SUPPLEMENTARY MATERIAL

Two new glycosides isolated from *Sapindus mukorossi* fruits: Effects on cell apoptosis and caspase-3 activation in human lung carcinoma cells

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Two new glycosides (**1**, **2**) and two saponins (**3**, **4**) were isolated from the fruits of *Sapindus mukorossi* Gaertn. The two glycosides were designated as sapindoside G (**1**) and 4'',4''''-*O*-diacetylmukurozioside IIa (**2**). All four compounds exhibited inhibitory effects against A549 human lung adenocarcinoma cells with inhibition rates up to 69.2%–83.3% at a concentration of 100 µg/mL. Flow cytometric analysis revealed that compounds **1**–**4** could suppress A549 cell growth by promoting cell apoptosis, which was related to the activation of caspase-3.

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Experimental

General

Optical rotation in MeOH (20°C) was measured in a Perkin Elmer341 polarimeter (Perkin-Elmer, Norwalk, Connecticut, USA). IR spectra were obtained with a TENSOR37 Fourier Transform Infrared spectrometer (Bruker Co., Karlsruhe, Baden-Württemberg, Germany). The 1D and 2D NMR spectra were recorded on a Bruker Avance III 400-MHz spectrometer (Bruker Co., Karlsruhe, Baden-Württemberg, Germany) in pyridine-*d*₅ (Cambridge Isotope Laboratories, Tewksbury, Massachusetts, USA). ESI-MS/MS and HR-ESI-MS were performed in a Finnigan-TSQ-Quantum instrument (Thermo Fisher, Woburn, Massachusetts, USA) and Thermo LTQ Orbitrap Elite (Thermo Fisher, Woburn, Massachusetts, USA), respectively. GC-MS was performed in a TRACE DSQ spectrometer (Thermo Fisher, Woburn, Massachusetts, USA) equipped with a DB-5MS quartz capillary column (30 m × 0.25 mm); the injection temperature was 250°C and the column temperature was 150–250°C (4°C/min). HPLC separation was carried out on an Waters HPLC system (Waters 600 Quat Pump, 2487 Dual λ Absorbance Detector). Silica gel (100–200 mesh, Qingdao Haiyang Chemical Co., Shandong, China), Sephadex LH-20 (Lanji Biosciences, Shanghai, China), and silica gel RP-18 (Spherical C18, 120 Å, 30–50 μ m, Daiso, Tokyo, Japan) were used for chromatography experiments; pre-coated silica gel plates (GF-254, Qingdao Haiyang Chemical Co., Shandong, China) were used for TLC. Cell apoptosis and cell cycle distribution were determined by flow cytometry (EPICS XL, Beckman Coulter, Brea, California, USA). MTT and caspase-3 (C1116, Beyotime Institute of Biotechnology, Jiangsu, China) were measured in a Microplate reader FlexStation 3 (Molecular Devices Co., Sunnyvale, California, USA).

Plant materials

S. mukorossi fruits were collected in October 7, 2012 from Guangzhou City, Guangdong Province, People's Republic of China. The plant was identified by Professor Depo Yang at Sun Yat-sen University; a voucher specimen (No. 20121007) was deposited at the Institute of Natural Products and TCM, Sun Yat-sen University, China.

Extraction and isolation

Dried fruits of *S. mukorossi* (3.5 kg) were extracted three times with MeOH (7 L $\times$ 3). After removing the solvent, the residue was successively partitioned with petroleum ether, EtOAc, and *n*-butanol (each 2.5 L $\times$ 3). The *n*-butanol layer (308.3 g) was subjected to silica gel CC and eluted with a continuous gradient of CHCl₃/MeOH (20:0.5, 20:1, 20:2, 20:3, and 20:4), yielding five fractions (Fr. 1–5). Fr. 2 (10.2 g) was partitioned into three fractions (Fr. 2.1–Fr. 2.3) by Sephadex LH-20 using methanol as eluent. Fr. 2.2 was subjected to silica gel separation (CHCl₃/MeOH, 20:0.5 to 20:5), yielding six fractions. Fr. 2.2.3 was further purified by HPLC (MeOH/H₂O, 40:60 to 100:0) yielding compound **3** (31.2 mg). Fr. 3 (40.2 g) was partitioned into three fractions by Sephadex LH-20 column with CHCl₃/MeOH (1:1) as eluent. Fr. 3.2 was chromatographed over a silica gel column, which was eluted with CHCl₃/MeOH/H₂O (10:0.5:0.1 to 10:5:0.1) to give six fractions. Fr. 3.2.6 was further purified by HPLC (MeOH/H₂O, 40:60 to 100:0) yielding compounds **1** (12.1 mg) and **4** (16.1 mg). Fr. 5 (20.5 g) was separated by chromatography on a Sephadex LH-20 column with CHCl₃/MeOH (1:1); three fractions (Fr. 5.1–5.3) were obtained. Fr. 5.2 was repeatedly subjected to silica gel column chromatography with CHCl₃/MeOH/H₂O as eluent (10:1:0.1 to 10:8:0.1), yielding six fractions; compound **2** (14.3 mg) was isolated from fraction 5.2.2 by HPLC (MeOH/H₂O, 40:60 to 100:0).

Compound 1

A white amorphous powder; $[\alpha]_D^{20}$ -3.8° (c=0.2, MeOH); IR $\gamma_{\max}$ KBr cm⁻¹: 3273, 2941, 1733, 1134, 1053; HR-ESI-MS *m/z* 865.4969 (calcd 865.4949, C₄₆H₇₃O₁₅ [M–H–Glc–Xyl–Rha][–]); Negative ESI-MS/MS: *m/z*=1351 [M+HCOO][–], 866, 855, 733, 713, 588, 130; ¹H NMR (400MHz, pyridine-*d*₅) δ : 1.49 (1H, overlapped ‘os’, H-1 α), 1.53 (1H, os, H-1 β), 1.85 (1H, os, H-2 α), 2.13 (1H, os, H-2 β), 3.32 (1H, os, H-3), 0.83 (1H, os, H-5), 1.49 (1H, os, H-6 α), 1.52 (1H, os, H-6 β), 1.26 (1H, os, H-7 α), 1.83 (1H, os, H-7 β), 1.68 (1H, m, H-9), 1.93 (1H, m, H-11 α), 2.16 (1H, os, H-11 β), 5.50 (1H, br s, H-12), 1.22 (1H, os, H-15 α), 1.29 (1H, os, H-15 β), 1.87 (1H, os, H-16 α), 2.01 (1H, m, H-16 β), 3.32 (1H, os, H-18), 1.29 (1H, os, H-19 α), 1.83 (1H, os, H-19 β), 1.25 (1H, os, H-21 α), 1.48 (1H, os, H-21 β), 1.86 (1H, os, H-22 α), 2.06 (1H, m, H-22 β), 1.34 (**3H**, s, **H-23**), 1.15 (3H, s, H-24), 0.87 (3H, s, H-25), 1.02 (3H, s, H-26), 1.33 (3H, s, H-27), 1.33 (3H, s, H-29), 1.04 (3H, s, H-30), 4.88 (1H, d, *J*=7.8 Hz, H-1’), 6.23 (1H, br s, H-1’'), 5.36 (1H, d, *J*=7.4 Hz, H-1’’'), 4.90 (1H, d, *J*=8.0 Hz, H-1’’’’), 5.30 (1H, d, *J*=6.8 Hz, H-1’’’’’), 6.25 (1H, br s, H-1’’’’’’). ¹³C NMR (100MHz, pyridine-*d*₅) δ : 39.4 (C-1), 27.1 (C-2), 89.3 (C-3), 40.1 (C-4), 56.5 (C-5), 19.0 (C-6), 33.7 (C-7), 40.3 (C-8), 48.6 (C-9), 37.6

(C-10), 24.2 (C-11), 123.0 (C-12), 145.3 (C-13), 42.7 (C-14), 28.8 (C-15), 24.3 (C-16), 47.2 (C-17), 42.5 (C-18), 47.0 (C-19), 31.5 (C-20), 34.8 (C-21), 33.8 (C-22), 28.7 (C-23), 17.7 (C-24), 16.1 (C-25), 17.7 (C-26), 26.7 (C-27), 180.7 (C-28), 28.7 (C-29), 24.3 (C-30), 105.6 (C-1'), 75.9 (C-2'), 73.7 (C-3'), 70.2 (C-4'), 65.9 (C-5'), 102.0 (C-1''), 72.4 (C-2''), 83.5 (C-3''), 73.5 (C-4''), 69.7 (C-5''), 18.9 (C-6''), 108.0 (C-1'''), 75.0 (C-2'''), 89.3 (C-3'''), 69.6 (C-4'''), 67.6 (C-5'''), 105.7 (C-1'''), 78.9 (C-2'''), 75.1 (C-3'''), 71.6 (C-4'''), 76.0 (C-5'''), 67.9 (C-6'''), 107.8 (C-1'''), 83.3 (C-2'''), 76.1 (C-3'''), 70.2 (C-4'''), 66.0 (C-5'''), 102.1 (C-1'''), 72.5 (C-2'''), 73.5 (C-3'''), 74.9 (C-4'''), 70.0 (C-5'''), 18.9 (C-6''').

Compound 2

A white amorphous powder; $[\alpha]_D^{20}$ -42.9° ($c=0.2$, MeOH); IR $\gamma_{\max}$ KBr cm^{-1} : 3341, 2934, 1729, 1256, 1131, 1034; HR-ESI-MS m/z 1231.5619 (calcd 1231.5595, $\text{C}_{55}\text{H}_{91}\text{O}_{30}$ $[\text{M}-\text{H}]^-$); Negative ESI-MS/MS: $m/z=1231$ $[\text{M}-\text{H}]^-$, 1171, 1129, 1001, 983, 855, 837; ^1H NMR (400MHz, pyridine- d_5) δ : 4.28 (1H, os, H-1 α), 4.56 (1H, os, H-1 β), 5.60 (1H, os, H-2), 2.07 (1H, os, H-3 α), 2.16 (1H, os, H-3 β), 2.11 (1H, os, H-5 α), 2.20 (1H, os, H-5 β), 5.21 (1H, m, H-6), 1.97 (1H, os, H-8 α), 2.21 (1H, os, H-8 β), 1.38 (1H, m, H-9 α), 1.47 (1H, os, H-9 β), 1.10 (1H, m, H-10 α), 1.52 (1H, os, H-10 β), 1.90 (1H, os, H-11), 3.34 (1H, m, H-12 α), 3.84 (1H, os, H-12 β), 1.63 (3H, s, H-13), 1.60 (3H, s, H-14), 1.01 (3H, d, $J=7.2$ Hz, H-15), 4.73 (1H, d, $J=7.2$ Hz, H-1'), 5.74 (1H, br s, H-1''), 5.63 (1H, br s, H-1'''), 4.68 (1H, d, $J=7.2$ Hz, H-1'''), 5.69 (1H, br s, H-1'''), 5.62 (1H, br s, H-1'''). ^{13}C NMR (100MHz, pyridine- d_5) δ : 66.3 (C-1), 121.2 (C-2), 141.4 (C-3), 40.4 (C-4), 27.3 (C-5), 124.9 (C-6), 136.3 (C-7), 40.8 (C-8), 26.3 (C-9), 34.3 (C-10), 34.5 (C-11), 76.1 (C-12), 17.1 (C-13), 16.6 (C-14), 17.9 (C-15), 101.8 (C-1'), 78.8 (C-2'), 88.0 (C-3'), 70.5 (C-4'), 78.7 (C-5'), 62.8 (C-6'), 102.4 (C-1''), 72.9 (C-2''), 71.1 (C-3''), 76.2 (C-4''), 67.8 (C-5''), 18.9 (C-6''), 171.3 (4''-OAc), 21.3 (4''-OAc), 104.3 (C-1'''), 73.3 (C-2'''), 73.1 (C-3'''), 74.1 (C-4'''), 71.2 (C-5'''), 19.0 (C-6'''), 102.5 (C-1'''), 78.7 (C-2'''), 87.8 (C-3'''), 70.6 (C-4'''), 78.6 (C-5'''), 62.8 (C-6'''), 103.2 (C-1'''), 72.8 (C-2'''), 71.1 (C-3'''), 76.3 (C-4'''), 67.8 (C-5'''), 18.9 (C-6'''), 171.4 (4''''-OAc), 21.7 (4''''-OAc), 104.3 (C-1'''), 73.1 (C-2'''), 73.0 (C-3'''), 74.1 (C-4'''), 70.5 (C-5'''), 19.3 (C-6''').

Acid hydrolysis and GC analysis

Compounds **1** and **2** (4.0 mg each) in 2 mL of 1 M HCl-dioxane (1:1, v/v) were heated at 80°C for 4 h. The reaction mixture was neutralized with Na_2CO_3 and filtered. The resulting filtrate was successively partitioned

with CHCl_3 and H_2O . The aqueous layer was evaporated to dryness and the dried residues were diluted in 1.0 mL pyridine without water; subsequently, 3 mg L-cysteine methyl ester hydrochloride in 0.5 mL pyridine was added. The mixture was heated at 60°C for 1 h; 0.5 mL hexamethyl disilazane-trimethyl chlorosilane (HMDS–TMCS) was added and the mixture was heated at 60°C for another 30 min. After drying the solution under a stream of N_2 , the residue was extracted with hexane (1.5 mL) and analyzed by GC-MS. Sugar units were identified by comparing the retention times of the corresponding derivatives with those of standards. Retention times of standards were 13.89 min (D-glucose), 11.90 min (L-glucose), 9.22 min (D-xylose), 8.20 min (L-xylose), 6.90 min (D-arabinose), 6.36 min (L-arabinose), and 6.58 min (L-rhamnose). L-Ara, L-Rha, D-Glc, and D-Xyl were detected in compound **1**; L-Rha and D-Glc were detected in compound **2**.

In vitro cytotoxicity assays

The cytotoxicity of compounds **1–4** was assessed by the MTT method in A-549 cancer cell lines. Cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum and cultured at a density of 5×10^4 cells/mL per well in a 96-well microtiter plate. Six different concentrations of each compound were added to each well in triplicate. Adriamycin was used as the positive control. Following incubation at 37°C in 5% CO_2 for 24 h, 20 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h. Following the 4 h incubation, medium was removed and replaced with DMSO (100 μL). Absorbance at 490 nm was measured in a microplate reader. Cytotoxicity was expressed as the concentration of drug that inhibited cell growth by 50% (IC_{50}).

Flow cytometry analysis for cell apoptosis and cell cycle

Flow cytometry was used to analyze the effects of compounds **1–4** on cell apoptosis and cell cycle distribution of A549 cells. A549 cells (1×10^6 cells/mL per well) were seeded on six-well culture plates and incubated for 24 h. Subsequently, the cells were treated for 24 h with 33.61 $\mu\text{g/mL}$ of **1**, 98.35 $\mu\text{g/mL}$ of **2**, 46.75 $\mu\text{g/mL}$ of **3**, or 33.19 $\mu\text{g/mL}$ of **4**; these concentrations inhibited cell growth by 50%. Each compound was tested in triplicate. For cell apoptosis analysis, the cells were rinsed three times in phosphate buffer saline (PBS), stained with 5 μL propidium iodide (PI) and 5 μL Annexin V-FITC in the dark and at room temperature for 15 min, and analyzed by flow cytometry. For cell cycle analysis, the cells were washed with PBS, fixed overnight with 70% EtOH at -20°C , treated with RNase A for 30 min at 37°C , stained with PI in the dark for 10 min, and analyzed by flow cytometry.

Caspase-3 assay

The assay was performed using a commercial caspase-3 assay kit. A549 cells (1×10^6 cells/mL) were treated with 6.25, 12.5, 25, 50, 75, and 100 $\mu\text{g/mL}$ of compounds **1–4** for 24 h. The treated cells were harvested and lysed with buffer. Lysates were centrifuged at $24328 \times g$ for 1 min. The resulting supernatants were collected and the protein content in the cell lysates was determined by Bradford protein assay using bovine serum albumin (BSA) as a standard. In this experiment, supernatant containing 2 mg/mL protein was incubated in a 96-well microplate with acetylated tetrapeptide (Ac-DEVD) labeled with the chromophore p-nitroaniline (pNA) and in the presence of caspase-3 buffer. After incubation for 2 h at 37°C , the absorbance at 405 nm of the liberated pNA chromophore was measured in a microplate reader. Results were expressed as caspase-3 specific activity.

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Fig. S1: Key HMBC correlations ($\text{H} \rightarrow \text{C}$) of compound **1**

Fig. S2: Significant ROESY Correlations of Compound **1**

Fig. S3: Key HMBC correlations ($\text{H} \rightarrow \text{C}$) of compound **2**

Fig. S4: Significant ROESY Correlations of Compound **2**

Fig. S5 Flow cytometric analysis of human A549 cells apoptosis induced
by compounds **1–4** using Annexin V/PI staining

Fig. S6: Effects of compounds **1–4** on cell cycle distribution

Fig. S7: Effects of compounds **1–4** on caspase-3 activity in A549 cells.

Fig. S8: LR-ESI-MS Spectrum of Compound **1**

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Fig. S10: ^1H -NMR (400 MHz, pyridine- d_5) Spectrum of Compound **1**

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Fig. S12: HSQC Spectrum of Compound **1**

Fig. S13: HMBC Spectrum of Compound **1**

Fig. S14: COSY Spectrum of Compound **1**

Fig. S15: ROESY (400 MHz) Spectrum of Compound **1**

Fig. S16: IR Spectrum of Compound **1**

Fig. S17: LR-ESI-MS Spectrum of compound **2**

Fig. S18: MS² Spectrum of compound **2**

Fig. S19: ¹H-NMR (400 MHz, pyridine-d₅) Spectrum of Compound **2**

Fig. S20: ¹³C-NMR (100 MHz, pyridine-d₅) Spectrum of Compound **2**

Fig. S21: HSQC Spectrum of Compound **2**

Fig. S22: HMBC Spectrum of Compound **2**

Fig. S23: COSY Spectrum of Compound **2**

Fig. S24: ROESY (400 MHz) Spectrum of Compound **2**

Fig. S25: IR Spectrum of Compound **2**

Fig. S1 Key HMBC correlations (H $\rightarrow$ C) of compound **1**

Fig. S2 Significant ROESY Correlations of Compound **1**

H $\rightarrow$ C HMBC

Fig. S3 Key HMBC correlations (H $\rightarrow$ C) of compound **2**

H $\rightarrow$ H ROESY

Fig. S4 Significant ROESY Correlations of Compound **2**

Fig. S5 Flow cytometric analysis of human A549 cells apoptosis induced by compounds **1–4** using Annexin V/PI staining

Fig. S6 Effects of compounds **1–4** on cell cycle distribution

Fig. S7 Effects of compounds **1–4** on caspase-3 activity in A549 cells.

Fig. S8 LR-ESI-MS Spectrum of Compound **1**

Fig. S9 MS² Spectrum of Compound **1**

Fig. S10 ¹H-NMR (400 MHz, pyridine-d₅) Spectrum of Compound **1**

Fig. S11 ^{13}C -NMR (100 MHz, pyridine- d_5) Spectrum of Compound **1**

Fig. S12 HSQC Spectrum of Compound **1**

Fig. S13 HMBC Spectrum of Compound 1

Fig. S14 COSY Spectrum of Compound 1

Fig. S15 ROESY (400 MHz) Spectrum of Compound **1**

Fig. S16 IR Spectrum of Compound **1**

Fig. S17 LR-ESI-MS Spectrum of compound **2**

S#: 564 SRM: 1231 CE: 53 CT: 6.07 #C: 20

1.34e1

Fig. S18 MS² Spectrum of compound **2**

Fig. S19 ^1H -NMR (400 MHz, pyridine- d_5) Spectrum of Compound 2

Fig. S20 ^{13}C -NMR (100 MHz, pyridine- d_5) Spectrum of Compound 2

Fig. S21 HSQC Spectrum of Compound 2

Fig. S22 HMBC Spectrum of Compound 2

Fig. S23 COSY Spectrum of Compound 2

Fig. S24 ROESY (400 MHz) Spectrum of Compound 2

Fig. S25 IR Spectrum of Compound **2**

Table S1 Inhibition of compounds **1–4** on A549 cells

	Inhibition ratios (%)			
	1	2	3	4
100 µg/mL	83.3 ± 0.15	70.6 ± 0.16	72.2 ± 0.18	69.2 ± 0.08
50 µg/mL	62.8 ± 0.48	42.6 ± 0.23	40.3 ± 0.03	58.1 ± 0.06
25 µg/mL	59.8 ± 0.03	29.8 ± 0.05	38.9 ± 0.15	48.1 ± 0.13
12.5 µg/mL	47.0 ± 0.12	26.8 ± 0.06	22.2 ± 0.02	42.4 ± 0.08
6.25 µg/mL	35.2 ± 0.02	24.1 ± 0.28	—	10.4 ± 0.07

Adriamycin is used for positive control (IC₅₀ 7.97 ± 0.25 µg/mL)