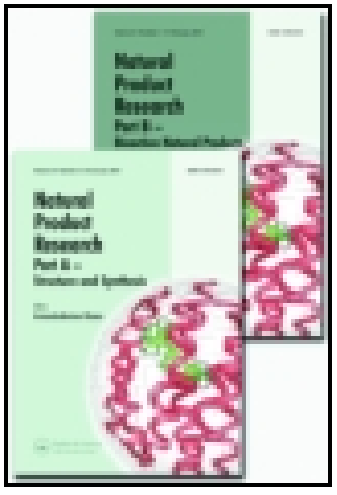

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Two new glycosides isolated from Sapindus mukorossi fruits: effects on cell apoptosis and caspase-3 activation in human lung carcinoma cells

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

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ABSTRACT

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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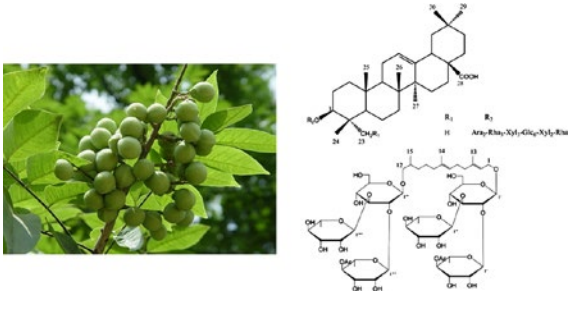
Sapindus mukorossi; glycosides; spectroscopic analysis; flow cytometric; apoptosis; caspase-3

GRAPHICAL ABSTRACT



1. Introduction

The genus *Sapindus* belongs to the Sapindaceae family, which consists of 2000 species (Rao et al. 2012). *Sapindus mukorossi* Gaertn, commonly known as Wu-huan-zi, is prevalent in southern China and has been used in the treatment of asthma, dermatological disorders and hepatic disorders (Sharma et al. 2011). According to the literature, there are several phytochemicals present in the pericarp, seeds, leaves, roots, stems and galls. The major constituents in the fruit are saponins (10.0–11.5%) and sugars (10%) (Verma 2012). The glycosides isolated

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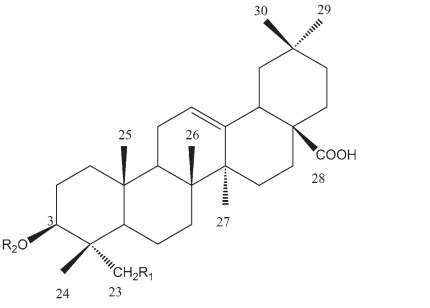
from *S. mukorossi* are mainly sesquiterpene oligoglycosides and triterpenoidal saponins of hederagenin, dammarane and tirucullane (Upadhyay & Singh 2012). These glycosides have antimicrobial (Ibrahim et al. 2006), cytotoxic (Chen et al. 2010), molluscicidal (Huang et al. 2003; Upadhyay & Singh 2011), insecticidal (Rahman et al. 2007), fungicidal (Supradip et al. 2010) and hepatoprotective (Peng et al. 2014) properties. In this study, we isolated and characterized two new glycosides and evaluated the *in vitro* antiproliferative activity of all compounds against A549 cells.

2. Results and discussion

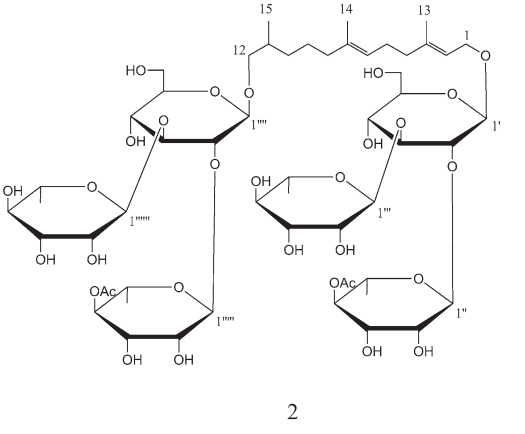
2.1. Structural elucidation

Compound **1** was a white amorphous powder. Its molecular formula was $C_{63}H_{102}O_{28}$ based on HR-ESI-MS and MS/MS data (Figure 1). The ^{1}H NMR spectrum had signals characteristic of seven tertiary singlet methyl groups [δ_H 0.87, 1.02, 1.04, 1.15, 1.33, 1.33, and 1.34], two secondary methyl groups [δ_H 1.57 (3H, d, J = 5.4 Hz) and 1.57 (3H, d, J = 5.4 Hz)], an olefinic proton [δ_H 5.50 (1H, br s)] and an oxygen-bearing methine proton [δ_H 3.32 (1H, overlapped 'os')]. The ^{1}H NMR spectrum of 1 revealed the presence of six anomeric proton signals at δ_H 4.88 (1H, d, J = 7.8 Hz, Ara H-1′), 6.23 (1H, br s, Rha H-1′′), 5.36 (1H, d, J = 7.4 Hz, Xyl H-1′′′), 4.90 (1H, d, J = 8.0 Hz, Glc H-1′′′′), 5.30(1H, d, J = 6.8 Hz, Xyl H-1′′′′′) and 6.25 (1H, br s, Rha H-1′′′′′′), which were correlated with the ^{13}C NMR signals for anomeric carbons at δ_C 105.6 (C-1′), 102.0 (C-1′′), 108.0 (C-1′′′), 105.7 (C-1′′′′), 107.8 (C-1′′′′′) and 102.1 (C-1′′′′′′), respectively. The corresponding seven angular methyl groups [δ_C 16.1, 17.7, 17.7, 24.3, 26.7, 28.7, and 28.7], olefinic signal [δ_C 123.0, 145.3] and carboxyl group [δ_C 180.7] were detected. These findings implied that compound **1** had an oleanane-type triterpene and six sugar moieties. The linkage points of the sugar units to each other and to the aglycone were determined by the following HMBC correlations (see supplementary material, Figure S1): δ_H 4.88 (Ara-1′) with δ_C 89.3 (aglycon C-3), δ_H 6.23 (Rha-1′′) with δ_C 75.9 (Ara C-2′), δ_H 5.36 (Xyl-1′′′) with δ_C 83.5 (Rha C-3′′), δ_H 4.90 (Glc-1′′′′) with δ_C 89.3 (Xyl C-3′′′), δ_H 5.30 (Xyl-1′′′′′) with δ_C 67.9 (Glc C-6′′′′) and δ_H 6.25 (Rha-1′′′′′′) with δ_C 83.3 (Xyl C-2′′′′′). The relative configuration of the aglucone and the sugar linkages were confirmed by ROESY spectrum (Figure S2). The relatively large coupling constants for the anomeric protons of compound **1** revealed an α-configuration of the arabinose unit and a β-configuration of the glucose and xylose units. Even though the anomeric protons of two rhamnose moieties were observed as singlets in the ^{1}H NMR spectrum, the ^{13}C NMR shifts of Rha C-5 at δ_C 69.7 and 70.0 indicated an α-configuration (Wang et al. 2013). Acid hydrolysis of compound **1** yielded L-arabinose, D-glucose, D-xylose and L-rhamnose, which were detected by GC-MS analysis of their derivatives. Accordingly, compound **1** was identified as 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl-(1→3)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl oleanolic acid, and was named sapindoside G.

Compound **2** was a white powder. The negative ion HR-ESI-MS spectra of compound **2** showed quasimolecular ion peaks at *m/z* 1231.56188 $[M-H]^-$; its molecular formula was $C_{55}H_{92}O_{30}$ (Figure 1). The ^{1}H and ^{13}C NMR spectra of compound **2** had signals characteristic of three methyl groups [δ_H 1.01 (3H, d, J = 7.2 Hz, Me-15), 1.60 (3H, s, Me-14) and 1.63 (3H, s, Me-13)]; two methylene groups bearing an oxygen function [δ_H 3.34 (1H, m, H-12α), 3.84 (1H, os, H-12β), 4.28 (1H, os, H-1α) and 4.56 (1H, os, H-1β)], two tri-substituted olefins [δ_H 5.21 (1H, m, H-6), 5.60 (1H, os, H-2)]; two β-D-glucopyranosyl moieties [δ_H 4.68 (1H, d, J = 7.2 Hz, Glc H-1′′′′) and 4.73 (1H, d, J = 7.2 Hz, Glc H-1′)]; four α-L-rhamnopyranosyl moieties [δ_H 1.61



	R_1	R_2
1	H	Ara_2-Rha_3-Xyl_3-Glc_6-Xyl_2-Rha
3	OH	Ara_2-Rha_3-Xyl_3-OAc
4	OH	Ara_2-Rha_2



2

Figure 1. Structure of compounds **1**–**4**.

(3H, Rha Me-6′′), 1.61 (3H, Rha Me-6′′′′′), 1.67 (3H, Rha Me-6′′′), 1.70 (3H, Rha Me-6′′′′′′), 5.62 (1H, br s, Rha-1′′′′′′), 5.63 (1H, br s, Rha-1′′′), 5.69 (1H, br s, Rha-1′′′′′) and 5.74 (1H, br s, Rha-1′′)]; and two acetyl groups [δ_H 2.00 (3H, s) and 2.09 (3H, s)]. Detailed analysis of 1D NMR, HSQC, ^{1}H-^{1}H COSY and HMBC spectra (Figure S3) established the assignment of an alkyl glycoside similar to mukurozioside IIa (Morikawa et al. 2010), except for the additional signals attributed to the acetyl groups. The binding sites for the acetyl groups of compound **2** were elucidated by HMBC and acetylation shifts. The presence of a correlation between δ_H 5.71 (Rha-4′′)/δ_C 171.3 and δ_H 5.71 (Rha-4′′′′′)/δ_C 171.4 revealed that the acetyl groups were located at 4′′ and 4′′′′′ position in the rhamnopyranosyl moiety. The acetylation shifts [δ_C 71.1 (Rha-3′′), 76.2 (Rha-4′′), 67.8 (Rha-5′′); δ_C 71.1 (Rha-3′′′′′), 76.3 (Rha-4′′′′′) and 67.8 (Rha-5′′′′′)] confirmed the assignment of the acetate groups. In the ROESY spectrum, correlations at H_2-1/H_3-13 and H_2-5/H_3-14 and the absence of any correlations at H-2/H_3-13 and H-6/H_3-14 indicated that the olefinic bonds in the aglucone were both *trans* relationships. The linkages between sugar units were also concluded from ROESY spectrum (Figure S4). Consequently, compound **2** was identified as 4′′,4′′′′′-*O*-diacetylmukurozioside IIa.

By comparing the physical and spectroscopic data with those reported in the literature (Chirva et al. 1970; Nakayamak et al. 1986), compounds **3** and **4** were identified as Hishoushisaponin Ee and Sapindoside A, respectively.

2.2. Cell viability assay

MTT assay results revealed that A549 cell growth was inhibited in a dose-dependent manner by compounds **1**–**4**. All four compounds exhibited inhibitory effects with inhibition rates up to 69.2–83.3% at a concentration of 100 μg/mL (Table S1). Compounds **1**, **3** and **4** had better inhibitory effects than compound **2** against A549 cells, with IC_{50} values of 33.61 ± 0.24, 46.75 ± 0.07 and 33.19 ± 0.13 μg/mL, respectively. Compound **2** had an IC_{50} value of 98.35 ± 0.18 μg/mL.

2.3. Cell cycle and cell apoptosis

To further confirm the induction of cell apoptosis, cells were stained with annexin V/PI for flow cytometry. As shown in Figure S5, the early apoptotic rates of A549 cells were 52.4%

(**1**), 4.9% (**2**), 52.4% (**3**) and 56.6% (**4**). Compared to untreated A549 cells, the percentage of late apoptotic cells increased by 1.8% (**1**), 3.3% (**2**), 13.9% (**3**) and 8.1% (**4**) following 24-h treatment. Compound **2** had a weaker effect on A549 cells apoptosis than the other three compounds. Late apoptosis was induced in the test concentration of compound **2**. Therefore, the inhibition of A549 cell growth by glycosides is attributed to an induction in cell apoptosis. Compound **2** has different inhibitory mechanisms than compounds **1**, **3** or **4**. For cell cycle analysis, the percentages of cells in G0/G1, S and G2/M phases were determined by flow cytometry. The effect of compounds **1**–**4** on the cell cycle distribution of A549 cells is shown (Figure S6). As a result, the compounds had little influence in the A549 cell cycle arrest.

2.4. Induction of apoptosis by activating caspase-3

Caspase family proteins play crucial roles in cell apoptosis. To assess whether compounds **1**–**4** activate the caspase-dependent cell death pathway, we studied the activation of caspase-3 using colorigenic tetrapeptide substrates such as Ac-DEVD-pNA, which is selective for caspase-3 enzymatic activities. Compared to the control group, compound-treated groups had higher absorbance measurements at 405 nm. After a 2-h treatment with compound **1**, caspase-3 activity increased from 1.44 ± 0.26 μM pNA/2 h of protein to 4.33 ± 0.48 μM pNA/2 h of protein in a concentration-dependent manner (Figure S7). The activity of the enzyme increased in the presence 6.25–75 μg/mL of compounds **2**–**4** and decreased in the presence of 100 μg/mL of compounds **2**–**4**. These results indicated that compounds **1**–**4** from *S. mukorossi* activated caspase-3. A high caspase-3 activity was observed during the early apoptotic stage. The highest caspase-3 enzymatic activities obtained were 4.33 ± 0.48 μM pNA/2 h (with 100 μg/mL of 1), 5.09 ± 0.17 μM pNA/2 h (with 75 μg/mL of **3**) and 3.47 ± 0.40 μM pNA/2 h (with 75 μg/mL of **4**). Compound **2** had a lower induction of caspase-3 activity, consistent with the apoptotic rate described in the flow cytometry results.

3. Conclusions

Two new glycosides (**1**, **2**) and two saponins (**3**, **4**) were isolated from the fruits of *S. mukorossi* Gaertn. Compounds **1**, **3** and **4** showed inhibition of A549 cell growth in a dose-dependent manner by promoting cell apoptosis via activation of caspase-3. Studies are in progress to elucidate the possible mechanism of action of these glycosides.

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Supplemental data and research materials

Supplemental data for this article can be accessed at http://dx.doi.10.1080/14786419.2015.1054283.

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