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Bioactive Constituents, Metabolites, and Functions

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Bioactivity-guided isolation and identification of anti-adipogenic compounds in Shiya tea (leaves of *Adinandra nitida*)

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Abstract

Obesity is a worldwide epidemic contributing to a higher risk of developing maladies such as type 2 diabetes, heart disease, and cancer. Shiya tea (leaves of *Adinandra nitida*), a traditional Chinese tea, is widely consumed due to its palatable flavor and various curative effects, such as reducing blood pressure and blood lipids, as well as anti-inflammation, etc. However, no relevant research on the anti-obesity effects of Shiya tea has been reported. In particular, no health benefiting compounds, other than flavonoids, in Shiya tea have been reported. Thus, 3T3-L1 preadipocytes have been used as a bioactivity-guided identification model to verify the inhibitory effects of Shiya tea on adipogenesis, as well as identify anti-adipogenic compounds. Four triterpenoid saponins (**1**–**4**), including one new compound (**2α, 3α-dihydroxy-ursolic acid-28-O-β-D-glucopyranosyl ester,** compound **1**), and a flavonoid (**5**) have been identified using NMR (1D and 2D NMR) and LC−MS techniques. Compound **1**, the major anti-adipogenic constituent with an IC_{50} value of 27.6 μg/mL, has been identified for the first time in Shiya tea. In order to understand the structure-activity relationship, three hydrolytic compounds (**1s**, **2s**, and **5s**) were obtained to provide an inhibitory effect on lipid accumulation during 3T3-L1 adipocyte differentiation. The inhibitory effect of the triterpenoid (**1s**) possessing no sugar group decreased significantly, while the flavonoid (**5s**) also without a sugar group showed increased activity. In addition, the hydroxyl group position may also play a role in inhibitory efficacy.

KEYWORDS: Shiya tea, anti-adipogenesis, bioactivity-guided isolation, triterpenoid saponins, structure-activity relationship

INTRODUCTION

Obesity is a worldwide epidemic contributing to a higher risk of developing maladies such as type 2 diabetes, heart disease, and cancer.[1-3] According to the World Health Organization (WHO), nearly two billion adults worldwide are overweight and over 500,000,000 are obese.[4] Previously, only five drugs have been approved by the FDA to treat obesity, but reports indicate various side effects from their usage.[5] Therefore, searching for alternative therapies or using natural resources to prevent obesity with little or no side effects has become a necessity.

Adinandra nitida (Theaceae), a shrub or tree, is mainly distributed in the southern part of China, such as Guangdong, Guangxi, Guizhou and Yunnan Provinces.[6] The leaves of *A. nitida*, have long been consumed as a traditional tea (Shiya tea) beverage in China, [6, 7] due to its palatable flavor and many curative effects, such as reducing blood pressure and blood lipids, as well as anti-inflammation, etc.[8,9] Previous studies indicated that Shiya tea is rich in flavonoids, such as camellianin A, camellianin B, and apigenin, with great radical scavenging ability for 1,1-diphenyl-2-picrylhydrazyl (DPPH) and antitumor activities.[7,8] Flavonoids in Shiya tea are considered the major active and functional chemical constituents.[10-13] However, there has been no studies indicating additional compounds, other than flavonoids, in Shiya tea demonstrate any bioactivities, in particular anti-obesity effects. In order to explore other potential bioactive compounds in Shiya tea, as well as potential to prevent obesity, its inhibitory effects of adipogenesis using 3T3-L1 preadipocytes has been conducted, and the bioactivity-guided identification of anti-adipogenic compounds has also been performed. Consequently, bioactivity-guided isolation of the methanol extract led to the isolation and identification of four triterpenoid saponins (**1**–**4**), including one new compound (**1**), and a flavonoid (**5**) using

NMR (1D and 2D NMR) and LC-MS techniques. The new triterpenoid saponin (**1**), the major active constituent in Shiya tea, reduced intracellular lipid accumulation in 3T3-L1 adipocytes. The structure-activity relationship of triterpenoid saponins is also discussed.

MATERIALS AND METHODS

Materials. Analytical (250 μm thickness, 2−25 μm particle size) TLC plates were purchased from Macherey-Nagel company (Macherey-Nagel, Germany). Silica gels for column chromatography (100−200 or 200−300 mesh, (Thermo Scientific, CA, USA) and Sephadex LH-20 (Thermo Scientific, CA, USA) were obtained from Thermo Scentific. Methoxyamine, N-methyl-N-(trimethylsily)trifluoracetamide, and pyridine were used for preparing derivative of the glucose (Sigma-Aldrich, USA). LC-MS grade acetonitrile, water, and formic acid and ACS-grade n-hexane, ethyl acetate, acetone, chloroform and methanol were purchased from Sigma-Aldrich. Shiya tea samples were produced in Zhaoping of Guangxi Province, China in May 2018.

LC−MS Analysis. LC−MS analysis was conducted using a Vanquish UHPLC system (UHPLC+ focused pump, autosampler, and column compartment) coupled with a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) via an electrospray ionization (ESI) interface. Chromatographic separation for crude extract and isolates were performed using an ACQUITY UPLC BEH C18 column (150 mm × 2.1 mm id, 5 μm, Waters, Milford, MA, USA). The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The sample was eluted with the following linear gradient: 25–65% B from 0–12 min, 65–98% B from 12–15 min, and maintain at 98% from 15–22 min. The flow rate was 0.2 mL/min, and the injection volume was set at 2 μL. The

column temperature was maintained at 40 °C. The MS parameters were as follows: negative ionization mode, capillary temperature of 250 °C, sheath gas (N_2) flow rate of 45 psi, aux gas (N_2) flow rate of 10 psi, ion spray voltage of 2.5 kV, and S-lens RF level of 50. Full MS scans in the FT cell were acquired in the range of *m/z* 65−975 with a mass resolution of 70,000, AGC target of 1,000,000 and maximum IT of 246 ms. The MS/MS experiments were set as data dependent scan (dd-MS^2) with a mass resolution of 17,500, AGC target of 200,000 and maximum IT of 54 ms. The isolation window was set at *m/z* 1.0, and the stepped collision energy was used at 20, 40 and 60 eV. Xcalibur (Ver. 4.0) was employed for data collection and analysis.

NMR Analysis. ^{1}H (600 or 400 MHz), ^{13}C (150 or 100 MHz), ^{1}H−^{1}H COSY (correlation spectroscopy), ^{1}H−^{13}C HMQC (heteronuclear multiple quantum correlation), ^{1}H−^{1}H ROESY (rotating frame overhauser effect spectroscopy), and HMBC (heteronuclear multiple bond correlation) NMR spectra were acquired on a Bruker 600 MHz instrument. Compounds **1**–**4**, **1s**, and **2s** were analyzed in CD_3OD, while compounds **5** and **5s** were measured in pyridine-d_5.

Purification of Compounds 1−5. Shiya tea (4.0 kg) was powdered and soaked in MeOH (20 L) three times at 25 °C. The combined extract was filtered and concentrated with a rotary evaporator to yield crude extract (0.7 kg), which was suspended in water and then extracted with EtOAc (3 × 5 L) resulting in the corresponding extracts: EtOAc (200 g) and water layer (500 g). The anti-adipogenic effects were evaluated by measuring inhibition of adipogenic differentiation in 3T3-L1 preadipocytes. The active EtOAc fraction was applied to a silica gel CC (7.62 cm × 65 cm id) and eluted with petroleum ether/acetone (50:1, 20:1, 10:1, 5:1, 2:1, 1:1, and 0:1 v/v, each 25 L) to create four fractions (Fr. 1–Fr.4), in which only Fr. 3 showed better anti-adipogenic activity. Then, Fr. 3 (40 g) was subjected to an ODS CC (6.35 cm × 48

cm id, MeOH/H_2O, 40, 50, 60, 70, 80, and 100% v/v, each 300 mL) resulting in five fractions (Fr. 3A–Fr.3E), in which only Fr.3D was active. Next, Fr.3D (20 g) was fractionated using a gradient of CH_2Cl_2/MeOH (50:1, 30:1, 10:1 v/v, each 3 L) to obtain subfractions. Fr. 3D.1–Fr.3D.5. Fr.3D.1 (7 g) was further purified on a silica gel CC using CH_2Cl_2/acetone (3:2, 1:1 v/v, each 10 L) to furnish **1** (2.0 g) and **2** (1.0 g). Fr.3D.2 (4 g) was subjected to a Sephadex LH-20 CC (145 cm × 4.5 cm id) flushed with MeOH giving rise to five subfractions (Fr. 3D.2.1–Fr. 3D.2.5), and Fr.3D.2.3 was then purified by a semi-preparative HPLC (52% MeOH/H_2O for 50 min, v/v, 4 mL/min) with an X-bridge column (250 mm × 20 mm, 5 μm) to afford **3** (200 mg) and **4** (210 mg) with retention times of 37.0 and 44.5 min, respectively. Fr.3D.5 (5 g) was repeatedly recrystallized in EtOAc to afford **5** (3.0 g).

2α, 3α-Dihydroxy-ursolic acid-28-O-β-D-glucopyranosyl ester (1). White, amorphous powder; $[\alpha]_D^{25}$ +27 (c 0.4, MeOH); IR ν_{max} 3397, 2939, 2864, 1753, 1458, 1379, 1067, 1027, 605, 583 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR-ESI-MS: *m/z* calcd. for $C_{37}H_{59}O_{11}$ [M + HCOO]$^-$ 679.4057 found 679.4064.

Kajiichigoside F1 (2). White amorphous powder; HR-ESI-MS: *m/z* calcd. for $C_{37}H_{59}O_{12}$ [M + HCOO]$^-$ 695.4007 found 695.4014. ^1H NMR (400 HMz, CD_3OD): δ_H 0.79 (s, 3H), 0.89 (s, 3H), 0.95 (d, 3H, *J* = 5.2 Hz, H-30), 1.01 (s, 3H), 1.01 (s, 3H), 1.23 (s, 3H), 1.37 (s, 3H), 3.70, (dd, 1H, *J* = 11.8, 4.2 Hz, H-6a), 3.82, (dd, 1H, *J* = 11.8, 4.2 Hz, H-6b), 3.95, (dt, 1H, *J* = 11.8, 3.5 Hz, H-2), 5.33 (t, 1H, *J* = 2.9 Hz, H-12), 5.34 (d, 1H, *J* = 8.6 Hz, H-1'); ^{13}C NMR (100 HMz, CD_3OD): δ_C 15.2 (CH_3, C-30), 15.6 (CH_3, C-25), 16.3 (CH_3, C-26), 17.9 (CH_2, C-6), 21.1 (CH_3, C-24), 23.4 (CH_2, C-11), 25.1 (CH_3, C-27), 25.7 (CH_2, C-16), 25.8 (CH_2, C-21), 27.9 (CH_3, C-29), 28.3 (CH_2, C-15), 29.3 (CH_3, C-23), 32.6 (CH_2, C-7), 36.9 (CH_2, C-22), 38.0 (C, C-10), 38.1 (C, C-4), 40.0 (C, C-8), 41.2 (C, C-14), 41.4 (CH, C-20), 41.6 (CH_2, C-1), 46.8

(CH, C-9), 47.9 (C, C-17), 48.1 (CH, C-5), 53.6 (CH, C-18), 61.0 (CH, C-6'), 65.8 (CH, C-2), 69.7 (CH, C-4'), 72.5 (CH, C-2'), 72.3 (CH, C-19), 76.9 (CH, C-5'), 77.2 (CH, C-3'), 78.7 (CH, C-3), 94.4 (CH, C-1'), 128.2 (CH, C-12), 138.3 (C, C-13), 177.1(C, C-28)

Glucosyl tormentate (3). White amorphous powder; HR-ESI-MS: *m/z* calcd. for $C_{37}H_{59}O_{12}$ $[M + HCOO]^-$ 695.4007 found 695.4014; ^{1}H NMR (400 HMz, CD_3OD): δ_H 0.80 (3H, s), 0.83 (3H, s), 0.95 (3H, d, J = 6.0 Hz), 1.03 (3H, s), 1.22 (3H, s), 1.35 (3H, s), 5.33 (1H, s, H-12), 5.34 (1H, d, J = 8.5, H-1 of glucose); ^{13}C NMR (100 HMz, CD_3OD): δ_C 15.2 (CH_3, C-30), 15.7 (CH_3, C-26), 16.0 (CH_3, C-25), 16.2 (CH_3, C-24), 18.3 (CH2, C-6), 23.3 (CH2, C-11), 23.4 (CH_3, C-27), 25.1 (CH_2, C-16), 25.7 (CH_2, C-21), 25.8 (CH_3, C-29), 27.9 (CH_2, C-15), 28.2 (CH_3, C-23), 32.7 (CH_2, C-7), 36.9 (CH_2, C-22), 37.8 (C, C-10), 39.1 (C, C-8), 39.9 (C, C-4), 41.3 (CH, C-20), 41.6 (C, C-14), 46.8 (CH, C-9), 48.0 (CH_2, C-1), 48.2 (C, C-17), 53.6 (CH, C-18), 55.3 (CH, C-5), 61.1 (CH, C-6'), 68.1 (CH_2, C-2), 69.7 (CH, C-4'), 72.2 (C, C-19), 72.5 (CH, C-2'), 76.9 (CH, C-5'), 77.2 (CH, C-3'), 83.2 (CH, C-3), 94.4 (CH, C-1'), 138.4 (CH, C-12), 128.1 (C, C-13), 177.1 (C, C-28).

2α,3α,19α-Trihydroxy-olean-12-en-28-oic acid-28-O-β-D-glucopyranoside (4). White amorphous powder; HR-ESI-MS: *m/z* calcd. for $C_{37}H_{59}O_{12}$ $[M + HCOO]^-$ 695.4007 found 695.4019. ^{1}H NMR (400 HMz, CD_3OD): δ_H 0.77 (s, 3H, H-24), 0.83 (s, 3H, H-30), 0.96 (d, 3H, H-25), 0.97 (d, 3H, H-29), 1.02 (s, 3H, H-26), 1.03 (s, 3H, H-23), 1.31 (s, 3H, H-27), 3.70, (dd, 1H, J = 11.8, 4.2 Hz, H-6a), 3.84, (dd, 1H, J = 11.8, 4.2 Hz, H-6b), 3.95, (dt, 1H, J = 11.8, 3.5 Hz, H-2), 5.35 (t, 1H, J = 2.9 Hz, H-12), 5.40 (d, 1H, J = 8.6 Hz, H-1'); ^{13}C NMR (400 HMz, CD_3OD): δ_C 15.6 (CH_3, C-25), 16.0 (CH_3, C-26), 16.4 (CH_2, C-6), 18.3 (CH_3, C-24), 23.5 (CH_2, C-11), 23.6 (CH_3, C-30), 23.8 (CH_3, C-27), 27.2 (CH_2, C-16), 27.9 (CH_3, C-29), 28.0 (CH_2, C-21), 28.0 (CH_2, C-15), 31.9 (CH_3, C-23), 32.4 (CH_2, C-7), 34.6 (CH_2, C-22), 38.0

(C, C-20), 38.2 (C, C-9), 39.1 (C, C-10), 39.1 (C, C-4), 39.5 (C, C-8), 41.3 (C, C-14), 43.7 (CH_2, C-1), 45.7 (CH, C-18), 46.7 (C, C-17), 48.4 (CH, C-5), 61.0 (CH, C-6'), 68.1 (CH, C-2), 69.7 (CH, C-4'), 72.5 (CH, C-2'), 77.0 (CH, C-5'), 77.3 (CH, C-3'), 81.0 (CH, C-3), 83.2 (CH, C-19), 94.4 (CH, C-1'), 123.4 (CH, C-12), 142.6 (C, C-13), 177.2 (C, C-28).

Camellianin A (5). White amorphous powder; HR-ESI-MS: *m/z* calcd. for $C_{29}H_{31}O_{15}$ $[M-H]^-$ 619.1663 found 619.6154; ^{1}H NMR (400 HMz, pyridine-d_5): δ_H 1.66 (3H, d, J = 6.0 Hz, H-6'''), 1.90 (3H, s, H-8''), 6.41 (1H, s, H-6), 6.73 (1H, s, H-3), 6.97 (1H, s, H-8), 7.18 (2H, d, J = 8.6 Hz, H-3' and H-5'), 7.80 (2H, d, J = 8.6 Hz, H-2' and H-6'); ^{13}C NMR (100 HMz, CD_3OD): δ_C 20.1 (CH_3, C-6'''), 21.8 (CH_3, C-8''), 65.1 (CH_2, C-6''), 71.4 (CH, C-5'''), 72.8 (CH, C-2'''), 73.8 (CH, C-4'''), 73.9 (CH, C-3'''), 75.9 (CH, C-2''), 76.3 (CH, C-5''), 78.7 (CH, C-3''), 80.3 (CH, C-4''), 98.8 (C, C-8), 100.6 (CH, C-1'''), 102.2 (CH, C-6), 102.6 (CH, C-1''), 108.2 (CH, C-3), 109.8 (C, C-10), 118.0 (CH, C-3' and C-5'), 123.7 (C, C-1'), 129.8 (CH, C-2' and C-6'), 159.9 (C, C-5), 161.3 (C, C-9), 162.8 (C, C-7) , 164.0 (C, C-4'), 165.5 (C, C-2), 172.0 (C, C-7''), 178.5 (C, C-4).

Verification of D-glucose isomer for compound 1. Compound **1** (2 mg) was heated with 2 M HCl (water, 2 mL) at 80 ℃ for 2 h. After cooling, the reaction mixture was extracted with $CHCl_3$ (3 × 2 mL). The aqueous layer was then evaporated to dryness using MeOH until neutral.[14] The dried residue was mixed with 90 μL of methoxyamine hydrochloride (20 mg/mL in pyridine) and vortexed for 2 h at room temperature. Afterward, 240 μL of N-methyl-N-(trimethylsily)trifluoracetamide were added to the sample, and the mixture was vortexed for an additional 30 min.[15]

GC-MS analysis of the derivatized sample was conducted using a Clarus 680 GC (Perkin-Elmer, Inc., Waltham, MA) equipped with a Clarus SQ 8T MS in EI mode with 70 eV. The

sample solution (1 μL) was injected into an Rxi-5 MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness). The oven temperature was programmed at 70 °C for 5 min, rised to 270 °C at a rate of 4 °C/min, and then increased to 320 °C (5 min hold) at a rate of 20 °C/min. The flow rate of the carrier gas, helium, was 1.1 mL/min. The configuration of D-glucose for compound **1** was established by comparing the retention time and molecular weight of the corresponding derivative to that of the standard, D-glucose.[15]

Hydrolysis of compounds 1, 2 and 5 for structure-activity study. A solution of **1** (30 mg) or **2** (30 mg) in 5 mL MeOH was added to 1 M HCl (5 mL) and stirred at 70 °C for 2 h. The mixture solution was next added to 10 mL water and then extracted with ethyl acetate (3 × 30 mL). The organic layer was concentrated using a rotary evaporator and then purified using flash chromatography with a gradient of CH_2Cl_2/acetone (9:1, 8:2, 6:4 v/v, each 50 mL) to obtain the pure compounds **1s** (20 mg) or **2s** (21 mg).[14]

2 M HCl (water, 3 mL) and compound **5** (30 mg) in MeOH (3 mL) were heated at 80 ℃ for 2 h. After cooling, the reaction mixture was extracted with ethyl acetate (3 × 10 mL). The organic layer was concentrated using a rotary evaporator, which then was purified using flash chromatography with a gradient of CH_2Cl_2/acetone (8:2, 6:4:1 v/v, each 50 mL) to yield **5s** (18 mg). The spectroscopic data of compounds **1s**, **2s**, and **5s** are listed below. The compounds **1**, **2**, and **5** and their hydrolyzed compounds were applied to 3T3-L1 cell cultures to understand the structure-activity relationship.

2α, 3α-Dihydroxy-ursolic acid (1s). White amorphous powder; HR-ESI-MS: *m*/*z* calcd. for $C_{30}H_{47}O_4$ $[M - H]^-$ 471.3474 found 471.3484; ^1H NMR (400 HMz, CD_3OD): δ_H 0.86 (s, 3H, H-26), 0.89 (s, 3H, H-24), 0.91 (br s, 3H, H-29), 1.00 (d, 3H, J = 6.8 Hz, H-30), 1.01 (s, 3H, H-23), 1.03 (s, 3H, H-25), 1.16 (s, 3H, H-27), 2.23 (d, 1H, J = 11.0 Hz, H-18), 3.34 (m, 1H,

H-3), 3.95 (dt, 1H, J = 11.8, 3.5 Hz, H-2), 5.26 (t, 1H, J = 3.6 Hz, H-12); ^{13}C NMR (100 HMz, CD_3OD): δ_C 15.6 (CH_3, C-25), 16.3 (CH_3, C-29), 16.4 (CH_3, C-26), 17.7 (CH_2, C-6), 20.2 (CH_3, C-30), 21.1 (CH_3, C-24), 22.8 (CH_3, C-27), 23.0 (CH_2, C-11), 23.9 (CH_2, C-16), 27.8 (CH_3, C-23), 27.9 (CH_2, C-15), 30.4 (CH_2, C-21), 32.8 (CH_2, C-7), 36.7 (CH_2, C-22), 38.0 (C, C-10), 38.1 (C, C-4), 39.0 (CH, C-20), 39.0 (CH, C-19), 39.6 (C, C-8), 41.2 (CH_2, C-1), 42.0 (C, C-14), 47.2 (CH, C-9), 47.9 (CH, C-5), 48.2 (C, C-17), 52.9 (CH, C-18), 65.7 (CH, C-2), 78.7 (CH, C-3), 125.4 (CH, C-12), 138.3 (C, C-13), 180.2 (C, C-28).

Euscaphic acid (2s). HR-ESI-MS: m/z calcd. for $C_{30}H_{47}O_5$ $[M-H]^-$487.3423 found 487.3419. ^{1}H NMR (400 HMz, CD_3OD): δ_H 0.81 (s, 3H, H-24), 0.89 (s, 3H, H-25), 0.95 (d, 3H, J = 5.2 Hz, H-30), 1.01 (s, 3H, H-26), 1.01 (s, 3H, H-23), 1.22 (s, 3H, H-29), 1.37 (s, 3H, H-27), 3.35, (m, 1H, H-3), 3.95, (dt, 1H, J = 11.8, 3.5 Hz, H-2), 5.32 (t, 1H, J = 4.0 Hz, H-12); ^{13}C NMR (100 HMz, CD_3OD): δ_C 15.2 (CH_3, C-30),15.5 (CH_3, C-25), 16.1 (CH_3, C-26), 17.9 (CH_2, C-6), 21.1 (CH_3, C-24), 23.3 (CH_2, C-11), 23.5 (CH_3, C-27), 25.2 (CH_2, C-16), 25.7 (CH_2, C-21), 25.9 (CH_3, C-29), 27.9 (CH_2, C-15), 28.2 (CH_3, C-23), 38.0 (C, C-10), 38.1 (C, C-4), 39.9 (C, C-8), 32.7 (CH_2, C-7), 37.6 (CH_2, C-22), 41.1 (CH, C-20), 41.3 (CH_2, C-1), 41.7 (C, C-14), 46.8 (CH, C-9), 47.3 (C, C-17), 47.9 (CH, C-5), 53.7 (CH, C-18), 65.8 (CH, C-2), 72.2 (CH, C-19), 78.7 (CH, C-3), 128.0 (CH, C-12), 138.7 (C, C-13), 180.9 (C, C-28).

Apigenin (5s). White amorphous powder; HR-ESI-MS: m/z calcd. for $C_{37}H_{59}O_{12}$ $[M-H]^-$ 269.0450 found 269.0449; ^{1}H NMR (400 HMz, pyridine-d_5): δ_H 6.75 (1H, s, H-6), 6.84(1H, s, H-3), 6.90 (1H, s, H-8), 7.23 (2H, d, J = 8.6 Hz, H-3' and H-5'), 7.90 (2H, d, J = 8.6 Hz, H-2' and H-6'); ^{13}C NMR (100 HMz, CD_3OD): δ_C 94.7 (C, C-8), 99.8 (CH, C-6), 103.7 (CH, C-3), 104.8 (C, C-10), 116.6 (CH, C-3' and C-5'), 122.0 (C, C-1'), 128.7 (CH, C-2' and C-6'), 158.3 (C, C-5), 162.5 (C, C-9), 162.9 (C, C-7) , 164.3 (C, C-4'), 165.7 (C, C-2), 182.5 (C, C-4).

Cell culture and adipocyte differentiation. 3T3-L1 cells (American Type Culture Collection; ATCC CL-193, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum (BS), 100 units/mL penicillin, and 50 μg/mL streptomycin in an incubator with 5% CO_2 at 37 °C. For differentiation experiments, 3T3-L1 preadipocytes were cultured in 24-well plates until confluent, and then maintained for two additional days. Afterwards, differentiation was induced with a mixture of 1 μM dexamethasone (Dex), 5 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and the sample in DMEM containing 10% fetal bovine serum (FBS). Two days later, medium was replaced with fresh DMEM supplemented with 10% FBS and 5 μg/mL insulin for 2 days. Then, the medium for the maturing adipocytes was replaced with 10% FBS in DMEM for another 2 days. Finally, cells were stained with Oil Red O. All samples were dissolved in dimethyl sulfoxide (DMSO) before apply to preadipocytes. The final concentration of DMSO was less than 0.2%.

Oil Red O staining. Differentiated 3T3-L1 adipocytes were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, and washed twice with PBS. Fixed cells were stained with Oil Red O solution (0.3% Oil Red O in 60% isopropyl alcohol) for 20 min, followed by two PBS rinses. Stained lipid droplets of the 3T3-L1 adipocytes were photographed using an Olympus IX73 microscope (Tokyo, Japan) at 200 × magnification. Finally, the dye retained in the cells was dissolved in isopropyl alcohol and quantified by measuring the absorbance at 490 nm using a microplate reader (FlexStation 3™, Molecular Devices, Sunnyvale, CA, USA).

Cytotoxicity assay. The cytotoxic effects of samples on 3T3-L1 cells were estimated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, cells

were cultured in 96-well plates until the full confluence, and incubated with the presence or absence of sample in differentiation medium for 4 days, and then exposed to 10% FBS in DMEM for an additional day without the sample. The effective fractions and samples didn't show cytotoxicity. Then, the MTT was added into each well at a final concentration of 0.5 mg/mL and placed in an incubator for 4 hours. After removal of medium and MTT solution, the formazan was dissolved using 200 μL DMSO and quantified by measuring the absorbance at 570 nm by using a microplate reader.

Statistical analysis. All results of bioactivity testing were expressed as the mean ± standard deviation (SD) (n = 3). Statistical comparisons were determined by student T-test. A *P*-value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

Bioassay-guided Isolation of Potential Anti-adipogenic Compounds from Shiya tea. Each fraction isolated from MeOH extracts of Shiya tea was screened for potential anti-adipogenic effects (Figure 1). Only the active fractions were used for the further isolation. The ethyl acetate layer was subjected to a silica gel column to obtain different fractions, and only Fr.3 was active. Fractions 3B and 3B1, which were fractionated/sub-fractionated from Fr. 3, significantly inhibited intracellular lipid accumulation at 100 μg/mL during 3T3-L1 adipocyte differentiation without cytotoxicity. From Fr. 3B1, five compounds were isolated.

Structure Elucidation of Compounds (1−5) from Shiya tea. Bioactivity-guided isolation of MeOH extracts of Shiya tea led to the isolation of four triterpenoid saponins (**1**–**4**), including one new compound (**1**), and a flavonoid (**5**) (Figure 2), which were elucidated using

spectroscopic methods (1D, 2D NMR, and LC−Orbitrap−MS). Four known compounds, kajiichigoside F1 (**2**),[17] glucosyl tormentate (**3**),[18] 2α, 3α, 19α-trihydroxy-olean-12-en-28-oic acid-28-O-beta-D-glucopyranoside (**4**),[19] and camellianin A (**5**)[20] were identified by comparing their spectroscopic data with those reported in the literature.

2α, 3α-Dihydroxy-ursolic acid-28-O-β-D-glucopyranosyl ester (**1**), of which structure has been not reported, was white amorphous powder with a molecular formula of $C_{36}H_{58}O_9$ based on *m/z* 679.4064 $[M + HCOO]^-$ (calcd. for $C_{37}H_{59}O_{11}$, 679.4057) with 8 degrees of unsaturation. The ^1H NMR spectra (Table 1) revealed the presence of seven methyls [δ_H 0.85 (s), 0.88 (s), 0.92 (d, J = 6.2 Hz), 0.99 (d, J = 5.2 Hz), 1.00 (s), 1.03 (s), 1.15 (s)], and an olefinic proton at δ_H 5.28 (d, J = 2.9 Hz). The 1D NMR data along with the HMQC spectra showed 36 carbon signals, including a characteristic glucose, two hydroxyls, a double bond and a carbonyl. The above-mentioned information indicated that **1** was an ursane-type triterpenoid saponin.

Further analysis of the 1D and 2D NMR data of **1** suggested a high similarity between **1** and glucosyl tormentate (**3**) except for the relative configuration of OH-3 and absence of a hydroxyl at C-19.[17] In the ^1H-^1H COSY spectrum (Figure 3), two isolated spin systems were observed as H_2-1/H-2/H-3 and Me-29/H-19/H-20/Me-30, which revealed the presence of two fragments as indicated by the highlighted bold bonds in Figure 3. The fragments were further confirmed from the HMBC correlations of Me-23 to C-3, C-4, and C-5, Me-29 to C-18, C-19, and C-20, and Me-30 to C-19, C-20, and C-21 (Figure 3). The glucose was connected to the carbonyl of C-28 by the key HMBC correlation from the anomeric proton of H-1' (δ_H 5.36, d, J = 8.6 Hz) to C-28 (δ_C 176.6), and the coupling constant indicated that the glucopyranose was β-oriented. D-glucose was obtained from acid hydrolysis of **1**, which was determined by GC analysis of corresponding derivatives.[14] Thus, the planar structure of **1** was assigned.

The relative configuration of **1** was assigned from the ROESY correlations and coupling constant, in which cross-peaks of H-2/H-3, H-2/Me-25, H-3/Me-24, Me -25/ M-26, and Me-29/H-20 implied that those groups were cofacial and randomly assigned as β-oriented (Figure 3). The coupling constant between H-2 and H-3 (J = 3.5 Hz) along with the chemical shift of C-2 (δ_C 65.8) and C-3 (δ_C 78.8) further confirmed the β-orientation of H-2 and H-3.[17,19] In addition, H-5, H-9, H-19, and Me-30 were α-oriented due to the ROESY correlations of H-5/H-9, H-9/Me-27, and Me-30/H-19. Therefore, the relative configuration of **1** was constructed as 2α, 3α-dihydroxy-ursolic acid-28-O-β-D-glucopyranosyl ester.

MS Fragmentation Patterns of Authentic Isolates (1–5). Four triterpenoid saponins had the predominant $[M - H]^-$ and $[M + HCOO]^-$ precursor ions due to the presence of formic acid in the mobile phase (Table 2). The cleavage of sugar moieties from the compound structures were the characteristic fragmentation in all isolates (Table 2). Compounds (**1**–**4**) generated fragment ions with the loss of glucose (*m/z* 162), and compound (**5**) formed a fragment ion with the loss of glucose (*m/z* 162) and rhamnose (*m/z* 146). Compound **1**, the new triterpenoid saponin, without the hydroxyl at C-19 had only one fragment ion $[M - Glu - H]^-$ in the MS^2 spectra, while others were found to generate more fragments, such as the loss of a carboxylic acid at C-28 and H_2O. The LC−MS chromatogram of the isolates (**1**–**5**) in crude extract was shown in Figure 4.

Potential Anti-adipogenic Effects of Purified Compounds and their hydrolyzed compounds. All the isolates (**1**−**5**) were evaluated by measuring the inhibitory effect on lipid accumulation in 3T3-L1 adipocytes (Figure 5). The intracellular lipid accumulation and lipid droplets were significantly suppressed by **1** in a dose-dependent manner (Figure 6) with an IC_{50} value of 27.6 μg/mL without cytotoxicity. As for the ursane- and oleanane-type triterpenoids

(**1**–**4**), the absence of the hydroxyl at C-19 was important for inhibitory effect on lipid accumulation in 3T3-L1 adipocytes. Therefore compound **1**, without a hydroxyl at C-19 revealed the most effective inhibition of 3T3-L1 adipocyte differentiation. In order to discuss the effect of a sugar group on the bioactivity, three aglycones of 2α, 3α-dihydroxy-ursolic acid (**1s**),[21] euscaphic acid (**2s**)[17], and apigenin (**5s**)[22] were obtained by hydrolysis of compounds **1**, **2**, and **5**, respectively. When β-D-glucopyranose was removed by acid hydrolysis from compound **1**, the percentage of lipid accumulation increased from 47.6 % to 85.4% at 40 μg/mL. Both aglycones of **1s** and **2s** showed no obvious anti-adipogenic activity indicating the effect of the position of a hydroxyl was not conclusive in the absence of a sugar group. Ursolic acid, which exhibited a better inhibitory effect of preadipocyte differentiation, possesses a similar structure as compound **1s** with the beta-hydroxyl at C-3 instead of alfa-hydroxyl.[23] Thus, in addition to glycoside, the numbers and the orientation of the hydroxyl groups play an important role in the inhibitory effect of ursolic acid type tritepenoid on lipid accumulation. In addition, ursane-type triterpenoids exhibited multiple biological functions, such as antioxidant, antimicrobial, antiplatelet aggregation, and reducing serum total cholesterol activities.[24-27] Apigenin (**5s**), an aglycone of compound **5**, showed an enhanced inhibitory effect with an IC_{50} value of 34.6 μg/mL when compared to compound **5**. This complements the literature in that flavonoids without a sugar group provide stronger anti-adipogenic effects.[28] However, this result opposes that of triterpenoid saponin. Therefore, the triterpenoid saponin (**1**) and flavonoid (**5s**) both showed anti-adipogenic potential for obesity prevention.

In conclusion, bioactivity-guided isolation of Shiya tea led to the isolation and identification of four triterpenoid saponins (**1**–**4**), including one new compound, and a flavonoid (**5**) using NMR (1D and 2D NMR) and tandem liquid chromatography mass

spectrometry (HPLC-ESI/MS^2) techniques. A comprehensive profile of isolates in Shiya tea was outlined by LC-MS. Compound **1** could reduce intracellular lipid accumulation in 3T3-L1 adipocytes in a dose-dependent manner with an IC_{50} value of 27.6 μg/mL. Previous studies indicated that flavonoids in Shiya tea are considered the major active and functional chemical constituents. However, this study found a new triterpenoid, saponin, to be the major anti-adipogenic constituent in Shiya tea. Three hydrolytic compounds (**1s**, **2s**, and **5s**) were obtained and screened for their effect on lipid accumulation inhibition. The inhibitory activity of the triterpenoid (**1s**) without a sugar group decreased significantly, while the flavonoid (**5s**) without a sugar showed great increased activity.

ASSOCIATED CONTENT

Supporting Information

The 1D, 2D NMR, HRESIMS of compound **1**; Table of anti-adipogenic effects of the fractions.

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Notes

The authors declare no competing financial interest.

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445 **Table and figure captions**

446 **Table 1**. ^{1}H and ^{13}C NMR spectroscopic data of compound **1** in CD_3OD.

447 **Table 2.** MS precursor and fragment ions of the isolates (**1**−**5**) in Shiya tea.

448 **Figure 1.** Effects of the fractions of *A. nitida* on lipid accumulation during 3T3-L1 adipocyte
449 differentiation (quercetin was used as positive control. **p < 0.01 vs. control, *p < 0.05 vs.
450 control). Data are expressed as a mean ± standard deviation (n = 3). The cells were treated with
451 the extracts of ethyl acetate and water, the fractions of Fr.1–4 at 200 μg/mL, the fractions of
452 Fr.1–4, Fr.3A–3E, and Fr.3B.1–3B.4 at 100 μg/mL, and the fractions of Fr.2 and Fr.3A, and
453 quercetin at 50 μg/mL.

454 **Figure 2.** Chemical structures of **1**−**5**.

455 **Figure 3.** Key 2D NMR correlations of **1**

456 **Figure 4.** Total ion chromatogram (TIC) of the isolates (**1**–**5**) in Shiya tea extract.

457 **Figure 5.** Effect of compounds **1**–**5** on lipid accumulation during 3T3-L1 adipocytes
458 differentiation. (quercetin was used as positive control. **p < 0.01 vs. control) Data are
459 expressed as a mean ± standard deviation (n = 3).

460 **Figure 6.** Inhibitory effects of compound **1** and **5s** on intracellular lipid accumulation and
461 lipid droplets during adipocytes differentiation. Quantification of intracellular lipid in Oil red
462 O stained adipocytes (A). Representative images of Oil red O staining after various amounts
463 of compounds were added (B). Data are expressed as a mean ± standard deviation (n = 3).

464 **For Table of Contents Only.**

465 **Table 1.** ^{1}H and ^{13}C NMR spectroscopic data of compound **1** in CD_3OD.

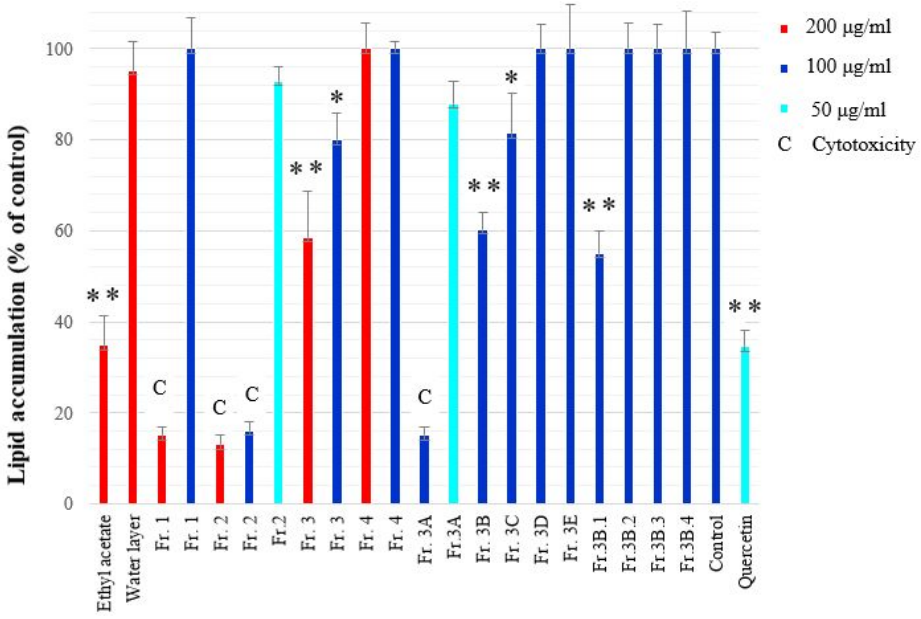
No.	δ_H (J in Hz)	δ_C, type
1	α: 1.28, m β:1.63, m	41.3, CH_2
2	3.94, dt (11.8, 3.5)	65.8, CH
3	3.34, d (3.5)	78.7, CH
4		38.1, C
5	1.25, m	47.9, CH
6	α: 1.46, m β:1.39, m	17.7, CH_2
7	α: 1.58, m β:1.35, m	32.8, CH_2
8		39.8, C
9	1.72, t (8.4)	47.2, CH
10		37.9, C
11	α: 1.98, m β:1.99, m	23.0, CH_2
12	5.28, t (2.9)	125.7, CH
13		137.8, C
14		42.0, C
15	α: 1.11, m β:1.96, m	27.9, CH_2
16	α: 1.78, m β:2.10, td (12.5, 4.0)	23.8, CH_2
17		48.1, C
18	2.26, d (11.0)	52.8, CH
19	1.42, m	39.1, CH
20	1.02, m	38.9, CH
21	α: 1.53, m β:1.37, m	30.3, CH_2
22	α: 1.65, m β:1.78, m	36.1, CH_2
23	1.00, s	27.9, CH_3
24	0.88, s	21.1, CH_3
25	1.03, s	15.7, CH_3
26	0.85, s	16.5, CH_3
27	1.15, s	22.7, CH_3
28		176.6, C
29	0.92, d (6.2)	16.2, CH_3
30	0.99, d (5.2)	20.1, CH_3
1'	5.36, d (8.6)	93.4, CH
2'	3.34, m	72.5, CH
3'	3.35, m	77.2, CH
4'	3.38, m	69.8, CH
5'	3.42, m	76.9, CH

6'	a:3.70, dd (11.8, 4.2) b:3.82, dd (11.8, 2.0)	61.1, CH_2

466

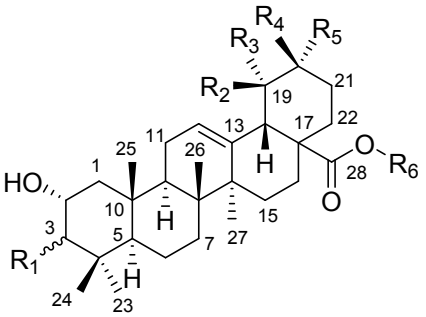
467 **Table 2.** MS precursor and fragment ions of the isolate (**1**−**5**) in Shiya tea.

Com.	$[M - H]^-$	$[M + HCOOH - H]^-$	MS^2
1	633.3985	679.4064	633.3985/471.3471 $[M - Glu - H]^-$
2	649.3881	695.4014	649.3881/487.3424 $[M - Glu - H]^-$, 425.3475 $[M - Glu - CO_2 - H_2O - H]^-$, 407.3346 $[M - Glu - CO_2 - 2H_2O - H]^-$
3	649.3881	695.4014	649.3881/487.3426 $[M - Glu - H]^-$, 469.3304 $[M - Glu - H_2O - H]^-$
4	649.4072	695.4019	649.4072/487.3427 $[M - Glu - H]^-$, 469.3325 $[M - Glu - H_2O - H]^-$
5	619.1654	Not detected	619.1654/601.1686 $[M - H_2O - H]^-$, 577.1569 $[M - CH_3CO - H]^-$, 269.0456 $[M - Glu - CH_3CO - Rha - H]^-$

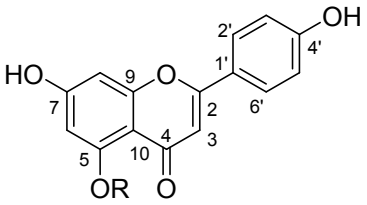


469

470 **Figure 1.**

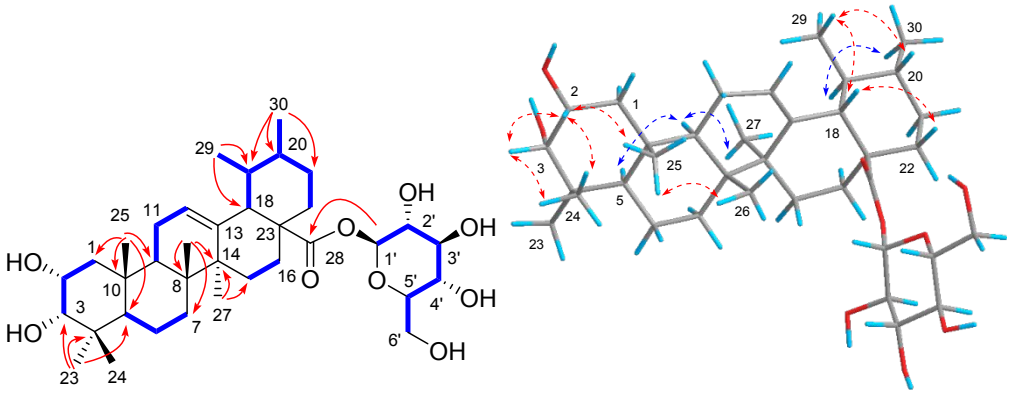


	R_1	R_2	R_3	R_4	R_5	R_6
1	α-OH	CH_3	H	H	CH_3	β-D-Glucopyranosyl
1s	α-OH	CH_3	H	H	CH_3	H
2	α-OH	CH_3	OH	H	CH_3	β-D-Glucopyranosyl
2s	α-OH	CH_3	OH	H	CH_3	H
3	β-OH	CH_3	OH	H	CH_3	β-D-Glucopyranosyl
4	α-OH	H	OH	CH_3	CH_3	β-D-Glucopyranosyl



5 R = -Rham-Glc-$OCCH_3$
5s R = H

Figure 2.

473 

474 **Figure 3.**

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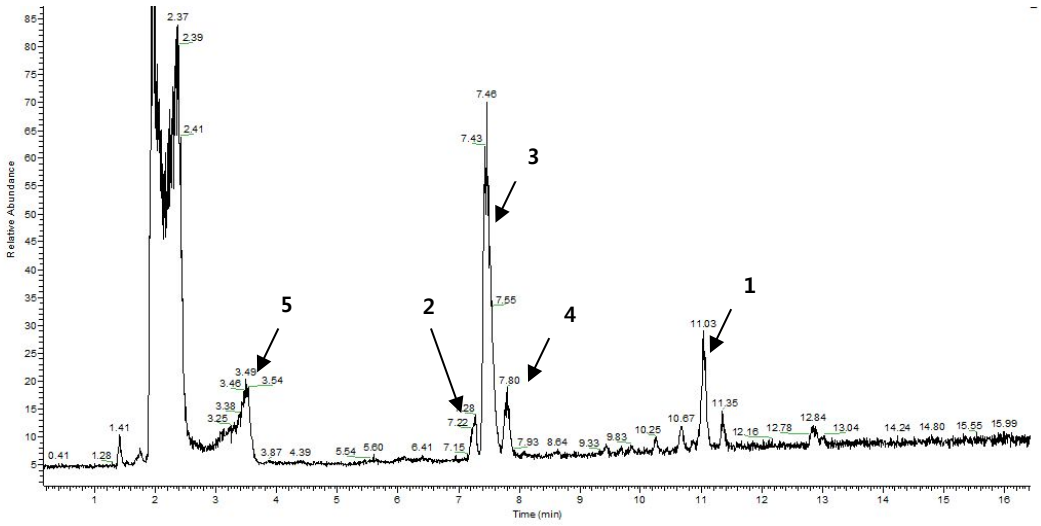

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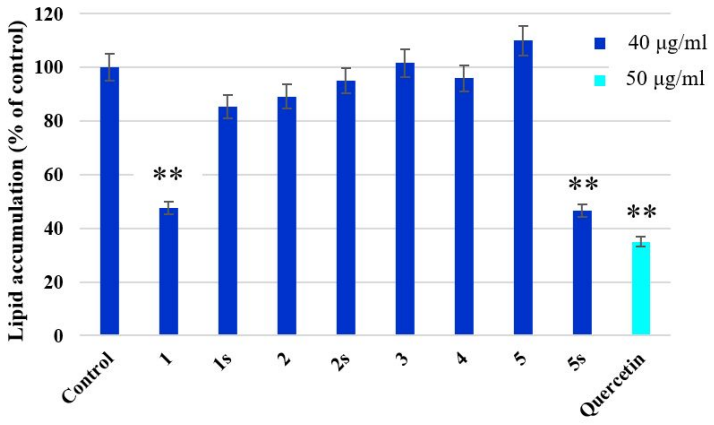
477 **Figure 4**.



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479 **Figure 5.**

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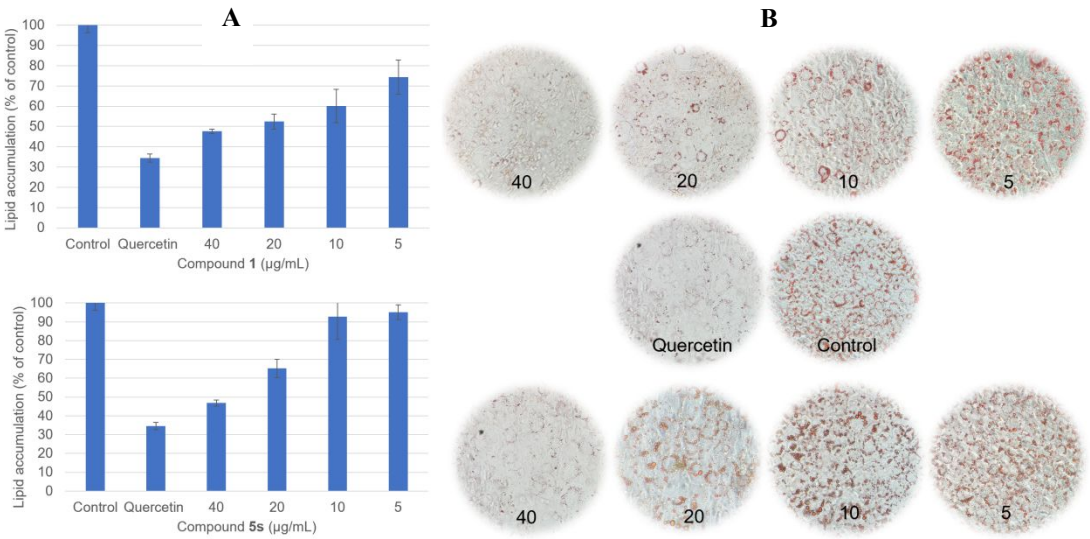

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483 **Figure 6.**

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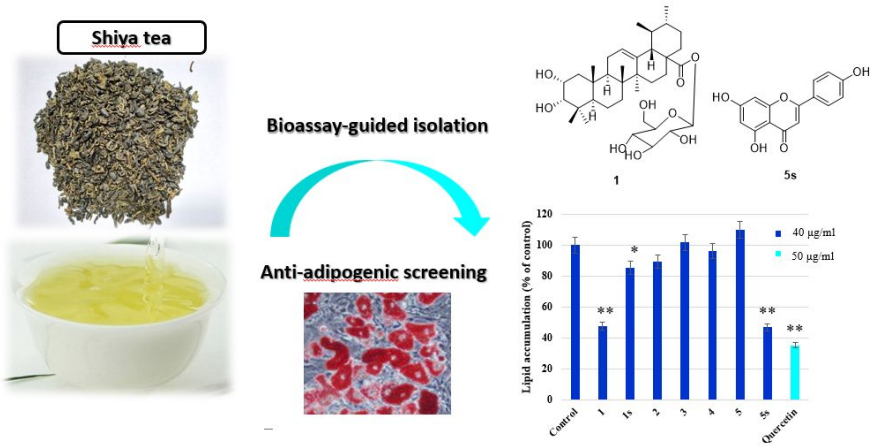

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