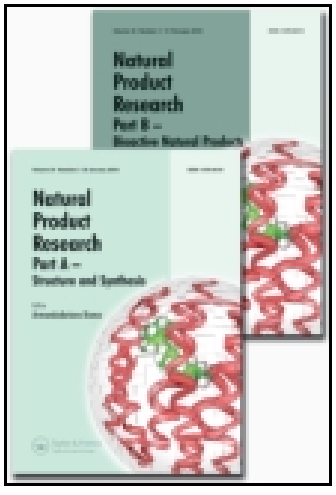

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A new triterpenoid saponin and an oligosaccharide isolated from the fruits of Sapindus mukorossi

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A new triterpenoid saponin and an oligosaccharide isolated from the fruits of *Sapindus mukorossi*

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A new triterpenoid saponin (**1**) and a new oligosaccharide (**2**), together with three known saponins (**3**–**5**), have been isolated from *n*-BuOH extract of the fruits of *Sapindus mukorossi* Gaertn. The structures were elucidated using detailed analysis of one- and two-dimensional nuclear magnetic resonance spectra along with their mass spectrometric data and the results of acid hydrolysis. Of the isolated compounds **1** and **3**–**5** displayed cytotoxic effects against human cancer cell lines in A-549 (lung carcinoma), MDA-231 (breast carcinoma) and PC-3 (prostatic carcinoma).

Keywords: *Sapindus mukorossi*; triterpene saponin; oligosaccharide; cytotoxicity

1. Introduction

The Sapindaceae family, represented by 150 genera containing approximately 2000 species, is distributed in tropical and subtropical regions. It has been reported that 56 species of 25 genera have been investigated in China (Takagi et al. 1980; Nakayama et al. 1986; Tanaka et al. 1996). The fruits of *Sapindus mukorossi* Gaertn., well known as natural surfactants, exhibited significant antihelminthic, antimicrobial (Tamura et al. 2001; Ibrahim et al. 2006; Supradip et al. 2010) and molluscicidal (Huang et al. 2003; Upadhyay & Singh 2011) activities. Many saponins have been isolated and identified from the pericarps, stems, roots and galls of *S. mukorossi*, including sesquiterpene oligoglycosides (Kasai et al. 1986) and hederagenin-type (Kimata et al. 1983), tirucallane-type (Ni et al. 2004, 2006) and dammarane-type saponins (Teng et al. 2003; Kuo et al. 2005). In addition, fatty acids (Wu et al. 2012) and flavonoids (Zikova & Krivenchuk 1970) from *S. mukorossi* were also investigated. In this work, we describe the isolation of one new triterpenoid saponin and oligosaccharide from *S. mukorossi*. Their structures were elucidated on the basis of nuclear magnetic resonance (NMR) analyses, including one-dimensional (1D) and two-dimensional (2D) NMR spectroscopy and mass spectrometry. All compounds were evaluated for their cytotoxicity against A-549, MDA-231 and PC-3 cell lines.

2. Results and discussion

Air-dried fruits of *S. mukorossi* were crushed and extracted with MeOH. The concentrated extract was suspended in H_2O and successively extracted with petroleum ether, EtOAc and

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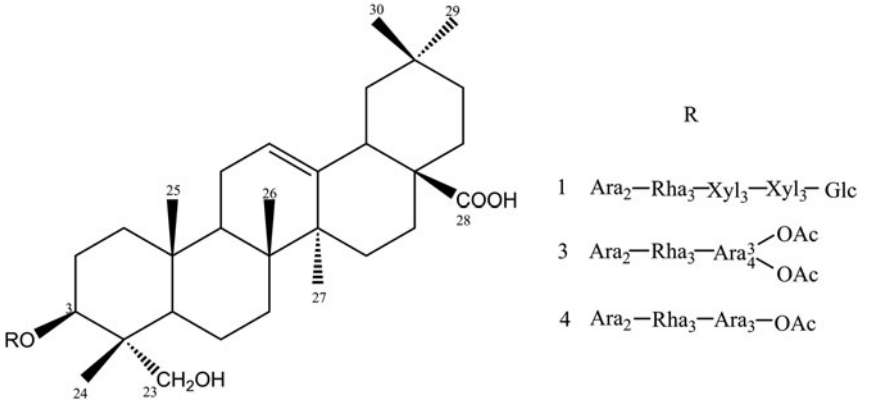

Figure 1. Structure of compound **1**, **3** and **4**

BuOH. The BuOH extract was subjected to sequential column chromatography over silica gel, Sephadex LH-20 and RP-18 gel to yield five compounds (**1**–**5**).

Compound **1** (Figure 1) was isolated as a white amorphous powder and its molecular formula was determined to be $C_{57}H_{92}O_{25}$ on the basis of the negative-ion high-resolution electrospray ionisation mass spectrometry (HR-ESI-MS). The ^{1}H NMR spectrum of **1** revealed six singlet methyl groups [δ_H 0.95, 0.97, 1.04, 1.06, 1.14 and 1.28], a secondary methyl group [δ_H 1.59 (3H, d, $J = 5.8$ Hz)], an olefinic proton [δ_H 5.50 (1H, br s)], an oxygen bearing methine proton [δ_H 4.30 (1H, dd, $J = 12.0$, 4.2 Hz)] and two hydroxymethyl protons [δ_H 3.93 (1H, d, $J = 10.1$ Hz) and 4.32 (1H, d, $J = 10.1$ Hz)]. The ^{13}C NMR and DEPT spectra revealed 57 signals comprising 7 methyls, 15 methylenes, 27 methines and 8 quaternary carbons. Six signals, at δ_C 14.7, 16.6, 18.0, 24.3, 26.7 and 33.8, have been assigned to angular methyl groups. Two signals, at δ_C 123.1 (C-12) and 145.3 (C-13), were assigned to olefinic carbons. The shielded chemical shift at δ_C 180.8 (C-28) suggests a carboxy group. The structure of **1** is characteristic of the oleanolic acid skeleton, by comparing its ^{1}H and ^{13}C NMR data (Tables S1 and S2) with those of congeners from *S. mukorossi* (Huang et al. 2008). In addition, five anomeric protons at δ_H 5.07 (1H, d, $J = 7.3$ Hz, Ara H-1′), δ_H 6.33 (1H, br s, Rha H-1″), δ_H 5.31 (1H, d, $J = 7.1$ Hz, Xyl-1‴), δ_H 5.35 (1H, d, $J = 7.4$ Hz, Xyl-1⁗) and δ_H 5.09 (1H, d, $J = 6.8$ Hz, Glc-1⁗′) were observed coupled with their corresponding anomeric carbons at δ_C 105.1 (C-1′), 101.9 (C-1″), 107.9 (C-1‴), 108.0 (C-1⁗) and 105.2 (C-1⁗′), respectively. Compound **1** was assumed to contain five sugar units. Evidence for the sugar linkages to the aglycone at C-3 is given by the HMBC cross-peaks δ_H 5.07 (Ara H-1′)/δ_C 81.7 (Agly C-3). Moreover, the sequence of the 3-*O*-oligosaccharidic moiety was assigned by HMBC experiments. HMBC cross-peaks (see Supplementary Material, Figure S1) at δ_H 6.33 (Rha H-1″)/δ_C 75.6 (Ara C-2′), at δ_H 5.31 (Xyl H-1‴)/δ_C 83.4 (Rha C-3″), at δ_H 5.35 (Xyl H-1⁗)/δ_C 83.5 (Xyl C-3‴) and at δ_H 5.09 (Glc H-1⁗′)/δ_C 81.7 (Xyl C-3⁗) suggests a Glc-(1 → 3)-Xyl-(1 → 3)-Xyl-(1 → 3)-Rha-(1 → 2)-Ara sequence. This was confirmed by the ROESY cross-peaks at δ_H 6.33 (Rha H-1″)/δ_H 4.58(Ara H-2′), at δ_H 5.31 (Xyl H-1‴)/δ_H 4.73 (Rha H-3″), at δ_H 5.35 (Xyl H-1⁗)/δ_H 4.75 (Xyl H-3‴) and at δ_H 5.09 (Glc H-1⁗′)/δ_H 4.27 (Xyl H-3⁗). The sugars were determined by GC–MS analysis to be D for glucose and xylose and L for arabinose and rhamnose. On the basis of the previous results, the structure of compound **1** was elucidated as hederagenin-3-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside.

Compound **2** was obtained as a white amorphous powder. A molecular formula of $C_{36}H_{62}O_{27}$ was deduced by HR-ESI-MS. The ^{1}H and ^{13}C NMR spectra were superimposable, and indicated signals that can be assigned to two β-D-glucopyranosyl [δ_H 4.75 (1H, d,

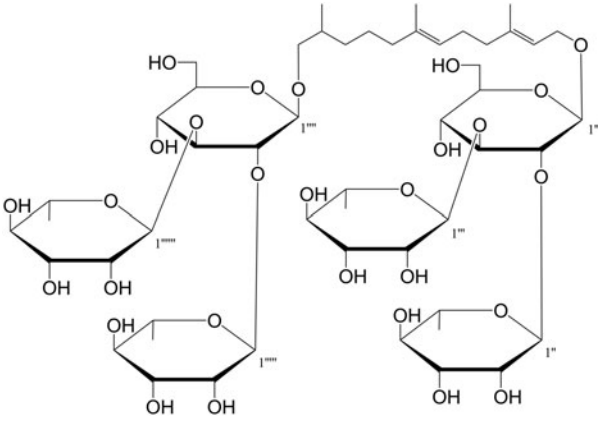

Figure 2. Structure of compound **5**.

$J = 7.8$ Hz, Glc H-1′), 4.67 (1H, d, $J = 7.8$ Hz, Glc H-1‴″)] and four α-L-rhamnopyranosyl moieties [δ_H 5.72 (1H, br s, Rha H-1″), 5.70 (1H, br s, Rha H-1‴), 5.74 (1H, br s, Rha H-1⁗), 5.68 (1H, br s, Rha H-1⁗″); 1.69 (3H, Rha Me-6″), 1.63 (3H, Rha Me-6‴), 1.67 (3H, Rha Me-6⁗) and 1.63 (3H, Rha Me-6⁗″)]. The comparison between the ^{13}C NMR data for **2** (Table S3) and **5** allows the identification of the same sugar moieties. Compound **5** (Figure 2) was identified to be the known mukurozioside IIa (Morikawa et al. 2010). Compound **2** consists only of sugar units, which is the essential difference between **2** and **5**. The HMBC spectrum of **2** (see Supplementary Material, Figure S2) indicated correlations at δ_H 5.72 (Rha H-1″)/δ_C 78.6 (Glc C-2′), at δ_H 5.70 (Rha H-1‴)/δ_C 87.7 (Glc C-3′), at δ_H 5.74 (Rha H-1⁗)/δ_C 78.6 (Glc C-2‴′) and at δ_H 5.68 (Rha H-1⁗″)/δ_C 87.7 (Glc C-3‴′), which permitted the assignment of rhamnopyranosyl moieties at C-2 and C-3 of both glucosepyranose units. The presence of these linkages was also concluded from the observed cross-peaks at δ_H 5.72 (Rha H-1″)/δ_H 4.05 (Glc H-2′), at δ_H 5.70 (Rha H-1‴)/δ_H 4.08 (Glc H-3′), at δ_H 5.74 (Rha H-1⁗)/δ_H 4.02 (Glc H-2‴′) and at δ_H 5.68 (Rha H-1⁗″)/δ_H 4.11 (Glc H-3‴′) in the ROESY spectrum. The sugars were determined to be D for glucose and L for rhamnose, by GC–MS analysis. On the basis of the above-mentioned evidence, the structure of **2** was determined to be α-L-rhamnopyranosyl-(1 → 3)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranosyl-(1 → 1)-β-D-glucopyranosyl-[(2 → 1)-α-L-rhamnopyranosyl]-(3 → 1)-α-L-rhamnopyranose (Figure 3).

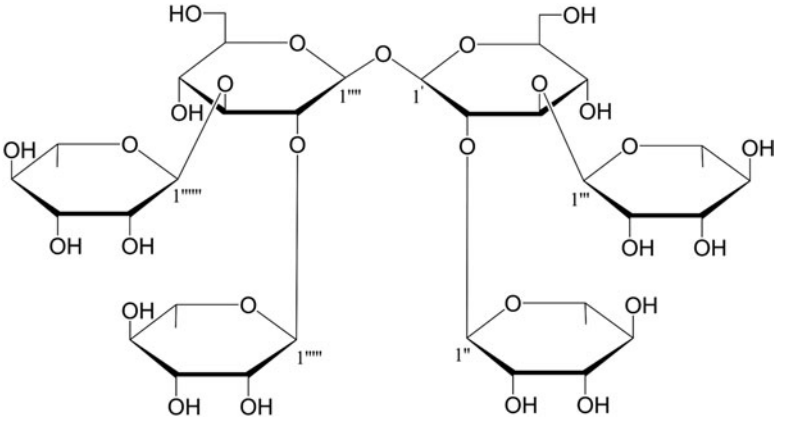

Figure 3. Structure of compound **2**.

Compounds **3**–**5** were identified as the known compounds, mukurozisaponin G (**3**), rarasaponin III (**4**) and mukuroziosides IIa (**5**). The cytotoxic activities of these glycosides against three human cancer cell lines (A-549, MDA-231 and PC-3) were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Table S4). Earlier report on this plant proved that *S. mukorossi* extracts exhibited cytotoxic effects on various human cancer cell lines (Chen et al. 2010). Herein, compounds **1** and **3–5** were able to inhibit the growth of the three cell lines at a test concentration. In particular, compound **1** displayed a high inhibitory activity, with the IC_{50} values of 17.19, 51.49 and 41.83 $\mu g\,mL^{-1}$ in A-549, MDA-231 and PC-3 cells, respectively. Compounds **3** and **5** exhibited moderate cytotoxicity ($IC_{50} > 100\ \mu g\,mL^{-1}$), while compound **2** was inactive on the three cell lines mentioned earlier.

3. Experimental

3.1. *General procedures*

Pre-coated silica gel plates (GF-254, Qingdao Haiyang Chemical Co., Qingdao, China) were used for TLC testing of the glycosides. Silica gel (100–200 mesh, Qingdao Marine Chemical Ltd, Qingdao, China), Sephadex LH-20 (Lanji Biosciences, Shanghai, China), silica gel RP-18 (Spherical C18, 120 Å, 30–50 μm, DAISO Co., Tokyo, Japan) and HPLC separation was carried out on an Waters HPLC system (Waters 600 Quat Pump, 2487 Dual λ Absorbance Detector, Waters Co., San Diego, CA, USA). Optical rotation was measured with a Perkin Elmer 341 polarimeter (Perkin-Elmer, Norwalk, CT, USA). IR spectra were obtained on a TENSOR37 Fourier Transform Infrared spectrometer (Bruker Co., Karlsruhe, Baden-Württemberg, Germany). ESI-MS data were obtained using a Finnigan-TSQ-Quantum instrument (Thermo Fisher, Woburn, MA, USA), and GC–MS data using a TRACE DSQ spectrometer (Thermo Fisher, Woburn, MA, USA). HR-ESI-MS was detected on a Thermo LTQ Orbitrap Elite (Thermo Fisher, Woburn, MA, USA). Deuterated solvents (used for recording NMR spectra) were pyridine-d_5 of the Cambridge Isotope Laboratories (Tewksbury, MA, USA). The 1D and 2D NMR spectra were recorded on a Bruker Avance III 400-MHz spectrometer (Bruker Co., Karlsruhe, Baden-Württemberg, Germany). MTT assay was measured in Microplate reader FlexStation 3.

3.2. *Plant material*

The fruits of *S. mukorossi* were collected from Guangdong Province, China, in October 2011. The plant was identified by Professor Yangdepo at Sun Yat-Sen University, and a voucher specimen (No. 20111001) has been deposited at the Institute of Natural Products and TCM, Sun Yat-Sen University, China.

3.3. *Extraction and isolation*

The dried fruits of *S. mukorossi* (3.5 kg) were extracted with MeOH (7 L × 3) at 60°C. The methanol extract was suspended in water and successively partitioned with petroleum ether, EtOAc and *n*-butanol (each 2.5 L × 3). The *n*-butanol layer was concentrated under reduced pressure and the residue was first separated by chromatography on a silica gel column with gradient mixture of $CHCl_3$/MeOH (20:0.5, 20:1, 20:2, 20:3, 20:4) to yield five fractions (1–5). Fr. 2 (10.2 g) was applied to Sephadex LH-20 ($CHCl_3$/MeOH, 1:1) to afford three fractions (Frs 2.1–2.3). Fr. 2.2 was further subjected to silica gel separation ($CHCl_3$/MeOH, 20:0.1 to 20:2) to yield **3** (13.1 mg) and **4** (15.2 mg). Fr. 5 (20.5 g) was applied to a silica gel column using a mixture of $CHCl_3$/MeOH (20:0.3 to 20:4) to yield four fractions (Frs 5.1–5.4). Frs 5.2 and 5.4 were purified by preparative HPLC, and eluted with stepwise gradient mixture of MeOH/H_2O (40–100%). As a result, Fr. 5.2 afforded **1** (12.7 mg) and Fr. 5.4 yielded **2** (17.1 mg) and **5** (33.1 mg).

3.4. *Acid hydrolysis and GC analysis*

Compounds **1** and **2** (4.0 mg each) were refluxed with 1 M HCl–dioxane (1:1 v/v, 2 mL) at 80°C (in a water bath) for 4 h. The reaction mixture was neutralised with Na_2CO_3 and filtered. The filtrate was successively extracted with $CHCl_3$ and H_2O. The aqueous layer was evaporated to dryness. The dried sugar residues were diluted in 1.0 mL pyridine without water, and then 3 mg L-cysteine methyl ester hydrochloride in 0.5 mL pyridine was added to the sugar solution. The mixture was stored at 60°C for 1 h, and trimethylsilylated with hexamethyldisilazane–trimethylchlorosilane (0.5 mL) at 60°C for another 30 min. After drying the solution in a stream of N_2, the residue was extracted with hexane (1.5 mL). The hexane layer was analysed by GC–MS using the following conditions: DB-5MS quartz capillary column (30 m × 0.25 mm); injection temperature: 250°C; column temperature: 150–250°C; rate: 4°C/min. Under these conditions, the sugars of each reactant were identified by comparing with authentic samples: t_R (min) D-glucose (13.89), L-glucose (11.90), D-xylose (9.22), L-xylose (8.20), D-arabinose (6.90), L-arabinose (6.36) and L-rhamnose (6.58).

Hederagenin-3-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside (**1**): white amorphous powder; $[\alpha]_D^{20} + 11.0°$ ($c = 0.2$, MeOH); IR γ_{max} KBr cm^{-1}: 3389, 3367, 2941, 1698, 1051, 1033; HR-ESI-MS *m/z* 881.4903 $[M-H-Xyl-Glc]^-$; ESI-MS/MS(–): *m/z* = 1221 $[M + HCOO]^-$, 1174, 923, 904, 881, 603; ^{1}H NMR (400 MHz, pyridine-d_5) δ: 1.11 (1H, overlapped 'os', H-1α), 1.60 (1H, m, H-1β), 2.02 (1H, os, H-2α), 2.25 (1H, m, H-2β), 4.30 (1H, dd, $J = 12.0, 4.2$ Hz, H-3), 1.83 (1H, os, H-5), 1.49 (1H, os, H-6α), 1.52 (1H, os, H-6β), 1.29 (1H, os, H-7α), 1.67 (1H, os, H-7β), 1.79 (1H, os, H-9), 1.96 (1H, os, H-11α), 2.10 (1H, m, H-11β), 5.50 (1H, br s, H-12), 1.17 (1H, os, H-15α), 2.15 (1H, m, H-15β), 1.83 (1H, os, H-16α), 2.13 (1H, os, H-16β), 3.31 (1H, br dd, $J = 11.2, 4.2$ Hz, H-18), 1.28 (1H, os, H-19α), 1.80 (1H, os, H-19β), 1.25 (1H, os, H-21α), 1.46 (1H, m, H-21β), 1.82 (1H, os, H-22α), 2.06 (1H, os, H-22β), 3.93 (1H, d, $J = 10.1$ Hz, H-23α), 4.32 (1H, d, $J = 10.1$ Hz, H-23β), 1.14 (3H, s, H-24), 0.97 (3H, s, H-25), 1.06 (3H, s, H-26), 1.28 (3H, s, H-27), 0.95 (3H, s, H-29), 1.04 (3H, s, H-30), 5.07 (1H, d, $J = 7.3$ Hz, H-1′), 6.33 (1H, br s, H-1″), 5.31 (1H, d, $J = 7.1$ Hz, H-1‴), 5.35 (1H, d, $J = 7.4$ Hz, H-1⁗), 5.09 (1H, d, $J = 6.8$ Hz, H-1⁗′). ^{13}C NMR (100 MHz, pyridine-d_5) δ: 39.6 (C-1), 26.9 (C-2), 81.7 (C-3), 44.1 (C-4), 48.7 (C-5), 20.1 (C-6), 33.4 (C-7), 40.3 (C-8), 48.2 (C-9), 37.4 (C-10), 24.2 (C-11), 123.1 (C-12), 145.3 (C-13), 42.7 (C-14), 28.9 (C-15), 24.4 (C-16), 47.2 (C-17), 42.5 (C-18), 47.0 (C-19), 31.5 (C-20), 34.8 (C-21), 33.7 (C-22), 64.6 (C-23), 14.7 (C-24), 16.6 (C-25), 18.0 (C-26), 26.7 (C-27), 180.8 (C-28), 33.8 (C-29), 24.3 (C-30), 105.1 (C-1′), 75.6 (C-2′), 73.7 (C-3′), 70.0 (C-4′), 66.6 (C-5′), 101.9 (C-1″), 71.6 (C-2″), 83.4 (C-3″), 73.5 (C-4″), 70.1 (C-5″), 18.9 (C-6″), 107.9 (C-1‴), 75.0 (C-2‴), 83.5 (C-3‴), 72.5 (C-4‴), 67.9 (C-5‴), 108.0 (C-1⁗′), 75.7 (C-2⁗′), 81.7 (C-3⁗′), 72.5 (C-4⁗′), 67.6 (C-5⁗′), 105.2 (C-1⁗′), 75.6 (C-2⁗′), 78.9 (C-3⁗′), 70.2 (C-4⁗′), 76.1 (C-5⁗′), 62.3 (C-6⁗′).

α-L-Rhamnopyranosyl-(1 → 3)-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranosyl-(1 → 1)-β-D-glucopyranosyl-[(2 → 1)-α-L-rhamnopyranosyl]-(3 → 1)-α-L-rhamnopyranose (**2**): white amorphous powder; $[\alpha]_D^{20} - 52.3°$ ($c = 0.2$, MeOH); IR γ_{max} KBr cm^{-1}: 3400, 3349, 2945, 1032; HR-ESI-MS *m/z* 1017.4729 $[M - H + 2CH_3CH_2OH]^-$; ESI-MS/MS(–): *m/z* = 1017, 926, 870, 724, 162; ^{1}H NMR (400 MHz, pyridine-d_5) δ: 4.75 (1H, d, $J = 7.8$ Hz, H-1′), 5.72 (1H, br s, H-1″), 5.70 (1H, br s, H-1‴), 4.67 (1H, d, $J = 7.8$ Hz, H-1⁗′), 5.74 (1H, br s, H-1⁗′), 5.68 (1H, br s, H-1⁗′). ^{13}C NMR (100 MHz, pyridine-d_5) δ: 103.6 (C-1′), 78.6 (C-2′), 87.7 (C-3′), 70.5 (C-4′), 78.8 (C-5′), 62.8 (C-6′), 104.3 (C-1″), 73.1 (C-2″), 72.9 (C-3″), 73.3 (C-4″), 70.5 (C-5″), 19.4 (C-6″), 104.3 (C-1‴), 73.3 (C-2‴), 73.1 (C-3‴), 74.3 (C-4‴), 71.2 (C-5‴), 19.0 (C-6‴), 103.5 (C-1⁗′), 78.6 (C-2⁗′), 87.7 (C-3⁗′), 70.5 (C-4⁗′), 78.8 (C-5⁗′), 62.8 (C-6⁗′), 104.3 (C-1⁗′), 73.2 (C-2⁗′), 73.3 (C-3⁗′), 74.1 (C-4⁗′), 70.5 (C-5⁗′), 19.2 (C-6⁗′), 104.3 (C-1⁗′), 73.1 (C-2⁗′), 73.1 (C-3⁗′), 74.3 (C-4⁗′), 71.1 (C-5⁗′), 19.0 (C-6⁗′).

3.5. In vitro *cytotoxicity assays*

A-549, MDA-231 and PC-3 were utilised in the MTT assay. All cells were obtained from the American Type Culture Collection (USA). The cell lines were grown in RPMI-1640 supplemented with 10% foetal bovine serum under a humidified atmosphere of 5% CO_2 at 37°C. Cell suspensions (100 μL) at a density of 5×10^4 cells mL^{-1} were plated in 96-well cell culture plates and incubated for 24 h. The test compound solutions (100 μL), at concentrations of 6.25, 12.5, 25, 50 and 100 μg mL^{-1}, were added to each well and further incubated for 24 h under the same conditions. MTT solution (20 μL of a 5 mg mL^{-1} solution in phosphate buffer saline) was added to each well and incubated for 4 h. The formazan crystals produced were dissolved in 100 μL of DMSO. Absorbance was then determined on a Multi-Mode Microplate Reader at 490 nm. Cytotoxicity was expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}), and the given values were calculated from the mean of three different experiments.

4. Conclusion

We conclude that a new hederagenin-type saponin (**1**) and a new oligosaccharide (**2**), together with three known saponins (**3**–**5**), were isolated from the fruits of *S. mukorossi* Gaertn. Compounds **1** and **3**–**5** exhibited moderate cytotoxicity against cancer cells.

Supplementary material

NMR and ESI-MS/MS spectra for the compounds **1** and **2** are available as Supplementary material, alongside Tables S1–S4 and Figures S1–S2.

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