cells: U) were allowed to differentiate into adipocytes (differentiated cells: D) for 6 days in medium with or without orientin, isoorientin, isorhamnetin 3-O-rutinoside or glucoluteolin (50 μM). Bar = 50 μm. (B) Intracellular triacylglycerol level in 3T3-L1 cells. The cells (undifferentiated cells: U; *white column*) were caused to differentiate (differentiated cells: D) into adipocytes for 6 days in the absence (*grey column*) or presence of orientin, isoorientin, isorhamnetin 3-O-rutinoside or glucoluteolin (50 μM; *black columns*). Data are presented as the means ± S.D. *$p < 0.01$, as indicated by the brackets.

glucoluteolin (Fig. 7B). The expression level of the FAS gene was reduced when the cells were allowed to differentiate in the presence of glucoluteolin (Fig. 7B). However, the mRNA level of the ACC and SCD genes in the differentiated cells was not changed even in the presence of glucoluteolin (Fig. 7B).

Then, we investigated the transcription level of the lipolytic ATGL, HSL, and MGL genes. The expression level of all of these genes was increased when the cells were made to differentiate for 6 days in the absence of the flavonoid (Fig. 7C). However, in its presence, the mRNA level of the ATGL gene, but not that of the HSL and MGL genes, was decreased as compared with that in the vehicle-treated differentiated cells (Fig. 7C). In addition, the level of glycerol released from the cells was not changed when the cells were allowed to differentiate into adipocytes in the medium with glucoluteolin (Fig. 7D). These results reveal that glucoluteolin suppressed adipogenesis, but not lipogenesis or lipolysis, in adipocytes.

3.9. *Glucoluteolin-induced suppression of GLUT4-mediated glucose uptake in 3T3-L1 cells*

Next, we investigated the expression of the GLUT4 glucose transporter in glucoluteolin-treated adipose cells, as it is dominantly expressed in adipocytes and muscle (Muretta & Mastick, 2009). In the absence of this flavonoid, the expression level of the GLUT4 gene was enhanced during adipogenesis, as compared with that in the undifferentiated cells, whereas in its presence this level was reduced (Fig. 8A).

Finally, we measured glucose uptake into the glucoluteolin-treated cells by the use of 2-NBDG, a fluorescent glucose analogue. When 3T3-L1 cells were allowed to differentiate into adipocytes for 6 days in the presence of glucoluteolin, the level of 2-NBDG uptake was significantly decreased, as compared with that by the vehicle-treated differentiated cells (Fig. 8B and C). These results indicate that glucoluteolin suppressed GLUT4-mediated glucose uptake into adipocytes.

4. Discussion

Adipocyte differentiation and fat accumulation are associated with the occurrence and development of obesity. Obesity is a risk factor for a number of diseases such as cardiovascular diseases, hypertension, and diabetes mellitus (Attie & Scherer, 2009; Cornier et al., 2008). Hyperplastic obesity is caused by an increase in the number or size of adipocytes or both. Treatment and prevention of obesity are critical in the clinical field. Although, today, there are several anti-obesity drugs that decrease food intake by reducing appetite, they may be utilised only in patients with excessive obesity (BMI ≥ 27–30) (Cunningham & Wiviott, 2014; Jensen et al., 2014). Moreover, some of these drugs have the side effects on psychiatric and/or cardiovascular systems (Cunningham & Wiviott, 2014; Dietrich & Horvath, 2012). Therefore, the development of novel anti-obesity drugs without side-effects is strongly needed clinically. To solve this problem, elucidation of the molecular mechanism of regulating obesity is absolutely needed.

Some dietary supplements and natural products have a variety of therapeutic effects on many disorders without causing side effects or, if so, those causing very little inconvenience, although they generally have weaker therapeutic effects than those of the pharmaceutical medicines. Epidemiological studies have demonstrated that some dietary plant constituents are associated with a reduced risk of developing metabolic diseases (Meydani & Hasan, 2010; Wang et al., 2014). Flavonoids are well-known natural polyphenolic compounds found in vegetables, fruits, and beverages and have various biological activities (Di Carlo et al., 1999; Nishiumi et al., 2011; Pandey & Rizvi, 2009; Rathee et al., 2009). *In vitro* studies have

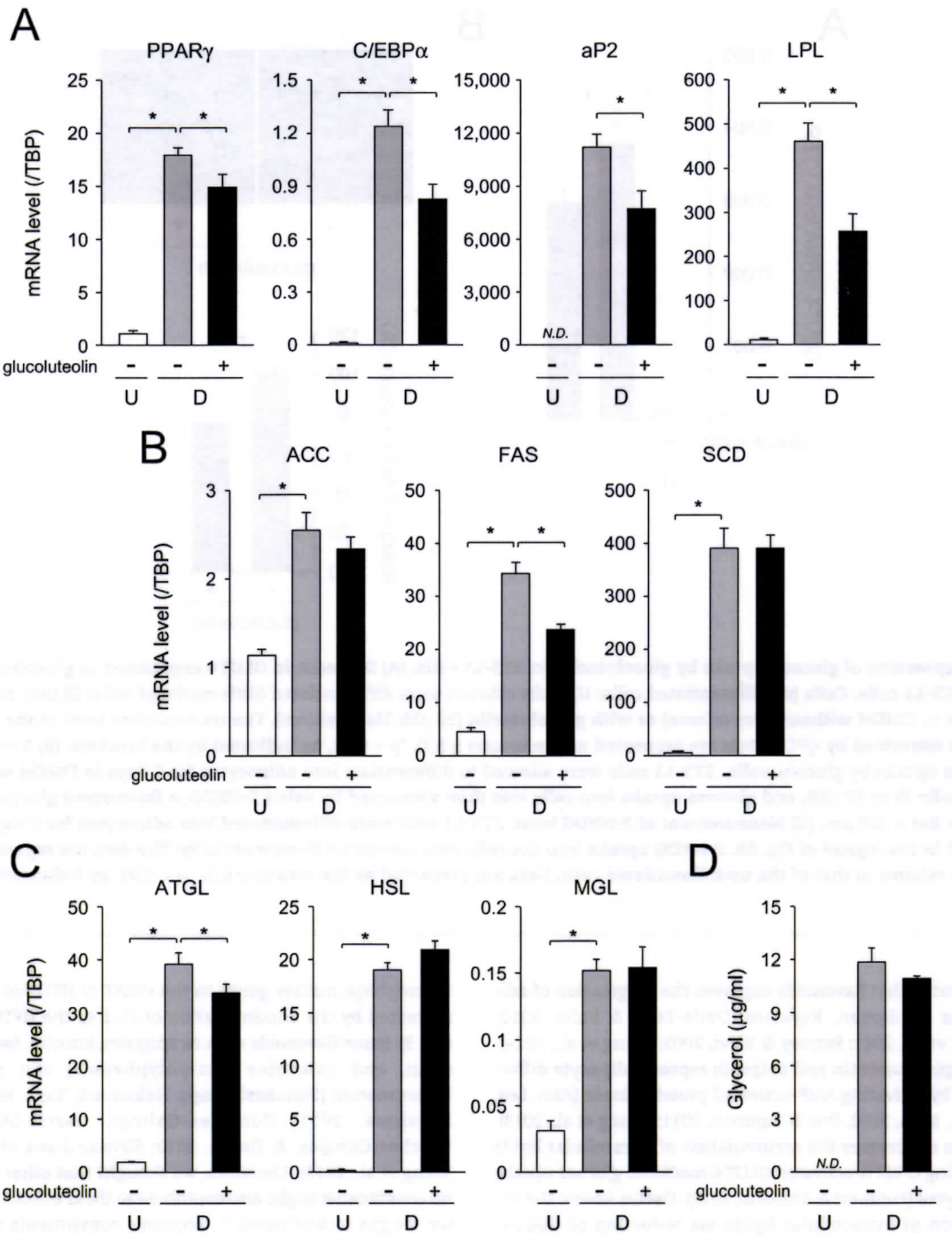

Fig. 7 – Suppression of expression of adipogenic, lipogenic, and lipolytic genes in glucoluteolin-treated 3T3-L1 cells. (A) Expression level of the adipogenic genes in glucoluteolin-treated 3T3-L1 cells. Cells (undifferentiated cells: U; *white columns*) were caused to differentiate (differentiated cells: D) into adipocytes for 6 days in the absence (*grey columns*) or presence of glucoluteolin (50 μM; *black columns*). Data are the means ± S.D. *$p < 0.01$, as indicated by the brackets. (B) Suppression of the transcription level of the lipogenic genes in glucoluteolin-treated 3T3-L1 cells. Undifferentiated cells (U; *white columns*) were caused to differentiate (D) into adipocytes for 6 days in DMEM without (*grey columns*) or with glucoluteolin (50 μM; *black columns*). *$p < 0.01$, as indicated by the brackets. (C) Reduction in the mRNA level of the lipolytic genes in glucoluteolin-treated 3T3-L1 cells. *$p < 0.01$, as indicated by the brackets. (D) Glycerol release assay. 3T3-L1 cells were differentiated and glycerol release was as described in the Materials and Methods. N.D.: not detected.

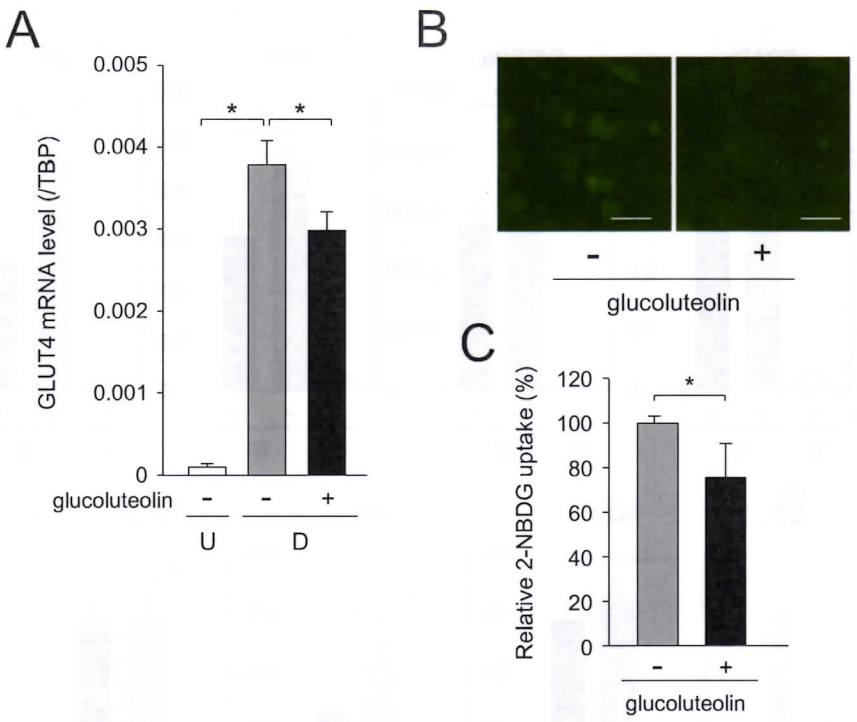

Fig. 8 – Repression of glucose uptake by glucoluteolin in 3T3-L1 cells. (A) Decrease in GLUT4 expression in glucoluteolin-treated 3T3-L1 cells. Cells (undifferentiated cells: U; *white column*) were differentiated (differentiated cells: D) into adipocytes for 6 days in DMEM without (*grey column*) or with glucoluteolin (50 μM; *black column*). The transcription level of the GLUT4 gene was examined by qPCR. Data are presented as the means ± S.D. *$p < 0.01$, as indicated by the brackets. (B) Suppression of glucose uptake by glucoluteolin. 3T3-L1 cells were allowed to differentiate into adipocytes for 6 days in DMEM with glucoluteolin (0 or 50 μM), and glucose uptake into cells was then visualised by using 2-NBDG, a fluorescent glucose analogue. Bar = 100 μm. (C) Measurement of 2-NBDG level. 3T3-L1 cells were differentiated into adipocytes for 6 days as described in the legend of Fig. 8B. 2-NBDG uptake into the cells was measured fluorometrically. The data are represented as the value relative to that of the undifferentiated cells. Data are presented as the means ± S.D. *$p < 0.01$, as indicated by the bracket.

demonstrated that flavonoids suppress the progression of adipogenesis (Andersen, Rayalam, Della-Fera, & Baile, 2010; Nishiumi et al., 2011; Pandey & Rizvi, 2009; Wang et al., 2014). For example, quercetin and apigenin repress adipocyte differentiation by activating AMP-activated protein kinase (Ahn, Lee, Kim, Park, & Ha, 2008; Ono & Fujimori, 2011; Zhang et al., 2013). Avicularin suppresses the accumulation of intracellular lipids by inhibiting C/EBPα-activated GLUT4-mediated glucose uptake in adipocytes (Fujimori & Shibano, 2013). Fisetin lowers the accumulation of intracellular lipids via reduction of GLUT4-mediated glucose uptake by inhibiting mTOR signalling (Watanabe, Hisatake, & Fujimori, 2015).

As mentioned earlier, DNJ and DMDP, as constituents of *C. communis*, have anti-diabetic effects by inhibiting the activity of α-glucosidase (Shibano et al., 2008; Youn et al., 2004), which digests starches and oligosaccharides in the small intestines. In line with that finding, we showed that CCT-administered mice on the HFD had lower serum glucose levels (Fig. 4A). We also found that CCT decreased the gain in body weight by reducing the fat of mice on the HFD (Fig. 1C and D), as well as slightly improved their insulin sensitivity (Fig. 4C). Moreover, the expression level of some inflammation-associated macrophage marker genes in the vWAT of HFD-fed mice was repressed by the administration of CCT to the HFD-fed mice (Fig. 3). Some flavonoids such as apigenin, luteolin, fisetin, quercetin, and catechins (tea-polyphenols) can modulate inflammation (Funakoshi-Tago, Nakamura, Tago, Mashino, & Kasahara, 2011; Gonzalez-Gallego, Garcia-Mediavilla, Sanchez-Campos, & Tunon, 2010; Siriwardhana et al., 2013; Wang et al., 2014). Therefore, we thought that other *C. communis* constituents might also contribute to these effects. Therefore, we sought to find novel *C. communis* constituents with anti-adipogenic effects, and focused on the flavonoids of *C. communis*. Finally, we found that glucoluteolin, a major flavonoid in *C. communis* (Shibano et al., 2008), had an anti-adipogenic effect in 3T3-L1 cells. The suppression mechanism of adipogenesis by glucoluteolin has never been defined, although a number of flavonoids such as quercetin have a number of beneficial effects such as inhibition of apoptosis and adipogenesis (Meydani & Hasan, 2010; Wang et al., 2014). The adipose tissue mass may be reduced due to a decrease in the number of adipose cells through their apoptosis or to inhibition of adipogenesis. However, glucoluteolin has no apparent toxic effect in 3T3-L1 cells (Fig. 5B), indicating that glucoluteolin decreased the

accumulation of intracellular triacylglycerol (Fig. 6A and B) through repression of GLUT4-mediated repression of glucose uptake by the cells (Fig. 8A–C).

Glucose transport into cells is the rate-limiting step of glucose uptake, and it is involved in the regulation of glucose homeostasis (Muretta & Mastick, 2009). Glucose uptake is tightly regulated by insulin, and GLUT4 is responsible for glucose uptake into adipose tissues and muscle (Muretta & Mastick, 2009). GLUT4-mediated glucose uptake is controlled at both transcriptional and post-transcriptional levels (Huang & Czech, 2007). Insulin stimulates the translocation of GLUT4 to the plasma membrane to initiate glucose uptake and to elevate its rate. An increase in transcription of the GLUT4 gene would be expected to elevate the level of GLUT4 and thus affect glucose homeostasis. The mRNA level of this gene in adipocytes is controlled by natural compounds, including flavonoids such as apigenin, avicularin, fisetin, genistein, and quercetin (Fujimori & Shibano, 2013; Nomura et al., 2008; Watanabe et al., 2015). In the present study, neither lipogenesis nor lipolysis was affected by administration of CCT to HFD-fed mice (Fig. 2B–D), although the nutrient-mediated body weight gain was repressed by CCT (Fig. 1C and D). In fact, CCT decreased the expression of the GLUT4 gene in the WAT (Fig. 2A). Moreover, glucoluteolin suppressed the accumulation of intracellular lipids that occurred via GLUT4-mediated glucose uptake into 3T3-L1 cells (Fig. 6A and B). Therefore, we propose that the observed CCT-reduced gain in body weight in the HFD-fed mice may have been mediated by the decreased glucose uptake in the WAT. Further *in vivo* analysis of glucoluteolin as an anti-obesity agent and of the improvement in insulin sensitivity mediated by glucoluteolin should be examined.

In summary, we found that CCT reduced the body weight gain observed in HFD-fed mice and its inhibitory mechanisms on adipogenesis in 3T3-L1 cells. Glucoluteolin, a major constituent of *C. communis*, repressed the accumulation of intracellular lipids by reducing GLUT4-mediated glucose uptake into 3T3-L1 cells. Thus, as *C. communis* contains constituents having anti-diabetic and anti-obesity effects, CCT, and containing these bioactive constituents would be beneficial as anti-obesity and anti-diabetes foods and beverage.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.02.012.

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Table S1. Primers used in this study

Gene	Acc.No.	Forward	Reverse
PPARγ	NM_011146	5'-CAAGAATACCAAAGTGCGATCAA-3'	5'-GAGCTGGGTCTTTTCAGAATAATAAG-3'
C/EBPα	NM_007678	5'-CTGGAAAGAAGGCCACCTC-3'	5'-AAGAGAAGGAAGCGGTCCA-3'
aP2	NM_024406	5'- GCCAGACACCCCTGCTA-3'	5'- GTTCTGGGCGTCACTCC-3'
CD36	NM_001159555	5'-GATGTGGAACCCATAACTGGATTCAC-3'	5'- GGTCCCAGTCTCATTTAGCCACAGTA-3'
GLUT4	NM_009204	5'-GACGGACACTCCATCTGTTG-3'	5'-GCCACGATGGAGACATAGC-3'
LPL	NM_008509	5'-GCTGGTGGGAAATGATGTG-3'	5'-TGGACGTTGTCTAGGGGGTA-3'
ACC	NM_133360	5'-GCGTCGGGTAGATCCAGTT-3'	5'-CTCAGTGGGGCTTAGCTCTG-3'
FAS	NM_007988	5'-GTTGGGGGTGTCTTCAACC-3'	5'-GAAGAGCTCTGGGGTCTGG-3'
SCD	NM_009127	5'-TTCCCTCCTGCAAGCTCTAC-3'	5'-CAGAGCGCTGGTCATGTAGT-3'
ATGL	NM_001163689	5'-TGACCATCTGCCTTCCAGA-3'	5'-TGTAGGTGGCGCAAGACA-3'
HSL	NM_010719	5'-GCACTGTGACCTGCTTGGT-3'	5'-CTGGCACCCTCACTCCATA-3'
MGL	NM_011844	5'-TCGGAACAAGTCGGAGGT-3'	5'-TCAGCAGCTGTATGCCAAAG-3'
TNFα	NM_013693	5'-TGCCTATGTCTCAGCCTCTTC-3'	5'-GAGGCCATTTGGGAACTTCT-3'
MCP-1	NM_011331	5'-CCATCAGTCCTCAGGTATTGG-3'	5'-CTTCCGGACGTGAATCTTCT-3'
IL-6	NM_031168	5'-AGTTGACGGACCCCAAAAG-3'	5'-AGCTGGATGCTCTCATCAGG-3'
TBP	NM_013684	5'-GTGATGTGAAGTTCCCCATAAGG-3'	5'-CTACTGAACTGCTGGTGGGTCA-3'

Supplementary materials

Spectrum data for flavonoids by NMR

Orientin: FAB-MS *m/z* 449 $[M+H]^+$. ^{1}H-NMR (Pyridine-d_5) δ: 7.87 (1H, d, *J*=2.0 Hz, H-2'), 7.50 (1H, dd, *J*=8.5, 2.0 Hz, H-6'), 7.27 (1H, d, *J*=8.5 Hz, H-5'), 6.90 (1H, s, H-3), 6.68 (1H, s, H-6), 5.85 (1H, d, *J*=9.5 Hz, Glc-1), 5.18 (1H, br.dd, *J*=8.5, 8.5 Hz, Glc-2), 4.57 (1H, dd, *J*=12.0, 2.3 Hz, Glc-6), 4.51 (1H, dd, *J*=8.5, 8.5 Hz, Glc-4), 4.48 (1H, dd, *J*=12.0, 5.0 Hz, Glc-6), 4.45 (1H, dd, *J*=8.5, 8.5 Hz, Glc-3), 4.20 (1H, ddd, *J*=8.5, 5.0, 2.3 Hz, Glc-5). ^{13}C-NMR (Pyridine-d_5) δ: 182.9 (C-4), 164.8 (C-7), 164.6 (C-2), 162.0 (C-9), 157.5 (C-5), 151.7 (C-4'), 147.8 (C-3'), 122.9 (C-1'), 119.5 (C-6'), 116.9 (C-5'), 114.6 (C-2'), 110.1 (C-8), 104.9 (C-10), 103.9 (C-3), 94.6 (C-6), 83.0 (Glc-5), 80.6 (Glc-3), 75.6 (Glc-1), 72.9 (Glc-2), 71.9 (Glc-4), 62.7 (Glc-6)

Isoorientin: FAB-MS *m/z* 449 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.28 (1H, dd, *J*=8.5, 2.0 Hz, H-6'), 7.27 (1H, d, *J*=2.0 Hz, H-2'), 6.80 (1H, d, *J*=8.5 Hz, H-5'), 6.45 (1H, s, H-3), 6.38 (1H, s, H-8), 4.82 (1H, d, *J*=7.8 Hz, Glc-1), 4.13 (1H, dd, *J*=8.5, 8.5 Hz, Glc-2), 3.84 (1H, dd, *J*=12.0, 5.0 Hz, Glc-6), 3.71 (1H, m, Glc-6), 3.52 (1H, m, Glc-3), 3.50 (1H, m, Glc-4), 3.40 (1H, m, Glc-5). ^{13}C-NMR (CD_3OD) δ: 182.7 (C-4), 163.6 (C-7), 164.5 (C-2), 161.0 (C-5), 156.2 (C-9), 150.2 (C-4'), 146.4 (C-3'), 122.8 (C-1'), 122.7 (C-6'), 116.3 (C-5'), 114.6 (C-2'), 107.3 (C-6), 104.7 (C-10), 103.0 (C-3), 94.0 (C-8), 83.0 (Glc-5), 79.5 (Glc-3), 74.0 (Glc-1), 71.4 (Glc-2), 71.9 (Glc-4), 62.4 (Glc-6).

Isorhamnetin-3-*O*-rutinoside: FAB-MS *m/z* 625 $[M+H]^+$. ^{1}H-NMR (CD_3OD) δ: 7.96 (1H, d, *J*=2.1 Hz, H-2'), 7.64 (1H, dd, *J*=8.5, 2.1 Hz, H-6'), 6.92 (1H, d, *J*=8.5 Hz, H-5'), 6.42 (1H, d, *J*=2.1 Hz, H-8), 6.21 (1H, d, *J*=2.1 Hz, H-6), 5.25 (1H, d, *J*=8.5 Hz, Glc-1), 4.54 (1H, d, *J*=2.1 Hz, Rham-1), 3.97 (1H, s, OMe), 3.72 (1H, dd, *J*=12.0, 2.5 Hz, Glc-6), 3.55 (1H, dd, *J*=12.0, 5.0 Hz, Glc-6), 3.46 (1H, m, Glc-2), 3.35 (Glc-3, 4), 3.22 (1H, m), 3.20-3.78 (Rham-2, 3, 4, 5), 1.10 (1H, d, *J*=6.5 Hz, Rham-6). ^{13}C-NMR (CD_3OD) δ: 180.0 (C-4), 166.3 (C-7), 163.5 (C-5), 158.9 (C-2), 159.1 (C-9), 151.2 (C-3'), 148.6 (C-4'), 135.7 (C-3), 124.3 (C-6'), 123.3 (C-1'), 116.5 (C-5'), 116.4 (C-2'), 104.9 (C-10), 103.0 (Glc-1), 102.9 (Rham-1), 100.4 (C-6), 95.3 (C-8), 78.5 (Glc-3) 77.7 (Glc-5), 76.4 (Glc-2), 74.4 (Rham-4), 72.8 (Rham-3), 72.4 (Rham-2), 71.2 (Glc-4), 70.1 (Rham-5), 68.8 (Glc-6), 57.0 (OMe), 18.2 (Rham-6).

Glucoluteolin: FAB-MS *m/z* 449 $[M+H]^+$. ^{1}H-NMR (Pyridine-d_5) δ: 7.90 (1H, d, *J*=2.5 Hz, H-2'), 7.52 (1H,

dd, *J*=8.5, 2.5 Hz, H-6'), 7.28 (1H, d, *J*=8.5 Hz, H-5'), 6.99 (1H, d, *J*=2.1 Hz, H-6), 6.94 (1H, s, H-3), 6.85 (1H, d, *J*=2.1 Hz, H-8), 5.83 (1H, d, *J*=7.6 Hz, Glc-1), 5.83 (1H, d, *J*=7.6 Hz, Glc-1), 4.58 (1H, dd, *J*=12.0, 2.2 Hz, Glc-6), 4.43 (1H, dd, *J*=8.0, 8.0 Hz, Glc-3), 4.42 (1H, dd, *J*=12.0, 5.3 Hz, Glc-6), 4.37 (1H, dd, *J*=8.0, 8.0 Hz, Glc-4), 4.36 (1H, dd, *J*=8.0, 7.6 Hz, Glc-2), 4.21 (1H, ddd, *J*=12.0, 5.3, 2.2 Hz, Glc-5). ^{13}C-NMR (Pyridine-d_5) δ: 182.9 (C-4), 165.4 (C-2), 164.0 (C-7), 162.7 (C-9), 157.9 (C-5), 152.0 (C-4'), 147.9 (C-3'), 122.8 (C-1'), 119.7 (C-6'), 116.9 (C-5'), 114.8 (C-2'), 106.6 (C-10), 104.2 (C-6), 101.9 (Glc-1), 100.7 (C-8), 95.3 (C-3), 79.3 (Glc-3), 78.6 (Glc-5), 74.9 (Glc-2), 71.2 (Glc-4), 62.4 (Glc-6).