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Active Compounds from *Lagerstroemia speciosa*, Insulin-like Glucose Uptake-Stimulatory/Inhibitory and Adipocyte Differentiation-Inhibitory Activities in 3T3-L1 Cells

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Seven ellagitannins, lagerstroemin (**1**), flosin B (**2**), stachyurin (**3**), casuarinin (**4**), casuariin (**5**), epipunicacortein A (**6**), and 2, 3-(*S*)-hexahydroxydiphenoyl-α/β-D-glucose (**7**), together with one ellagic acid sulfate, 3-*O*-methyl-ellagic acid 4′-sulfate (**8**), ellagic acid (**9**), and four methyl ellagic acid derivatives, 3-*O*-methylellagic acid (**10**), 3,3′-di-*O*-methylellagic acid (**11**), 3,4,3′-tri-*O*-methylellagic acid (**12**), and 3,4,8,9,10-pentahydroxydibenzo[b,d]pyran-6-one (**13**), were identified by the bioassay-directed isolation from the leaves of *Lagerstroemia speciosa* (L.) Pers. The chemical structures of these components were established on the basis of one- and two-dimensional NMR and high-resolution mass spectroscopic analyses. Other known compounds, including corosolic acid, gallic acid, 4-hydroxybenzoic acid, 3-*O*-methylprotocatechuic acid, caffeic acid, *p*-coumaric acid, kaempferol, quercetin, and isoquercitrin, were also isolated from the same plant. The obtained ellagitannins exhibited strong activities in both stimulating insulin-like glucose uptake (**1**−**5** and **7**) and inhibiting adipocyte differentiation (**1** and **4**) in 3T3-L1 cells. Meanwhile, ellagic acid derivatives (**10**−**13**) showed an inhibitory effect on glucose transport assay. This study is the first to report an inhibitory effect for methyl ellagic acid derivatives.

KEYWORDS: ***Lagerstroemia speciosa*; banaba; ellagitannins; glucose uptake; adipocyte differentiation**

INTRODUCTION

Lagerstroemia speciosa (L.) Pers. (Lythraceae) is a tree grown in tropical countries including the Philippines, Malaysia, India, southern China, and Australia. In the Philippines, this plant is commonly known as banaba, and its leaves have traditionally been used as an antidiabetic and weight loss herb (*1*−*3*). It has been reported that water and a methanolic extraction of banaba leaf possess potent hypoglycemic effects in vitro and in vivo (*4, 5*).

Previous chemical investigations indicated that *L. speciosa* contained terpenoids (*6, 7*), tannins (*8*−*10*), and ellagic acids (*11*). The corosolic acid and tannins were considered to be the active components responsible for the hypoglycemic activity of banaba (*4, 6, 12, 13*). It was observed that tannins showed an insulin-like glucose uptake-stimulatory effect by activating glucose transporter 4 (GLUT4), leading to the translocation of the GLUT4 from an intracellular vesicle pool to the plasma membrane (*5, 13*). Corosolic acid, however, showed no activity on this assay mode (*14*), although it was reported to induce GLUT4 translocation in vivo (*12*). Lagerstroemin, flosin B, and reginin A, the ellagitannins isolated from banaba, increased the rate of 2-deoxyglucose uptake of rat adipocytes (*15*). Gallotannins, such as tannic acid, possessed the same activity (*13, 16*).

GLUT4 is insulin dependent and is the major transporter used for uptake of glucose from the blood stream into muscle and fat tissues, so as to decrease the concentration of glucose in blood. Therefore, any components that activate GLUT4 have a potential to be developed as hyperglycemia-reducing and antidiabetic agents (*17, 18*). Antidiabetic drugs such as insulin, thiazolidinediones (TZDs), and other small insulin mimetic compounds have been reported to directly or indirectly enhance the insulin-GLUT4 signaling pathway (*19*−*23*).

In the marketplace, banaba extract is standardized to contain 1% or higher corosolic acid, and it is currently used as an herbal

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dietary supplement to reduce plasma blood sugar for diabetic people. We speculated that certain compound(s) in the banaba extract are responsible for the glucose transport stimulatory (GTS) activity, and either the same or different compound(s) are responsible for the antiadipogenic effect of the extract. Our approach was to use the glucose uptake assay to screen compounds for the GTS activity and to use the differentiation assay followed by the glucose uptake assay to screen for antiadipogenic compounds with the differentiation inhibitory activity. These assays were chosen based on the observations that overweight and obesity are closely associated with both adipocyte differentiation and type II diabetes and are a primary risk factor for the latter (*24, 25*). We report here the strong GTS activity of the isolated ellagitannins, **1**−**5** and **7**. We also observed that the methyl ellagic acid derivatives, **10**−**12**, as well as **13**, exhibited inhibitory effect on glucose transport. It is the first time that the in vitro inhibitory effect of banaba extract and the isolated compounds have been reported. At present, it remains unknown whether the observed inhibitory effect will affect the entire activity of banaba extract in vivo or not. The results of some compounds (**1**, **4**, **8**, **10**, and **12**) on adipocyte differentiation inhibitory activities in 3T3-L1 cells are also reported.

MATERIALS AND METHODS

General Experimental Procedures. Column chromatography was performed on the resins of Dowex Optipore L-285 (Supelco Co., Bellefonte, PA), MCI GEL CHP-20P, Diaion HP-20 (Mitsubishi Kasei Co.), Toyopearl HW-40 (Tosoh Co., Tokyo, Japan), Sephadex LH-20, and RP-18 (Sigma Chemical Co., St. Louis, MO), respectively. All of the solvents used were purchased from Fisher Scientific (Springfield, NJ). The ^{1}H and ^{13}C NMR spectra were recorded on an Inova-400 MHz (^{1}H at 400 MHz and ^{13}C at 100 MHz) instrument (Varian Inc., Palo Alto, CA) with CD_3OD, D_2O, and DMSO-d_6 as the solvent (Aldrich Chemical Co., Allentown, PA). The correlation spectra were obtained using standard gradient pulse sequences of Varian VNMR software and performed on four-nuclei PFG AutoSwitchable or PFG Indirect Detection probes. High-resolution fast atom bombardment−mass spectrometry (HRFAB-MS) was run on a JEOL HX-110 double focusing mass spectrometer. Both negative and positive electrospray ionization−mass spectrometric (ESI-MS) spectra were obtained on an LCQ ion trap (Thermo-Finnigan, San Jose, CA) mass spectrometer by setting the spray voltage to 3.5 kV and the capillary temperature at 200 °C. Both the sheath gas and the auxiliary gas were nitrogen and had flow rates of 80 and 10 units, respectively. A mass range of 50−2000 amu was scanned. Fourier transform infrared (FT-IR) was performed on a Perkin-Elmer spectrum BX system (PerkinElmer Instruments, Norwalk, CT). UV was acquired on a Cary 300 Bio UV−visible spectrophotometer (Varian). High-performance liquid chromatography (HPLC) analysis was performed on an Agilent 1100 LC Series using YMC ODS-AQ column (5 μm, 4.6 mm i.d. × 250 mm) with a flow rate of 1.0 mL/min. The solvent system consisted of a linear gradient that started with 5% (v/v) HPLC-grade acetonitrile in 0.1% triflouroacetic acid/water and increased to 20% acetonitrile over 40 min. At the end of the run, 100% of acetonitrile was allowed to flush the column for 5 min, and an additional 10 min of post run time was set to allow for equilibration of the column with the starting eluant. The UV detector was set at 254 nm wavelength, and the column temperature was ambient.

3T3-L1 fibroblasts were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's PBS (DPBS) were from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Insulin (IS), 3-isobutyl-1-methylxanthine (IBMX), and dexamethasone (DEX) were from Sigma Chemical. 2-Deoxy-D-[^{3}H] glucose and XK 50 column were from Amersham Pharmacia Biotech (Piscataway, NJ).

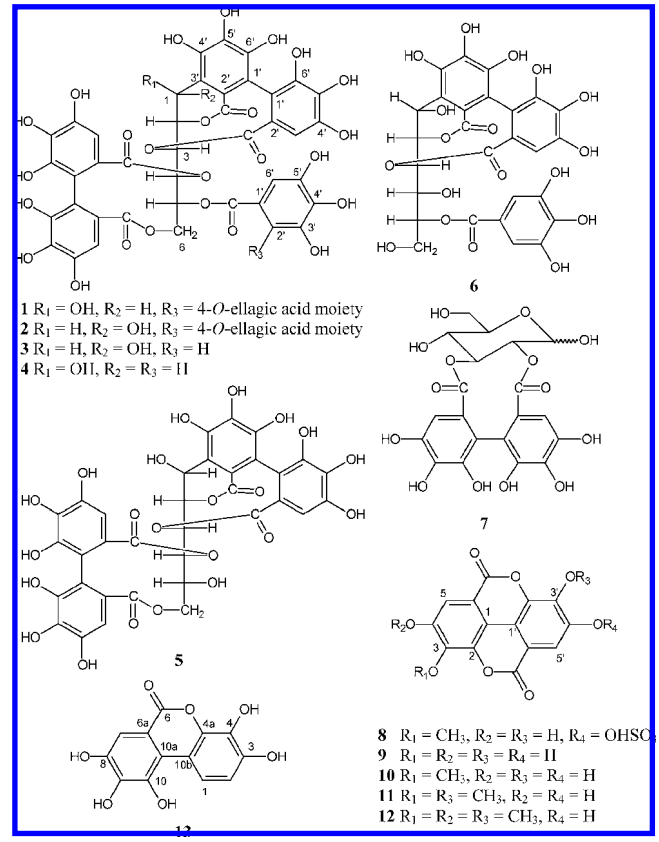

Figure 1. Chemical structures of ellagitannins and ellagic acid derivatives isolated from *L. speciosa*.

Plant Material. The dried banaba leaves of *L. speciosa* (L.) Pers. were purchased from the Philippines, and a voucher specimen (03D-5634) was deposited in the herbarium of Naturex.

Extraction and Isolation of Banaba Constituents. A total of 5 kg of the air-dried banaba leaf powder was percolated with 75 L of 60% acetone/water at 40 °C. The acetone was evaporated under vacuum, and the concentrated water solution (7 L containing 350 g of solid) was partitioned between ethyl acetate (7 L each) three times. The water solution containing 270 g of solid was column chromatographed over L-285 (8.0 i.d. × 70 cm) eluted with gradient aqueous MeOH system started from 10%, then 20, 30, 40, 60, and 80% MeOH, and to 100% MeOH last. The selected fractions were concentrated under reduced pressure and were further column chromatographed over Toyopearl HW-40 (6.0 cm i.d. × 20 cm) eluted with the same gradient aqueous MeOH system mentioned above. Each fraction was compared by HPLC analysis, and those elutes having similar HPLC patterns were combined. The combined fractions were subjected repeatedly to column chromatographies over MCI GEL CHP-20P and/or Sephadex LH-20 with aqueous water−MeOH system (started from H_2O and increased MeOH ratio to 20, 40, 60, 80, and 100% MeOH) to give **1**, 56 mg; **2**, 21 mg; **3**, 18 mg; **4**, 15 mg; **5**, 23 mg; **6**, 7 mg; **7**, 130 mg; **8**, 220 mg; **9**, 310 mg; **10**, 68 mg; **11**, 12 mg; **12**, 36 mg; and **13**, 66 mg. These structures (**Figure 1**) were determined by NMR, including two-dimensional correlation spectroscopic methods, and MS spectroscopic analyses, as well as comparison of the corresponding NMR data in literature.

Nuclear Magnetic Resonance Spectroscopy (NMR) and ESI-MS of Ellagitannins and Ellagic Acid Derivatives. *Lagerstroemin (**1**).* ^{1}H NMR (400 MHz, acetone-d_6 + D_2O): δ 7.47 (1H, s, galloyl H), 7.04, 7.02 (each 1H, s, valoneoyl H), 6.69, 6.48, 6.11 (each 1H, s, HHDP-H), 5.51 (1H,d, J = 4.8 Hz, H-1), 5.28 (1H, br s, H-3), 5.26 (1H, br s, H-4), 5.11 (1H, br s, H-5), 4.89 (1H, m, H-6), 4.52 (1H, d, J = 4.8 Hz, H-2), 3.61 (1H, d, J = 13.2 Hz, H-6). ^{13}C NMR (100 MHz, acetone-d_6 + D_2O): δ 169.3, 168.9, 168.7, 165.9 (HHDP COO^-), 161.0 (galloyl COO^-), 163.5, 160.5 (δ-lactone), 149.4, 148.4, 145.3, 145.1, 144.8, 144.8, 144.2, 142.9, 142.9, 142.8, 140.5, 139.9, 139.6, 138.7, 136.9, 136.6, 136.5, 135.4, 134.3 (HHDP and valoneoyl C-2′,

3′, 4′, 5′, 6′), 126.3, 125.9, 123.8, 119.1 (HHDP C-2′), 117.5, 115.6, 115.5, 115.4 (HHDP C-1′), 114.9, 114.3, 112.8, 112.6, 111.4, 109.5 (valoneoyl C-1′, -2′), 109.4 (galloyl C-6′), 108.6, 108.2 (valoneoyl C-3′), 107.4, 106.6, 104.6 (HHDP C-3′), 76.3 (C-2), 73.3(C-4), 69.5 (C-5), 68.7 (C-3), 65.9 (C-1), 64.4 (C-6) (*9*). ESI-MS (negative) *m/z* 1235 $[M - H]^-$, $C_{55}H_{32}O_{34}$.

Flosin B (**2**). ^{1}H NMR (acetone-d_6 + D_2O): δ 7.49 (1H, s, galloyl ArH), 7.06, 7.00 (each 1H, s, valoneoyl H), 6.73, 6.31, 6.14 (each 1H, s, HHDP H), 5.38 (1H, br s, H-4), 5.10 (1H, br s, H-3), 4.94 (1H, br s, H-1), 4.89 (1H, m, H-5), 4.69 (1H, br s, H-2), 4.55 (1H, m, H-6), 3.66 (1H, d, J = 13.2 Hz, H-6). ^{13}C NMR (acetone-d_6 + D_2O): δ 169.2, 168.5, 168.4, 164.0 (HHDP COO^-) 166.7 (galloyl COO^-), 161.1, 160.6 (δ-lactone), 149.3, 148.5, 146.4, 145.2, 144.8, 144.6, 144.0, 143.8, 143.0, 142.8, 140.5, 139.9, 139.7, 138.1, 136.8, 136.4, 136.3, 135.4, 134.4 (HHDP and valoneoyl C-2′, 3′, 4′, 5′, 6′), 126.6, 125.7, 124.0, 121.4 (HHDP C-2′), 118.1, 115.8, 115.3, 115.2, 115.0, 114.4, 113.0, 111.5, 109.1 (HHDP C-1′ and valoneoyl C-1′, C-2′), 112.7 (galloyl C-1′), 109.9 (galloyl C-6′), 108.8, 108.3 (valoneoyl C-3′), 107.8, 106.4, 104.8 (HHDP C-3), 80.8 (C-2), 72.3 (C-3), 71.1 (C-5), 69.9 (C-4), 64.1 (C-1), 64.0 (C-6) (*10*). ESI-MS (negative) *m/z*: 1235 $[M - H]^-$, $C_{55}H_{32}O_{34}$.

Stachyurin (**3**). ^{1}H NMR (acetone-d_6 + D_2O): δ 6.99 (2H, s, galloyl ArH), 6.78, 6.48, 6.41 (each 1H, s, HHDP H), 5.60 (1H, dd, J = 2.0, 8.8 Hz, H-4), 5.21 (1H, J = 3.2, 8.8 Hz, H-5), 4.85 (1H,d, J = 2.0 Hz, H-3), 4.82 (1H, br s, H-1), 4.76 (1H, br s, H-2), 4.71 (1H, m, H-6), 4.01 (1H, d, J = 12.8 Hz, H-6). ^{13}C NMR (acetone-d_6 + D_2O): δ 169.5, 169.1, 168.8, 166.8 (HHDP COO^-), 166.4 (galloyl COO^-), 146.7, 145.4, 145.3, 144.9, 144.2, 144.1, 143.7, 143.4 (HHDP C-4′,6′ and galloyl C-3′,5′), 139.2 (galloyl C-4′), 138.2, 136.5, 135.6, 134.7 (HHDP C-5′), 126.6, 125.9, 124.1, 121.6 (HHDP C-2′), 119.7 (galloyl C-1′), 117.7 (HHDP C-3′), 115.7, 115.4, 114.5 (HHDP C-1′), 110.0 (galloyl C-2′,6′), 108.3, 106.9, 105.2 (HHDP C-3′), 80.7 (C-2), 72.9 (C-4), 71. (C-3), 70.4 (C-5), 64.4 (C-6), 64.0 (C-1) (*26*). ESI-MS (negative) *m/z* 935 $[M - H]^-$, $C_{41}H_{28}O_{26}$.

Casuarinin (**4**). ^{1}H NMR (acetone-d_6 + D_2O): δ 6.99 (2H, d, J = 2.0 Hz, galloyl ArH), 6.74, 6.46, 6.41 (each 1H, s, HHDP H), 5.52 (1H, dd, J = 2.0, 5.2 Hz, H-1), 5.36 (1H, dd, J = 2.0, 9.0 Hz, H-4), 5.31 (1H, m, H-3), 5.22 (1H, m, H-5), 4.75 (1H, m, H-6), 4.55 (1H, br s, H-2), 4.02 (1H, d, J = 12.8 Hz, H-6). ^{13}C NMR (acetone-d_6 + D_2O): δ 169.5, 169.4, 168.7, 165.9 (HHDP COO^-), 166.3 (galloyl COO^-), 145.4 (galloyl C-3′, 5′), 145.6, 145.3, 144.9, 144.8, 144.2, 143.4, 143.0 (HHDP C-4′, 6′), 139.2 (galloyl C-4′), 138.6, 136.6, 135.5, 134.6 (HHDP C-5′), 126.6, 126.0, 124.0, 119.3 (HHDP C-2′), 119.8 (galloyl C-1′), 117.1 (HHDP C-3′), 115.8, 115.6, 115.5, 114.5 (HHDP C-1′), 109.8 (galloyl C-2′, 6′), 108.3, 106.7, 105.0 (HHDP C-3′), 76.6 (C-2), 73.6 (C-4), 70.6 (C-5), 69.2 (C-3), 66.2 (C-1), 64.3 (C-6) (*26*). ESI-MS (negative) *m/z* 935 $[M - H]^-$, $C_{41}H_{28}O_{26}$.

Casuariin (**5**). ^{1}H NMR (acetone-d_6 + D_2O): δ 6.64, 6.52, 6.35 (each 1H, s, HHDP ArH), 5.52 (1H, d, J = 5.2 Hz, H-1), 5.34 (1H, m, H-3), 4.95 (1H, dd, J = 8.0, 2.8 Hz, H-4), 4.57 (1H, dd, J = 4.8, 1.6 Hz, H-2), 4.53 (1H, dd, J = 12.4, 2.4 Hz, H-6), 4.03 (1H, d, J = 8.0 Hz, H-5), 3.85 (1H, d, J = 12.4 Hz, H-6). ^{13}C NMR (acetone-d_6 + D_2O): δ 170.3, 170.2, 168.9, 166.3 (COO^-), 145.7, 145.3, 144.9, 144.8, 144.1, 143.5, 143.2, 138.7, 136.4, 135.3, 134.5 (HHDP C-4′, 5′, 6′), 126.6, 126.4, 124.4, 119.4 (HHDP C-2′), 117.0 (HHDP C-3′), 115.7, 115.5, 115.4, 114.3 (HHDP C-1′), 108.3, 106.8, 104.9 (HHDP C-3′), 76.7 (C-2), 76.5 (C-4), 70.1 (C-3), 67.9 (C-6), 67.7 (C-5), 66.2 (C-1) (*26*). ESI-MS (negative) *m/z* 783 $[M - H]^-$, $C_{34}H_{24}O_{22}$.

Punicacortein A (**6**). ^{1}H NMR (acetone-d_6 + D_2O): δ 6.91 (2H, s, H-2, 6, galloyl ArH), 6.32 (1H, s, HHDP ArH), 5.54 (1H, d, 4.4, H-1), 5.18 (1H, brs., H-3), 4.78 (1H, m, H-2), 4.74 (1H, m, H-5), 4.02 (1H, d, J = 8.4 Hz, H-4), 3.81 (2H, m, H-6). ^{13}C NMR (acetone-d_6 + D_2O): δ 169.3, 167.0 (HHDP COO^-), 166.6 (galloyl COO^-), 145.3, 145.0 (2C), 143.3, 143.0, 138.6, 134.2 (HHDP C-4′, 5′, 6′ and galloyl C-3′, 4′, 5′), 127.3, 120.9 (HHDP C-2′), 119.4, 115.6 (HHDP C-1′), 110.0 (2C, galloyl C-2′, 6′), 117.7, 104.7 (HHDP C-3′), 77.1 (C-5), 73.1 (C-2), 71.5 (C-4), 69.8 (C-3), 66.5 (C-1), 60.2 (C-6) (*27*). ESI-MS (negative) *m/z* 633 $[M - H]^-$, $C_{27}H_{22}O_{18}$.

2,3-(S)-Hexahydroxydiphenoyl-(α/β)-D-glucose (**7**). ^{1}H NMR (acetone-d_6 + D_2O): δ 6.59, 6.58, 6.57, 6.55 (each 1H, s, HHDP ArH), β-form: 4.89 (1H, brs. H-3), 4.86 (1H, d, J = 2.0 Hz, H-1), 4.55 (1H, m, H-2), 3.64 (2H, m, H-6), 3.56 (1H, m, H-4), 3.42 (1H, m, H-5); α-form: 5.25 (1H, d, J = 3.6 Hz, H-1), 5.09 (1H, brs., H-3), 4.84 (1H, brs., H-2), 3.73 (1H, m, H-4), 3.70 (1H, m, H-5), 3.64 (2H, m, H-6). ^{13}C NMR (acetone-d_6 + D_2O): δ 170.8, 170.7, 170.1, 170.0 (COO^-), 144.9, 144.7, 143.7, 143.6, 135.7, 135.6 (HHDP C-4′, 5,′ 6′), 125.5, 125.1, 125.1, 125.0 (HHDP C-2′), 113.5, 113.5 (HHDP C-1′), 107.3, 107.0 (HHDP C-3′), β-form: 93.0 (C-1), 79.6 (C-3), 76.8 (C-2), 76.3 (C-4), 66.7 (C-5), 60.3 (C-6); α-form: 90.0 (C-1), 77.5 (C-3), 74.5 (C-2), 71.7 (C-4), 66.6 (C-5), 60.2 (C-6) (*28*). ESI-MS (negative) *m/z* 481 $[M - H]^-$, $C_{20}H_{18}O_{14}$.

3-O-Methylellagic Acid 4′-Sulfate (**8**). Amorphous white powder; UV (MeOH) λ_{max} (log λ) 249.2 (2.63), 346.6 (1.91), 358.6 (1.94) nm. IR (KBr): λ_{max} 3380, 2962, 2918, 2850, 2362, 2345, 1764, 1614, 1580, 1487, 1431, 1397, 1364, 1296, 1262 (S→O), 1167, 1101, 1035 (S→O), 972, 915, 894, 796 (S−O), 778, 756, 717, 677, 628, 583 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.12 (1H, s, H-5′), 7.53 (1H, s, H-5), 4.04 (3H, s, 3-OMe). ^{13}C NMR (DMSO-d_6): δ 158.8 (2C, C-7, 7′), 152.8 (C-4), 143.5 (C-4′), 142.5 (C-3′), 142.0 (C-2), 140.1 (C-3), 136.3 (C-2′), 117.0 (C-5′), 114.9 (2C, C-6, 1′), 113.3 (C-1), 111.4 (C-5), 106.9 (C-6′), 61.0 (3-OMe). HRFAB-MS *m/z* 396.9867 $[M]^+$ (calcd for $C_{15}H_8O_{11}S$, 396.9866).

Ellagic Acid (**9**). ^{1}H NMR (CD_3OD): δ 7.46 (2H, s, H-5, 5′). ^{13}C NMR (CD_3OD): δ 159.4 (2C, C-7, 7′), 148.1 (2C, C-4, 4′), 139.6 (2C, C-2, 2′), 136.5 (2C, C-3, 3′), 112.6 (2C, C-6, 6′), 110.3 (2C, C-5, 5′), 107.9 (2C, C-1, 1′) (*29*).

3-O-Methylellagic Acid (**10**). ^{1}H NMR (DMSO-d_6): δ 7.50 (1H, s, H-5), 7.44 (1H, s, H-5′), 4.02 (3H, s, 3-OMe). ^{13}C NMR (DMSO-d_6): δ 158.9 (C-7), 158.8 (C-7′), 152.1 (C-4), 148.3 (C-4′), 141.5 (C-2), 140.1 (C-3), 139.9 (C-3′), 136.1 (C-2′), 112.5 (C-1′), 112.2 (C-6), 111.9 (C-1), 111.3 (C-5), 110.2 (C-5′), 107.2 (C-6′), 60.9 (3-OMe) (*30*). ESI-MS (negative) *m/z* 315 $[M - H]^-$, $C_{15}H_8O_8$.

3,3′-Di-O-methylellagic Acid (**11**). ^{1}H NMR (CD_3OD): δ 7.32 (2H, s, H-5, 5′), 4.01 (6H, s, 3,3′-OMe). ^{13}C NMR (CD_3OD): δ 160.1 (2C, C-7, 7′), 152.5 (2C, C-4, 4′), 141.0 (2C, C-2, 2′), 140.6 (2C, C-3, 3′), 112.8 (2C, C-6, 6′), 112.2 (2C, C-5, 5′), 111.1 (2C, C-1, 1′), 61.8 (2C, C-3, 3′-OMe) (*29, 31*). ESI-MS (negative) *m/z* 329 $[M - H]^-$, $C_{16}H_{10}O_8$.

3,4,3′-Tri-O-methylellagic Acid (**12**). ^{1}H NMR (CD_3OD): δ 7.60 (1H, s, H-5), 7.51 (1H, s, H-5′), 4.05 (3H, s, 3′-OMe), 4.03 (3H, s, 3-OMe), 3.99 (3H, s, 4-OMe). ^{13}C NMR (CD_3OD): δ 158.6 (C-7), 158.4 (C-7′), 153.7 (C-4), 153.2 (C-4′), 141.5 (C-2), 141.1 (C-3), 140.6 (C-2′), 140.3 (C-3′), 113.5 (C-6), 112.5 (C-6′), 111.8 (C-5′), 107.4 (C-1), 107.4 (C-5), 61.3 (C-3-OMe), 60.9 (C-3′-OMe), 56.7 (C-4-OMe) (*29*). ESI-MS (negative) *m/z* 343 $[M - H]^-$, $C_{17}H_{12}O_8$.

3,4,8,9,10-Pentahydroxydibenzo[b,d]pyran-6-one (**13**). ^{1}H NMR (CD_3OD): δ 8.40 (1H, d, J = 8.8 Hz, H-1), 7.34 (1H, s, H-7), 6.73 (1H, d, J = 8.8 Hz, H-2). ^{13}C NMR (CD_3OD): δ 163.8 (C-6), 146.8 (C-3), 146.4 (C-8), 144.0 (C-10), 141.7 (C-9), 140.9 (C-4a), 133.3 (C-4), 119.1 (C-1), 118.5 (C-10a), 112.8 (C-10b), 112.4 (C-2), 112.0 (C-6a), 108.0 (C-7) (*32, 33*). ESI-MS (negative) *m/z* 275 $[M - H]^-$, $C_{13}H_8O_7$.

Cell Culture and Adipocyte Differentiation. Regarding differentiation assays, 3T3-L1 cells were maintained in DMEM and supplemented with 10% FBS at 37 °C in a 10% CO_2 cell incubator. Preadipocyte 3T3-L1 cells were grown in 12 well plates until 2 days postconfluence. The differentiation was induced as previously described (*5, 13*) by the addition of 1 mg/L insulin (IS), 0.5 mmol/L IBMX, and 0.25 mmol/L DEX. Two days after induction, the IS−IBMX−DEX-containing medium was replaced with a medium containing 1 mg/L IS. The medium was subsequently replaced again with fresh culture medium (DMEM supplemented with 10% FBS) after 2 days and eventually every other day thereafter. To determine the roles of compounds in adipocyte differentiation, different concentrations of individual compound were added to the medium along with IS−IBMX−DEX (BE−IS−IBMX−DEX). The compound-treated cells were assayed for their glucose uptake activity 9−12 days after the initiation of induction.

Glucose Uptake Activity Assay. The glucose uptake activity was analyzed by measuring the uptake of 2-deoxy-D-[^{3}H] glucose as described previously (*5, 13*). Briefly, confluent 3T3-L1 adipocytes grown in 12 well plates were washed twice with serum-free DMEM

and incubated with 1 mL of the same medium at 37 °C for 2 h. The cells were washed three times with Krebs−Ringer−Hepes (KRP) buffer and incubated with 0.9 mL of KRP buffer at 37 °C for 30 min. Insulin, banaba extracts, or compounds were then added, and adipocytes were incubated at 37 °C for 15 min. Glucose uptake was initiated by the addition of 0.1 mL of KRP buffer and 37 MBq/L 2-deoxy-D-[^{3}H] glucose and 1 mmol/L glucose as final concentrations. After 10 min, glucose uptake was terminated by washing the cells three times with cold PBS. The cells were lysed with 0.7 mL of 1% Triton X-100 at 37 °C for 20 min. The radioactivity retained by the cell lysates was determined by a scintillation counter. Assays were repeated at least once, and data were analyzed by comparison of experimental samples of the same treatment conditions as a group with negative control (untreated) samples, or positive (insulin-treated) samples, or with experimental samples with different treatment conditions. If below the negative control, the results were described as inhibitory.

Adipocyte Differentiation Assay. Undifferentiated 3T3-L1 preadipocytes were induced to differentiate into adipocytes as described above. The degree of the differentiation of the cells induced by different agents was evaluated by microscopic observation of lipid accumulation, as well as by the glucose uptake activities that they exhibited at the end of the induction as described above. The glucose uptake assay was chosen and performed here for the determination of the degree of adipocyte differentiation on the basis of the observation that differentiated adipocytes can be induced by insulin to take up glucose, whereas undifferentiated preadipocytes cannot (*5, 13*). In addition, a near linear relationship was found between the glucose uptake activity of differentiated adipocytes and the triglyceride contents of cells (unpublished data). Glucose uptake and adipocyte differentiation inhibition assays for each compound or fraction were performed at least twice. The result was normalized and expressed as a percentage by considering the activities of positive control, 1 nmol/mL of insulin, as 100 and negative control, methanol, as zero. Results were reported as means ± standard errors of means. Data were analyzed by comparing the compound-treated samples with untreated negative control samples or with insulin-treated positive samples using unpaired Student's *t* test and with one-way analysis of variance with Turkey's posthoc test, depending on the nature of the assays. The significance level was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Isolation and Structural Elucidation of Ellagitannins and other Compounds from Banaba. Following the GTS activity screening, it was found that the water layer that was obtained from partition between water and ethyl acetate of the original 60% acetone/water extract of banaba was more active than the extracts made from water, methanol, and ethyl alcohol alone. Further isolation and purification gave seven ellagitannins (**1**−**7**), ellagic acid and its derivatives (**8**−**13**), and some other known compounds, including corosolic acid, gallic acid, 4-hydroxybenzoic acid, 3-*O*-methylprotocatechuic acid, *p*-coumaric acid, acid, kaempferol, quercetin, and isoquercitrin.

Compound **8** was obtained as an amorphous powder. The molecular formula $C_{15}H_8O_{11}S$ was established by HRFAB-MS (observed, 396.9867; calcd, 396.9866, $[M]^+$). The existing sulfur atom was supported by the high isotopic intensity of molecular ions on negative ESI-MS at *m/z* 395 (100%) $[M - H]^-$, 396 (18%) $[M - H + 1]^-$, and 397 (8%) $[M - H + 2]^-$, as well as the fragment at *m/z* 315, attributed to the loss of the sulfate group $[M - SO_3H]^-$. The ^{1}H and ^{13}C NMR spectra combined with heteronuclear multiple-quantum coherence and distortionless enhancement by polarization transfer analyses showed that **8** was closely related to 3-*O*-methylellagic acid (**10**) (*30*). It was found that H-5′ (δ_H 8.12, 1H, s), C-3′ (δ_C 142.5), and C-5′ (δ_C 117.0) signals of **8** had downfield chemical shifts of the resonances due to the anisotropic effect of the sulfate group, indicating C-4′ as the position of the sulfate group attached. The product from acid hydrolysis of **8** was identified as 3-*O*-methyl-ellagic acid by NMR analysis. On the basis of the above evidence, **8** was elucidated as 3-*O*-methyl-ellagic acid 4′-sulfate, which is isolated from *L. speciosa* for the first time. Previously, a 4′-monosodium sulfate of ellagic acid-3-methyl ether was reported to have been isolated from *Frankenia laevis* L. (*34*). The structures of the other isolated compounds were elucidated on the basis of comparison with NMR to correspond to previously published data and mass spectroscopic analyses.

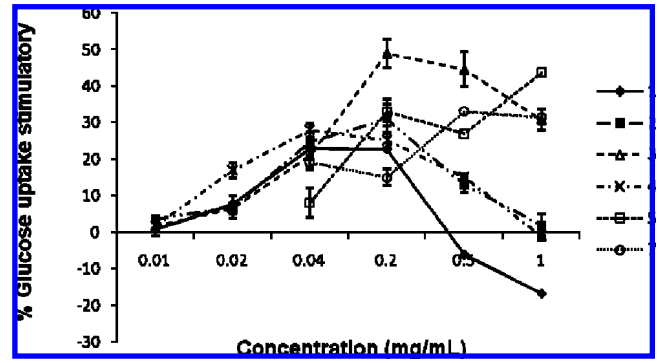

Figure 2. Glucose uptake activity of ellagitannins **1**−**5** and **7** in 3T3-L1 adipocytes.

Glucose Uptake Stimulatory Activity. Six isolated tannin constituents exhibited positive GTS activity (**Figure 2**). Particularly at the low concentration of 0.04 mg/mL, **1**−**4** and **7** showed strong GTS of 24, 25, 22, 29, and 20% equivalent to that of insulin at the concentration of 100 nM. The concentration to achieve maximal activity was 0.04 mg/mL for **1** (24%), 0.2 mg/mL for **2** (31%), 0.2 mg/mL for **3** (49%), 0.04 mg/mL for **4** (27%), 1.0 mg/mL for **5** (43%), and 0.5 mg/mL for **7** (34%). Among these tannins, **3** and **5** were the strongest two. Our results on the glucose transport activity of **1** and **2** are found to be similar at low compound concentrations but different at higher concentrations from those previously evaluated (*15*). This difference may be primarily due to the difference in how the adipocytes were used in the two studies. 3T3-L1 mouse adipocytes were used in our study, while rat adipocytes were used in Hayashi's study. Moreover, different concentration ranges were used in these two studies.

Tannins can be divided into two groups: hydrolyzable tannins (HTs) and proanthocyanidins (condensed tannins). Within the HTs are gallotannins and ellagitannins. The latter are the major tannins being isolated from banaba so far. It was reported that HTs were responsible for the stimulating activity of the uptake glucose transporter of banaba. In a study of the structure−activity relationship of antidiabetic gallotannins, it was found that only gallotanin having glucopyranose and xylopyranse cores showed the activity. In contrast, other polyol cores, such as D-galactopyranose, D-allopyranose, and D-mannopyranose were inactive, indicating a stereoconfiguration required for the GTS activity (*35*). In the seven ellagitannins isolated from banaba this time, in five of them, the D-glucose core is in its open chain. Compounds **1**−**5** and **7** exhibited strong GTS activity (do not have enough amount of **6** for GTS assay), indicating that the open chain configuration of glucose core maintained required stereochemistry for the GTS activity. Similar to insulin, the dose−response curves of ET showed that after reaching the maximum, the GTS activity tended to decrease when the concentration of ET was increased (*5, 13*). The GTS activity of **1** was found to be below the negative control level at high concentration, turning into an inhibitor on the GTS assay. The reason for this activity change is presently unknown. Other isolated compounds, including corosolic acid, gallic acid,

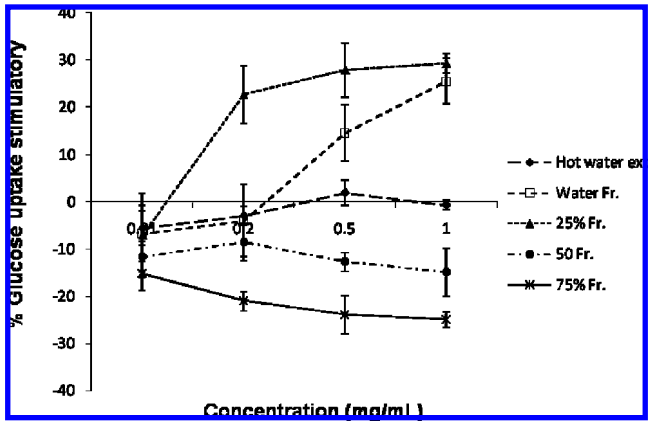

Figure 3. Glucose uptake activity for hot water extract of banaba and its four fractions in 3T3-L1 adipocytes. These four fractions separated components in banaba extract from polar to nonpolar, which were obtained from column chromatography eluted by decreasing solvent polarity.

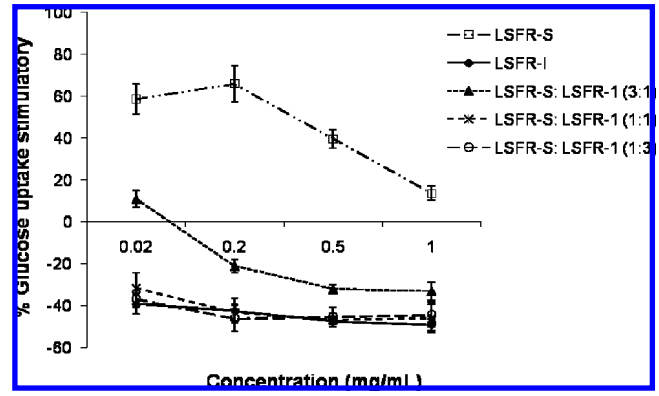

Figure 4. Glucose uptake inhibitory effect of banaba fractions in 3T3-L1 adipocytes. A strong GTS active fraction (LSFR-S) was mixed with a strong GTS inhibitory fraction (LSFR-I) in different proportions, and the GTS activity of LSFR-S was decreased with an increase of the portion of LSFR-I.

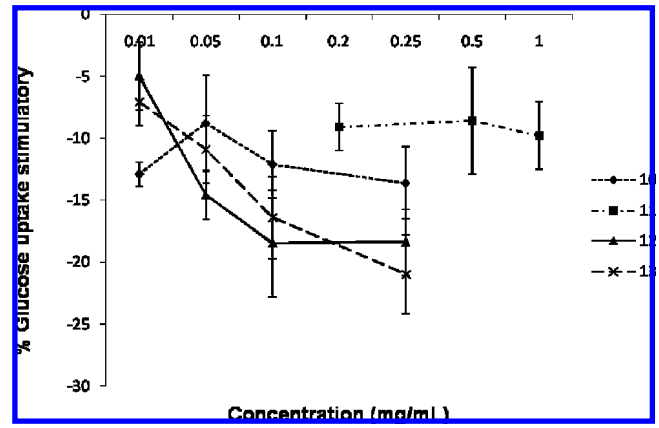

Figure 5. Glucose uptake inhibitory effect of ellagic acid derivatives **10**−**13** in 3T3-L1 adipocytes.

4-hydroxybenzoic acid, 3-*O*-methylprotocatechuic acid, caffeic acid, *p*-coumaric acid, ellagic acid, kaempferol, quercetin, and isoquercitrin, showed no activity in this study.

Glucose Uptake Inhibitory Activity. It was observed that the GTS activity of banaba extracts was decreased with the decreasing of polarity of extraction solvent. For example, the boiling water extract of banaba leaf showed no GTS activity. The boiling water extract was subjected to a column fractionation over Diaion HP-20 resin, and four equal amounts of eluent (water and 25, 50, and 75% of aqueous ethanol, respectively) were collected. The water and 25% ethanol elutes displayed positive GTS at a concentration of 1 mg/mL. The elute from 50% ethanol showed no activity, while the elute from 75% ethanol/water turned into inhibitory (result below negative control) on GTS result (**Figure 3**). The HPLC-UV chromatograms revealed ellagitannins **1** and **2** that appeared mainly in the 50 and 75% of ethanol fractions. Compounds **8** and **9** and many ellagic acid like derivatives were also the major peaks in these fractions. The observation that the higher ET content fraction showed no activity or inhibitory effect on the GTS assay (data not reported) suggested that some constituents in banaba possessed GTS inhibitory activity. To confirm this, a strong GTS active fraction (LSFR-S) and a strong GTS inhibitory fraction (LSFR-I) were prepared in a separate experiment (detail procedure not reported). LSFR-S and LSFR-I were mixed in different proportions of 3:1, 1:1, and 1:3, respectively, and each mixture was tested for GTS activity. As shown in **Figure 4**, the GTS activity of LSFR-S was decreased when the portion of LSFR-I was increased and was turned into inhibition of GTS when the ratio of LSFR-I became 1:1 and 1:3, respectively. The bioassay-guided isolation on a GTS inhibitory fraction led to the isolation and identification of three methyl ellagic acid derivatives (**10**−**12**) and one decarboxy ellagic acid, 3,4,8,9,10-pentahydroxydibenzo[b,d]pyran-6-one (**13**). All of these compounds showed a strong inhibitory effect on GTS assay (**Figure 5**). Hattori et al. reported that the glucose uptake action of lagerstroemin could be inhibited by wortmannin, a potent inhibitor of phosphoinositide 3-kinase (*36*). When one part of individual **10**−**13** mixed with three parts of **1**, respectively, they did not change the activity profile of **1** on GTS (data not shown). The mechanism of such an inhibitory effect of **10**−**13** is unknown. In screening ellagic acid derivatives for aldose reductase (AR) inhibitors, Terashima et al. reported that the sulfate group was the important substituent in increasing the AR inhibitory activity (*37*). In the present study, ellagic acid displayed no GTS activity, while its sulfate derivative **8** showed ambiguous results: two times moderate active and two times no activity. We have observed that **8** was unstable, and it lost sulfate easily to yield **10**, which is a GTS inhibitor. Therefore, we could not exclude the possibility that the inconsistent results for **8** obtained at different experimental period could be affected by the coexisted impurity **10**, the degraded product from **8**.

Activity of Inhibiting Adipocyte Differentiation. Banaba extract was reported to inhibit adipocyte differentiation in 3T3-L1 cells, and this activity was lost after tannin was removed (*5, 13*). In the present study, the activities of banaba extract and its isolated compounds on differentiation of preadipocytes into adipocytes induced by MDI cocktail were investigated. The result showed that **1** and **4** possessed a strong inhibitory effect on adipocyte differentiation. Compounds **8**, **10**, and **12** exhibited mild inhibitory activity (**Table 1**). The inhibition, as measured with glucose uptake assay, was found to correlate with the inhibition of differentiation. It was well-documented that only the differentiated adipocytes expressed high levels of the insulin receptor and GLUT4. The undifferentiated preadipocytes are incapable of glucose uptake due to lack of both IR and GLUT4 (*5, 13*). Preadipocytes that were treated with different compounds at different concentrations exhibited different degrees of morphological features of differentiated adipocytes such as cell shape (fibroblast-like vs round adipocyte-like) and intracellular triglyceride-containing vesicles (lacking fat vesicles vs with fat vesicles) (data not shown).

In conclusion, this study has demonstrated and confirmed that ellagitannins are one type of active constituent that is responsible

Table 1. Inhibitory Effect of Compounds **1**, **4**, **8**, **10**, and **12** on Adipocyte Differentiation[a]

	concentration (mg/mL)		
compound	0.04	0.1	0.5
1	100	100	100
4	100	100	100
8		64	99
10		38	90
12		62	

[a] 100% was assigned when there was complete inhibition of differentiation, and a value smaller than 100 indicates a partial inhibition.

for the antidiabetic properties of banaba. The GTS inhibitory effect of the methyl ellagic acid derivatives was reported here for the first time. The mechanism of this effect, and how it affects the entire hyperglycemia-correcting activities of banaba, is still unknown. Previous animal studies showed that banaba extract significantly reduced blood glucose levels in diabetic mice (*38*) and reduced the triglyceride content in the liver and the size of fat tissues in diabetic mice (*39*). In both studies, the long-term extract treatments did not induce significant side effects. In a small randomized clinical trial, the antidiabetic activity of Glucosol from banaba extract was observed in type II diabetic subjects (*40*).

Glucose uptake and antiadipogenic activities may be responsible for observed antidiabetic and antiobesity activities in studies using animals. Considering most antidiabetic pharmaceuticals cause weight gain in diabetic patients, this provides a potential to develop banaba-derived compounds as glucose-lowering and weight-controlling supplemental products, but more studies are necessary in the future.

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