Effects of Flavonoids and Phenolic Acids on the Inhibition of Adipogenesis in 3T3-L1 Adipocytes

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Obesity has become a global epidemic in both developed and developing countries, and it is a significant risk factor for various diseases such as diabetes, cancer, heart disease, and hypertension. In the present study, the effect of naturally occurring antioxidants (flavonoids and phenolic acids) on the inhibition of adipogenesis in 3T3-L1 adipocytes was investigated. The results showed that *o*-coumaric acid and rutin had the highest inhibition on intracellular triglyceride (61.3 and 83.0%, respectively) among 15 phenolic acids and 6 flavonoids tested. However, the oil red o stained material (OROSM) showed that cell number in 3T3-L1 adipocytes was not influenced by those compounds. For glycerol-3-phosphate dehydrogenase (GPDH) activity, the data indicated that *o*-coumaric acid and rutin had the highest inhibition on GPDH activity (54.2 and 66.8%, respectively) among the compounds tested. *o*-Coumaric acid and rutin also inhibited the expression of PPARγ, C/EBPα and leptin and then up-regulated expression of adiponectin at the protein level. Some naturally occurring antioxidants efficiently suppressed adipogenesis in 3T3-L1 adipocytes. These results suggest that *o*-coumaric acid and rutin targeted for adipocyte functions could be effective in improving the symptoms of metabolic syndrome.

KEYWORDS: Flavonoids; phenolic acis; *o*-coumaric acid; rutin; adipogenesis; 3T3-L1 adipocyte

INTRODUCTION

Currently, at the beginning of the 21st Century, obesity has become the leading metabolic disease in the world (*1*). It is a prevalent health hazard in industrialized countries and is closely associated with coronary heart disease, hypertension, type 2 diabetes mellitus, cancer, respiratory complications, and osteoarthritis (*2*). Obesity is a condition in which adipocytes accumulate a large amount of fat and become enlarged. It is characterized at the cellular level by an increase in the number and size of adipocytes differentiated from fibroblastic preadipocytes in adipose tissue (*3*).

The adipocyte is the primary site for energy storage and accumulates triglycerides during nutritional excess. Shimomura et al. (*4*) indicated that adipocyte synthesizes and secretes biologically active molecules called adipocytokines. During adipocyte differentiation, transcriptional factors such as peroxisome proliferator-activated receptor (PPAR)γ and CCAAT/enhancer-binding proteins (C/EBPs) are involved in the sequential expression of adipocyte-specific proteins (*5*). Adiponectin is an adipocytokine that has been shown to have antiatherogenic, anti-inflammatory, and antidiabetic roles (*6*). It has been found to be an important modulator of insulin sensitivity (*7*). Nakamura et al. (*8*) indicated that high circulating levels of adiponectin

might be protective against the development of coronary artery disease. Yamauchi et al. (*9*) indicated that adiponectin has emerged most recently as an important adipocytokine with insulin-sensitizing effects and represents a novel treatment target for insulin resistance and type 2 diabetes. Leptin is a secreted protein hormone that inhibits food intake and stimulates thermogenesis (*10*). The cytosolic enzyme GPDH appears to have an important role in the conversion of glycerol into triglyceride (*11*).

Flavonoids are constituents of fruits, vegetables, nuts and plant-derived beverages such as tea, wine, and traditional Eastern medicines. Phenolic acids and flavonoids have pharmacological properties such as antioxidant, antithromobosis, anti-inflammatory, anti-HIV-1, and anticancer (*12–14*). Our previous in vitro studies indicated that gallic acid and quercetin had the highest inhibition on 3T3-L1 preadipocyte population growths among 15 phenolic acids and six flavonoids (*15, 16*). However, the literature regarding the effect of those phenolic acids and flavonoids on inhibiting adipogenesis in 3T3-L1 adipocytes remains unclear. The chemical structures of flavonoids and phenolic acids being tested in the present study are shown in **Figure 1**.

In the present study, we investigated the effects of flavonoids and phenolic acids on the inhibition of intracellular triglyceride and GPDH activity in 3T3-L1 adipocytes. We also examined the effect of these compounds on protein expression of adipo-

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Phenolic acids

Hydroxybenzoic acids
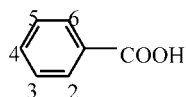
Salicylic acid   2 = OH

p-Hydroxybenzoic acid   4 = OH

Protocatechuic acid   3 = 4 = OH
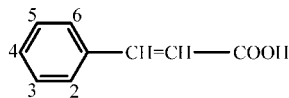
Gentisic acid   3 = 6 = OH

Gallic acid   3 = 4 = 5 = OH

Vanillic acid   3 = OCH_3, 4 = OH

3,4-Dimethoxybenzoic acid   3 = 4 = OCH_3

Syringic acid   3 = 5 = OCH_3, 4 = OH

Hydroxycinnamic acids

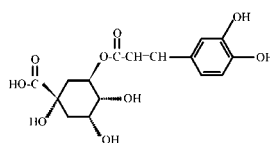*o*-Coumaric acid   2 = OH

m-Coumaric acid   3 = OH

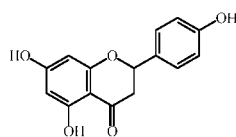*p*-Coumaric acid   4 = OH

Caffeic acid   3 = 4 = OH

Ferulic acid   3 = OCH_3, 4 = OH

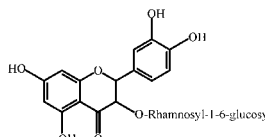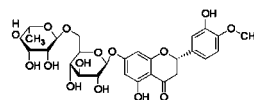Sinapic acid   3 = 5 = OCH_3, 4 = OH

Chlorogenic acid
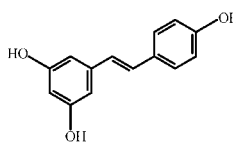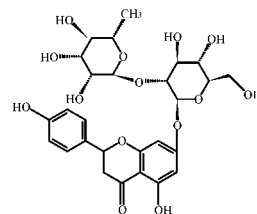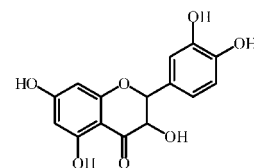
Flavonoids

Naringenin

Rutin

Hesperidin

Resveratrol

Naringin

Quercetin

Figure 1. Chemical structures of flavonoids and phenolic acids.

genesis in 3T3-L1 adipocytes. A murine 3T3-L1 cell line was used in this study due to it is widespread use as a cell model for adipose cell biology research over the course of several decades (*17*).

MATERIALS AND METHODS

Materials. Phenolic acids (salicylic, *p*-hydroxybenzoic, protocatechuic, gentisic, gallic, vanillic, 3,4-dimethoxybenzoic, syringic, *o*-coumaric, *m*-coumaric, *p*-coumaric, caffeic, ferulic, sinapinic, and chlorogenic acid), flavnoids (naringenin, rutin, hesperidin, resveratrol, naringin, and quercetin), dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin (INS), and oil red o were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum, and antibiotic mixture (penicillin-streptomycin) were purchased from the Invitrogen Co. (Carlsbad, CA). Polyvinyldifluoride (PVDF) membrane for Western blotting was obtained from Millipore (Bedford, MA). Anti-PPARγ antibody was obtained from Upstate (Lake Placid, NY). Anti-C/EBPα antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antiadiponectin antibody was obtained from BioVision (Mountain View, CA). Antileptin antibody was obtained from Chemicon (Temecula, CA). Antirabbit and antichicken secondary horseradish peroxidase antibodies were purchased from the Bethyl Laboratories, InC. (Montgomery, TX). Molecular mass markers for proteins were obtained from Fermentas Life Sciences (Burlington, Ontario, Canada). All other chemicals are reagent grade.

Cell Culture. 3T3-L1 preadipocytes (BCRC 60159) were purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). 3T3-L1 preadipocytes were planted into 6-well plates and maintained in DMEM supplemented with 10% bovine calf serum at 37 °C in a humidified 5% CO_2 incubator. Adipocytic differentiation was induced by the adipogenic agents (0.5 mM IBMX, 1 μM DEX, and 1 μM INS) that were added to culture medium. Afterwards, the medium was changed to normal culture medium and was freshly replaced every 48 h. The cells were harvested 8 days after the initiation of differentiation.

Oil Red O Staining of 3T3-L1 Adipocytes. Intracellular lipid accumulation was measured using oil red o. The oil red o working

A

B
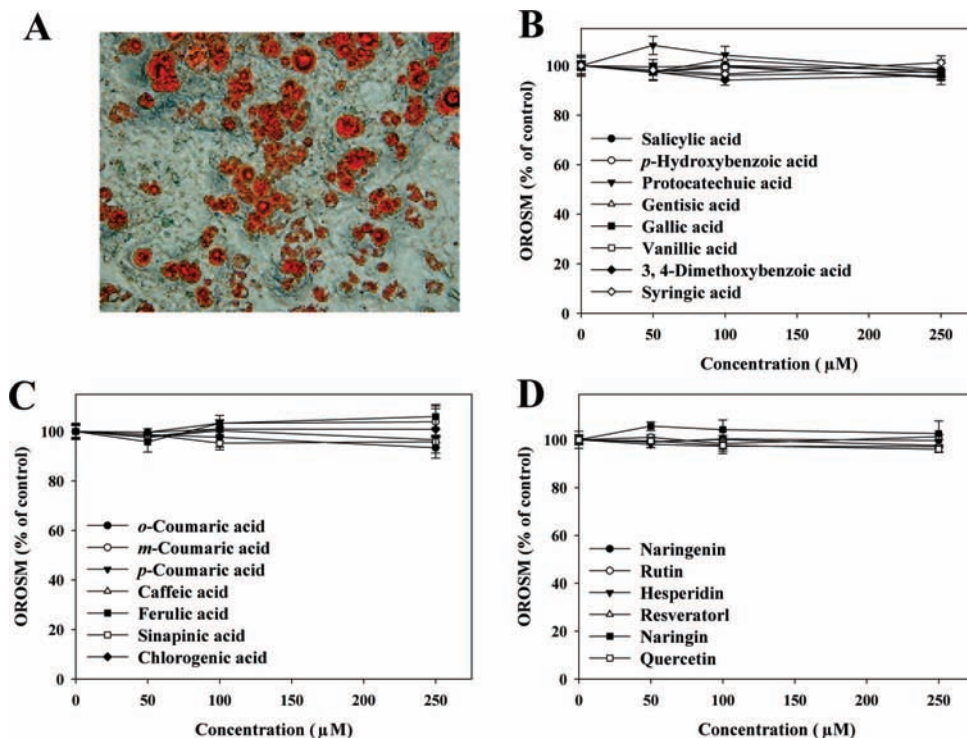
Figure 2. Effects of flavonoids and phenolic acids on oil red O stained material (OROSM) in 3T3-L1 adipocytes. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation and were stained with oil red O (**A**). Cells were treated with 0–250 μM of phenolic acids (**B** and **C**) and flavonoids (**D**) for 72 h at 37 °C in a humidified 5% CO_2 incubator. Cells were stained with oil red O. The reported values are the means $\pm$ SD ($n = 3$).

Table 1. Effect of Flavonoids and Phenolic Acids on the Inhibition of Intracellular Triglyceride in 3T3-L1 Adipocytes

compounds	inhibition (%)[a]
hydroxybenzoicacids	
salicylic acid	36.3 $\pm$ 5.9
p-hydroxybenzoic acid	nd[b]
protocatechuic acid	nd
gentisic acid	nd
gallic acid	29.2 $\pm$ 3.0
vanillic acid	43.5 $\pm$ 1.0
3,4-dimethoxybenzoic acid	nd
syringic acid	nd
hydroxycinnamic acids	
o-coumaric acid	61.3 $\pm$ 2.5
m-coumaric acid	25.3 $\pm$ 7.2
p-Coumaric acid	31.4 $\pm$ 4.1
caffeic acid	25.8 $\pm$ 3.3
ferulic acid	16.8 $\pm$ 1.1
sinapinic acid	2.3 $\pm$ 1.6
chlorogenic acid	25.1 $\pm$ 5.5
flavonoids	
naringenin	39.4 $\pm$ 7.8
rutin	83.0 $\pm$ 1.3
hesperidin	40.2 $\pm$ 3.2
resveratrol	41.8 $\pm$ 2.3
naringin	41.3 $\pm$ 8.4
quercetin	44.6 $\pm$ 3.9

[a] Inhibitions (%) are expressed as percentages of the inhibition of control at 0%. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were incubated with 250 μM of naturally occurring antioxidants for 72 h at 37 °C in 5% CO_2 incubator. The reported values are the means $\pm$ SD ($n = 3$). [b] nd = not detected.

Table 2. Effect of Flavonoids and Phenolic Acids on GPDH Activity in 3T3-L1 Adipocytes

compounds	inhibition (%)[a]
hydroxybenzoic acids	
salicylic acid	33.7 $\pm$ 6.2
p-hydroxybenzoic acid	5.6 $\pm$ 0.8
protocatechuic acid	3.9 $\pm$ 0.3
gentisic acid	4.5 $\pm$ 0.5
gallic acid	42.2 $\pm$ 1.9
vanillic acid	26.4 $\pm$ 5.7
3,4-dimethoxybenzoic acid	11.8 $\pm$ 1.5
syringic acid	13.7 $\pm$ 1.7
hydroxycinnamic acids	
o-coumaric acid	54.2 $\pm$ 6.9
m-coumaric acid	25.6 $\pm$ 4.2
p-coumaric acid	21.2 $\pm$ 3.6
caffeic acid	34.6 $\pm$ 4.3
ferulic acid	17.4 $\pm$ 5.9
sinapinic acid	15.3 $\pm$ 3.5
chlorogenic acid	32.0 $\pm$ 3.2
flavonoids	
naringenin	35.7 $\pm$ 1.4
rutin	66.8 $\pm$ 5.9
hesperidin	37.9 $\pm$ 4.6
resveratrol	36.8 $\pm$ 5.6
naringin	39.4 $\pm$ 5.6
quercetin	34.2 $\pm$ 2.6

[a] Inhibition (%) was expressed as the GPDH activity of control at 0%. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were incubated with 250 μM of naturally occurring antioxidants for 72 h at 37 °C in 5% CO_2 incubator. The reported values are the means $\pm$ SD ($n = 3$).
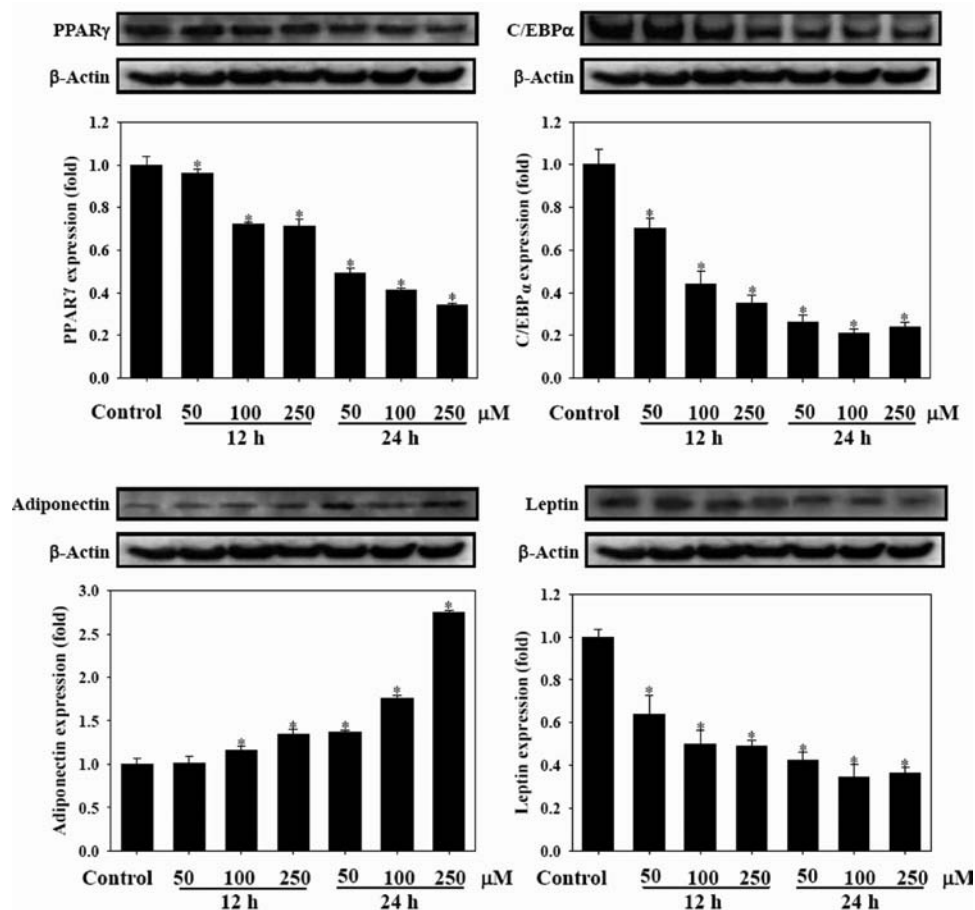
solution was prepared as described by Ramirez-Zacarias et al. (*18*). 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were incubated with 0–250 μM of flavonoids or phenolic acids for 72 h at 37 °C in a humidified 5% CO_2 incubator. Cells were washed twice with phosphate buffered saline (PBS, pH 7.4) and then fixed with 10% neutral formalin for at least 20 min at room

temperature. After the 10% neutral formalin was removed, 100% propylene glycol was added to each well for 3 min. Cells were decolorized with 60% propylene glycol before staining for 1 h with the oil red O working solution and then washed exhaustively with water. The staining dye of cells was extracted with isopropyl alcohol (1 mL/well) and measured spectrophotometrically at 510 nm in a FLUOstar Galaxy spectrophotometer (BMG Labtechnologies Ltd., Offenburg,

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Figure 3. Effect of *o*-coumaric acid on proteins levels of PPARγ, C/EBPα, adiponectin, and leptin in 3T3-L1 adipocytes. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were treated with 0–250 μM of *o*-coumaric acid for 12 and 24 h at 37 °C in a humidified 5% CO_2 incubator. The relative expression of PPARγ, C/EBPα, adiponectin, and leptin in 3T3-L1 adipocytes was quantified densitometrically using the software LabWorks 4.5, and calculated according to the reference bands of β-actin. Values are means for three replicated cultures and *$p < 0.05$ vs. control.

Germany). The oil red O-stained material (OROSM) was expressed on a per cell basis using the cell number determined from similar plates. The percentage of oil red O-stained material (OROSM, %) relative to control wells containing cell culture medium without compounds was calculated as $A_{510\ nm}$ [antioxidant]/$A_{510\ nm}$ [control] × 100.

Triglyceride Content. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were incubated with 250 μM of flavonoids or phenolic acids for 72 h at 37 °C in a humidified 5% CO_2 incubator. Cells were collected and lysed in lysis buffer (1% Triton X-100 in PBS). The total triglyceride content in cells was determined using a commercial triglyceride assay kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). The protein concentration was determined by using a BioRad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Inhibition (%) was expressed as percent decrease in triglyceride content against control (0%).

Glycerol-3-Phosphate Dehydrogenase Activity. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were incubated with 250 μM of flavonoids or phenolic acids for 72 h at 37 °C in a humidified 5% CO_2 incubator. Cells were washed twice carefully with ice-cold PBS on 3T3-L1 adipocytes, and lysed in 25 mM Tris/1 mM EDTA, pH 7.5 for the measurement of glycerol-3-phosphate dehydrogenase (GPDH) specific activity. GPDH activity was determined according to the procedure of Wise and Green (*11*). Protein concentration was determined by the BioRad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. Enzyme activity was expressed as units of activity/mg protein. Inhibition (%) was expressed as percent decrease in GPDH activity against control (0%).
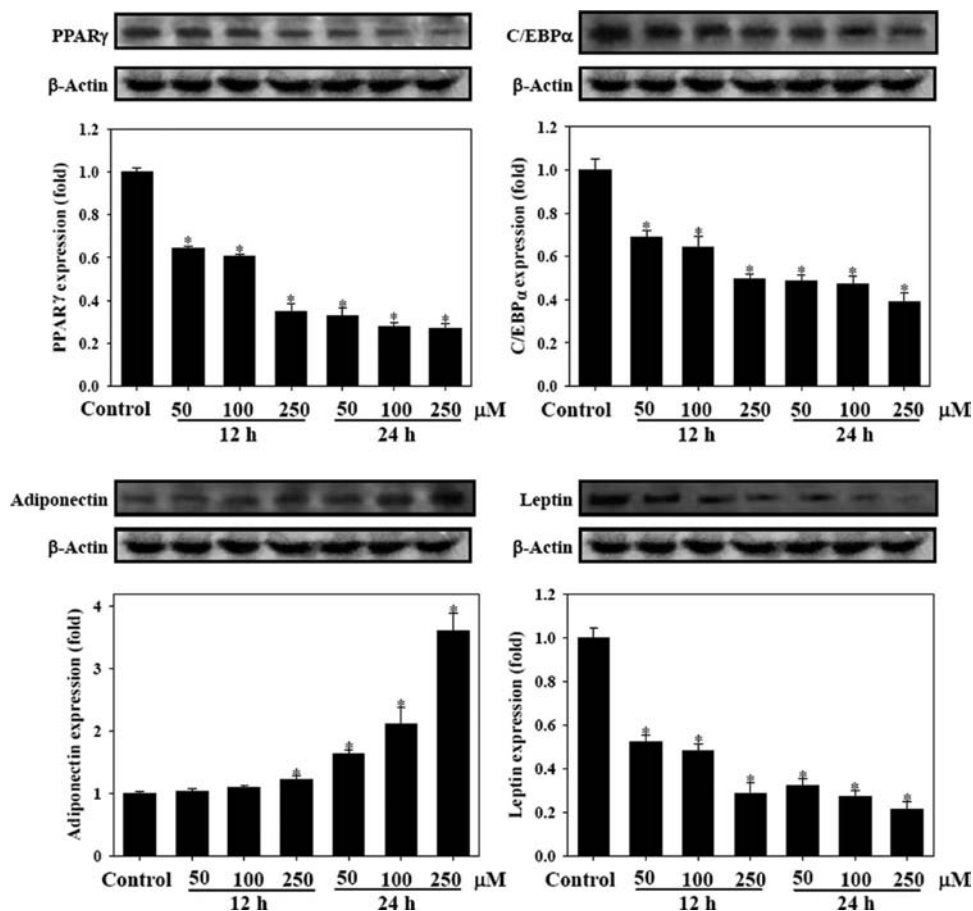
Western Blot Assay. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were incubated with 0–250 μM

of flavonoids or phenolic acids for 12 and 24 h at 37 °C in a humidified 5% CO_2 incubator. Cells were collected and lysed in ice-cold lysis buffer (20 mM tris-HCl (pH 7.4), 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin and 1 mM PMSF). The protein concentration was estimated with the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. Total protein (50–60 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel. The proteins in the gel were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in PBST (0.05% v/v Tween-20 in PBS, pH 7.2) for 1 h. Membranes were incubated with primary antibody at 4 °C overnight and then with secondary antibody for 1 h. Membranes were washed in PBST for 10 min three times between each step. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). The relative expression of proteins was quantified densitometrically using the software LabWorks 4.5 and calculated according to the reference bands of β-actin.

Statistical Analysis. Each experiment was performed in triplicate. The results are expressed as mean ± SD. Statistical analysis was performed using SAS software. Analysis of variance was performed using ANOVA procedures. Significant differences ($p < 0.05$) between means were determined by Duncan's multiple range tests.

RESULTS

Effects of Flavonoids and Phenolic Acids on the Content of Intracellular Triglyceride. Effects of flavonoids and phenolic acids on oil red O stained material (OROSM) in 3T3-L1

Figure 4. Effect of rutin on proteins levels of PPARγ, C/EBPα, adiponectin and leptin in 3T3-L1 adipocytes. 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were treated with 0–250 μM of rutin for 12 and 24 h at 37 °C in a humidified 5% CO_2 incubator. The relative expression of PPARγ, C/EBPα, adiponectin, and leptin in 3T3-L1 adipocytes was quantified densitometrically using the software LabWorks 4.5, and calculated according to the reference bands of β-actin. Values are means for three replicated cultures and *$p < 0.05$ vs. control.

adipocytes are shown in **Figure 2**. On the 8th day, the mature 3T3-L1 adipocytes accumulated many OROSM as indicated by the large intracellular droplets (**Figure 2A**). The OROSM showed that cell numbers in 3T3-L1 adipocytes was not influenced by the treatment with phenolic acids and flavonoids (**Figures 2B, C,** and **D**). The effects of flavonoids and phenolic acids on the inhibition of intracellular triglyceride in 3T3-L1 adipocytes are shown in **Table 1**. The results demonstrated that some phenolic acids and flavonoids caused an inhibition of intracellular triglyceride in the 3T3-L1 adipocytes. The data revealed that *o*-coumaric acid and rutin had the highest inhibition on intracellular triglycerides (61.3 and 83.0%, respectively) among the 15 phenolic acids and six flavonoids being tested.

Effects of Flavonoids and Phenolic Acids on GPDH Activity. The cytosolic enzyme GPDH appears to have an important role in the conversion of glycerol into triglyceride. GPDH occupies a central position in the triglyceride synthesis pathway, at the point where it branches from the glycolytic pathway (*11*). Effects of flavonoids and phenolic acids on GPDH activity in 3T3-L1 adipocytes are shown in **Table 2**. The results demonstrated that some phenolic acids and flavonoids caused the inhibition of GPDH activity in the 3T3-L1 adipocytes. Our data indicated that *o*-coumaric acid and rutin had the highest inhibition on GPDH activity (54.2 and 66.8%, respectively) among the 15 phenolic acids and 6 flavonoids being tested.

Effects of Flavonoids and Phenolic Acids on Adipocyte-Specific Protein Expression. Our data indicated that some flavonoids or phenolic acids have potential to inhibit intracellular

triglyceride and GPDH activity in 3T3-L1 adipocytes. *o*-Coumaric acid (phenolic acid) and rutin (flavonoid) had the greatest inhibitory effect in 3T3-L1 adipocytes. Here, we examined the effects of *o*-coumaric acid and rutin on protein expression of adipogenesis factors in 3T3-L1 adipocytes. PPARγ is known as a key station protein that is expressed early in the adipocyte differentiation of 3T3-L1 cells and prior to C/EBPα (*19*). Adiponectin is produced and secreted exclusively from adipocytes and plays a role in energy homeostasis and insulin sensitivity (*20*). Leptin plays a major role in the control of body fat storage through the regulation of food intake and total body energy expenditure (*21*). The effect of *o*-coumaric acid on the protein level of PPARγ, C/EBPα, adiponectin and leptin in 3T3-L1 adipocytes is shown in **Figure 3**>. The results showed that exposure of 3T3-L1 adipocytes to *o*-coumaric acid caused the down-regulation of PPARγ, C/EBPα and leptin as well as the upregulation of adopnectin in a time- and dose-dependent manner. Moreover, rutin also inhibited the expression of PPARγ, C/EBPα and leptin led to up-regulation of adiponectin at the protein level (**Figure 4**).

DISCUSSION

Over the past few decades, obesity has become a global epidemic in both developed and developing countries. It is characterized by an increased adipose tissue mass and is associated with high health risk (*3*). The prevalence of obesity and obesity-related disorders has led to major research interests

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in the influence of adipose tissue mass (*22*). The 3T3-L1 cell line is widely used as a model of adipocyte differentiation and adipose biology. Wang and Jones (*23*) indicated that the decreased adipocytic lipogenesis is one of the mechanisms of proposed antiobesity. In the present study, we focused on the effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes. This inhibitory effect resulted from the repression of adipocyte-specific protein expressions.

The goal of this research was to study the inhibition of adipogenesis and adipocyte differentiation with flavonoids and phenolic acids. The OROSM was expressed relative to the number of cells counted on comparable plates (*24*). However, the tested flavonoids and phenolic acids did not affect the cell number of 3T3-L1 adipocytes (**Figures 2B**, **C**, and **D**). Some reports indicated that CLA-treatment of rats did not decrease adipocyte number (*25*). We further investigated the effects of flavonoids and phenolic acids on the inhibition of intracellular triglyceride and GPDH activity in 3T3-L1 adipocytes. Fasting induces conversion of glycerol into triglyceride through an induction of several hepatic enzymes such as GPDH and glycerol kinase. Tomiyama et al. (*26*) indicated that the expression of GPDH is induced several fold upon conversion of preadipocytes to adipocytes, which is the predominant substrate for triglyceride synthesis in adipose tissue. Our data indicated that the exposure of 3T3-L1 adipocytes to some flavonoids and phenolic acids (*o*-coumaric acid and rutin) resulted in lower levels of intracellular triglycerides and GPDH than other compounds tested (**Tables 1** and **2**).

Adipose tissue is now known to produce and secrete a PPARγ and C/EBPα, which have roles in the early stage of adipocyte differentiation, because they are transcriptional factors for numerous genes (*27*). Some studies have addressed the important role that PPARγ plays in the regulation of insulin sensitivity and glucose homeostasis (*28*). The present experiment indicated that *o*-coumaric acid and rutin treatment inhibited the expression of PPARγ and C/EBPα protein levels (**Figures 3** and **4**), which demonstrated that compounds inhibited adipogenesis by affecting the transcriptional factor cascade upstream of PPARγ expression. Leptin (product of ob gene) is secreted from adipocytes, reduces food intake, and increases energy expenditure (*10*). Adiponectin is specifically expressed in white adipose tissues and is one of the most important adipocytokines. Adiponectin is an adipocytokine that has been shown to have antiatherogenic, anti-inflammatory and antidiabetic roles (*7*). In the present study, *o*-coumaric acid and rutin also inhibited the expression of leptin and then stimulated the up-regulation of adiponectin at the protein level (**Figures 3** and **4**). Adiponectin expression would, therefore, be regulated by PPARγ transcriptional activity (*29*).

Bioavailability refers to the extent to which a nutrient in a food constituent can be absorbed and used by the body after ingestion. Recent studies concerning the bioavailability of polyphenols are in agreement with their potential therapeutic effects. These health promoting effects have been mainly attributed to the content of polyphenols and plant secondary metabolites (*30*). Moreover, Azuma et al. (*31*) indicated that the fat content of a food constituent seems to affect the bioavailability of flavonols. In the present study, the concentrations of naturally occurring antioxidants were 0–250 μM. Our data presented here indicated that *o*-coumaric acid and rutin inhibited adipogenesis in 3T3-L1 adipocytes at 50 μM (Western blot assay). The range of concentrations used in the present study was consistent with those in many other studies on the effect

of phytochemicals on inhibiting adipogenesis in 3T3-L1 adipocytes (*32, 33*).

In conclusion, the inhibitory effects of flavonoids and phenolic acids on 3T3-L1 adipocytes, as indicated by the decrease in intracellular triglyceride content and GPDH activity have been elucidated. It appears to be mediated through the down-regulated expression of adipogenic transcription factors (PPARγ and C/EBPα) and adipocyte-specific proteins (leptin), and then the up-regulated expression of adiponectin. These results indicate that flavonoids and phenolic acids may play a role in the control of adipogenesis and they might have further implication in in vivo antiobesity effects.

ABBREVIATIONS USED

C/EBPα, CCAAT enhancer binding protein alpha; DEX, dexamethasone; DMEM, Dulbecco's modified eagle's medium; GPDH, glycerol-3-phosphate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine; INS, insulin; OROSM, oil red O-stained material; PBS, phosphate buffered saline; PPARγ, peroxisome proliferator-activated receptor-gamma; PVDF, polyvinyldifluoride.

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