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Steroidal saponins and cytoxicity of the wild edible vegetable—*Smilacina atropurpurea*

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ABSTRACT

Four new steroidal saponins, smilacinoside A (**1**), B (**2**), C (**3**), and D (**4**), together with three known saponins, funkioside D (**5**), aspidistrin (**6**) and 26-O-β-D-glucopyranosyl-22-methoxyl-(25R)-furost-5-en-3β,26-diol 3-O-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (**7**) were isolated from the dried tender aerial parts of *Smilacina atropurpurea* (Franch.) Wang et Tang. The structures of new compounds were elucidated as diosgenin 3-O-α-L-rhamnopyranosyl-(1 → 2)-O-β-D-galactopyranoside (**1**), diosgenin 3-O-α-L-rhamnopyranosyl-(1 → 3)-[6-O-palmitoxyl]-O-β-D-galactopyranoside (**2**), 26-O-β-D-glucopyranosyl-(25R)-furost-5-en-3β,22ξ,26-triol 3-O-α-L-rhamnopyranosyl-(1 → 2)}-β-D-galactopyranoside (**3**) and 26-O-β-D-glucopyranosyl-22-methoxyl-(25R)-furost-5-en-3β,26-diol 3-O-α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranoside (**4**) on the basis of chemical methods and detailed spectroscopic analysis, including 1D and 2D NMR techniques and single-crystal X-ray diffraction, respectively. Six of these compounds and MeOH extract were tested for their *in vitro* cytotoxicity toward K562 human tumor cells by an improved MTT method. Smilacinoside A, funkioside D and aspidistrin exhibited significant *in vitro* cytotoxicity against K562 with IC_{50} values of 1.09, 2.93 and 0.47 μg/mL, respectively.

1. Introduction

Smilacina atropurpurea (Franch.) Wang et Tang [1] (Liliaceae) is a traditional wild edible vegetable by the minority Tibetan, Lisu, Naxi people in southwest of the People's Republic of China. The tender aerial part like bamboo is called "Zhuye-cai", and a delicious dish as a soup or stir-frying together with meat. The nutrient components of it have been measured with rich amino acid and mineral elements abundantly [2–4]. The rhizome, as a folk traditional medicine, has been used for the treatment of lung ailment, rheumatism, menstrual disturbance, cuts, and bruises [5]. Three nucleosides from the tender aerial part [6] and eight steroidal saponins from the rhizome part [7] have been reported previously. But no chemical constituents have been reported on the steroidal saponins from the tender aerial part of it up to now. Our detailed chemical investigation on the tender aerial part of *S. atropurpurea* let to the isolation of four new steroidal saponins, smilacinoside A (**1**), B (**2**), C (**3**), and D (**4**), together with three known steroidal saponins, funkioside D (**5**), aspidistrin (**6**) and 26-O-β-D-glucopyranosyl-22-OCH_3-(25R)-furost-5-en-3β,26-diol 3-O-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (**7**). In this present paper, we report the isolation and structure elucida-

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tion of new compounds. Additionally, the *in vitro* cytotoxicity toward K562 human tumor cells of them are also described, and showed a significant cytotoxicity.

2. Experimental

2.1. General methods

Melting points were determined on an X-4 Digital Melting apparatus and are uncorrected. Optical rotations were measured with a Horiba SEAP-300 polarimeter. IR spectra were recorded on a Bio-Rad FTS-135 spectrometer with KBr pellets. ^{1}H and ^{13}C NMR experiments were performed in pyridine-d_5 on a Bruker AM-400 spectrometer, while 2D NMR spectra were recorded using a Brucker DRX-500 instrument. FAB MS and HRFAB MS were taken on a VG Auto Spec-3000 or on a Finning-MAT 90 instrument. Crystallographic data were measured on an MAC DIP-2030K diffractometer with a graphite monochromator, Mo Kα radiation. Column chromatography was performed using 200–300 mesh silica gel (Qingdao Marine Chemical Inc., Qingdao, PR China), Lichroprep RP-18 (40–63 μm, Merck, Darmstadt, Germany), and Lewapol macroporous resin (Bayer, Germany). Fractions were monitored by TLC with silica gel H (50 mm × 100 mm, Qingdao Marine Chemical Inc., Qingdao, PR China), and spots were visualized by heating silica plates sprayed with 1% vanillin–10% H_2SO_4 in EtOH (w/(v/v)).

2.2. Plant material

The dry tender aerial parts of *S. atropurpurea* (Franch.) Wang et Tang were collected in July 2002 from Nujiang, northwest of Yunnan, China, and identified by Professor Chun-Chao Lv of the Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (KIB200207102) has been deposited at the State Key laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Science.

2.3. Extraction and isolation

The air-dried and powdered tender aerial parts (1040 g) of *S. atropurpurea* (Franch.) Wang et Tang were extracted with MeOH (4 × 5 L) at room temperature and concentrated under vacuum to give a black syrup-like extract (202 g). The concentrated extract (202 g) was suspended in water (1000 mL) and divided into water-soluble part and -insoluble part (A, 2 g). The water-soluble part was directly subjected to column chromatography over Lewapol macroporous resin (1000 g) eluted with H_2O and MeOH (5000 mL eluent) to obtain two portions. The H_2O portion was extracted with *n*-BuOH to give to fraction C (40 g). The MeOH portion was concentrated to dryness (fraction B, 58 g) and further chromatographed over a silica gel (60 μm, 5000 g) column eluted with $CHCl_3$–MeOH–H_2O (7:3:0.5, 6000 mL) to give five fractions I–V by monitoring with silica gel TLC. Fractions III (2 g) and IV (11 g) were further chromatographed on a silica gel (200–300 mesh, 300 g) column eluted with $CHCl_3$–CH_3OH–H_2O (8:2:0.3) to yield compound **1** (2.648 g). Fraction V (34 g) was chromatographed on a silica gel (200–300 mesh, 700 g) column eluting with $CHCl_3$–CH_3OH–H_2O (7.5:2.5:0.5) and RP-18 column (40–63 μm, 300 g) eluted with MeOH–H_2O (70%) to yield compound **3** (1.74 g), compound **4** (2.32 g), compound **5** (12 mg), compound **6** (10 mg), compound **7** (105 mg), respectively. Fraction A was chromatographed on a silica gel (200–300 mesh, 50 g) column eluted with $CHCl_3$–CH_3OH–H_2O (9:1:0.1) to yield compound **2** (127 mg). Fraction C was chromatographed on a silica gel (200–300 mesh, 900 g) eluted with $CHCl_3$–CH_3OH–H_2O (7:3:1) to yield compounds **1** (1.68 g), compound **5** (64 mg), compound **6** (142 mg).

2.3.1. *Smilacinoside A (1)*

Colorless needle crystal, m.p. 298–300 °C (MeOH–$CHCl_3$); $C_{39}H_{62}O_{12}$; $\alpha_D^{28.7} - 86.73°$ (*c* 0.49, pyridine). IR ν_{max} (KBr) 3476 (OH), 1634, 1052, 981, 919, 899 (899 > 919), 865 cm^{-1}. FAB$^+$MS: *m/z* (%) 723 (85) $[M+H]^+$, 577 (13) $[M+H\text{-}146\ (Rha)]^+$, 415 (54) $[M+H\text{-}146\ (Rha)\text{-}162\ (Gal)]^+$, 397 (100) $[M+H\text{-}146\ (Rha)\text{-}162\ (Gal)\text{-}18\ (H_2O)]^+$. HRFABMS: *m/z* 721.4177 $[M(C_{39}H_{62}O_{12})-H]^-$ (calcd. for 721.4163). ^{1}H NMR (400 MHz, pyridine-d_5): δ 0.68 (d, J = 4.9 Hz, 3H, H-27), 0.81 (s, 3H, H-18), 0.95 (m, 1H, H-9), 1.05 (s, 3H, H-19), 1.08 (m, 1H, H-14), 1.13 (d, J = 6.8 Hz, 3H, H-21), 1.69 (d, J = 6.1 Hz, 3H, Rha CH_3-6), 1.78 (dd, J = 8.2, 6.6 Hz, 1H, H-17), 1.93 (dd, J = 6.6, 8.2 Hz, 1H, H-20), 3.51 (t, J = 10.1 Hz, 1H, H-26α), 3.58 (t, J = 10.1 Hz, 1H, H-26β), 3.96 (m, 1H, H-3), 4.41 (d, J = 6.4 Hz, 2H, Gal H-6), 4.53 (m, 1H, H-16), 4.98 (d, J = 7.7 Hz, 1H, Gal H-1), 5.28 (brs, 1H, H-6), 6.30 (brs, 1H, Rha H-1) ppm. ^{13}C NMR (400 MHz, pyridine-d_5), see Table 1.

2.3.2. *X-ray crystal structure analysis of 1*

Crystallographic data: $C_{39}H_{62}O_{12}\cdot 2H_2O$, monoclinic, space group C2, a = 23.464(6), b = 7.129(1), c = 25.466(6) Å, β = 95.846(10)°, V = 4200.6(6) Å^3, Z = 4, d = 1.200 g/cm^3, crystal dimensions 0.04 mm × 0.08 mm × 0.30 mm were used for measurements on an MAC DIP-2030K with a graphite monochromator (ω scans, $2\theta_{max}$ = 50.0°), Mo Kα radiation. The total number of reflections measured was 3053 unique, of which 2390 were observed. Final indices: R_f = 0.086. R_w = 0.082 ($w = 1/\delta|F|^2$) for observed reflections. The crystal structure was solved by the direct method SHELXS-97 and expanded using difference Fourier techniques, refined by the program and method SHELX-97 (Sheldrick, G.M., SHELX-97, Program for Crystal Structure Refinement; University of Gottingen, Germany, 1997) and the full-matrix least-squares calculations.

2.3.3. *Acid hydrolysis of* **1**

A solution of **1** (10 mg) in 1 M HCl–H_2O (20 mL) was heated at 95 °C for 5 h. The reaction mixture was directly monitored by silica gel TLC using MeOH or $CHCl_3$–MeOH (4:1) as development and anisaldehyde–H_2SO_4 as detection, comparing with the authentic samples: L-rhamnose (R_f 0.38, MeOH), D-galactose (R_f 0.30, MeOH) and diosgenin (R_f 0.81, $CHCl_3$/MeOH = 4:1), respectively. The result showed rhamnose, galactose and diosgenin were existed in the reaction mixture.

2.3.4. *Smilacinoside B (2)*

White amorphous powder, m.p. 236–238 °C (MeOH–$CHCl_3$); $C_{55}H_{92}O_{13}$; $\alpha_D^{28.7} - 86.73°$ (*c* 0.49, pyridine). IR ν_{max} (KBr) 3431 (OH), 2925, 2852, 1730 (C=O), 1631, 1455, 1376, 1242, 1173, 1054, 982, 919, 900 (900 > 919), 866, 837, 814, 781,

Table 1 – ^{13}C NMR data of smilicinoside A–D (in pyridine-d_5, ppm)

No.	2	1	3	4	Sugar	2	1	3	4
1	37.7t	37.6t	37.7t	37.6t	Gal				
2	32.4t	30.3t	30.4t	30.3t	1	101.0d	100.7d	100.9d	100.9d
3	78.6d	77.8d	78.0d	78.0d	2	73.4d	76.0d	76.1d	76.1d
4	39.2t	39.0t	39.1t	39.1t	3	75.7d	76.6d	76.6d	76.6d
5	141.0s	140.9s	141.1s	140.8s	4	70.8d	70.9d	70.9d	71.0d
6	121.7d	121.7d	121.9d	121.7d	5	76.2d	76.7d	76.7d	76.7d
7	32.3t	32.3t	32.5t	32.2t	6	64.5t	62.4t	62.4t	62.4t
8	31.8d	31.7d	31.8d	31.8d	Rha				
9	50.4d	50.3d	50.5d	50.4d	1	102.1d	102.1d	102.2d	102.0d
10	37.2s	37.2s	37.3s	37.2s	2	72.6d	72.6d	72.6d	72.6d
11	21.2t	21.1t	21.2t	21.1t	3	72.9d	72.9d	73.0d	72.9d
12	40.0t	39.8t	40.1t	39.8t	4	74.2d	74.2d	74.2d	74.3d
13	40.5s	40.5s	40.8s	40.6s	5	69.4d	69.4d	69.5d	69.4d
14	56.8d	56.6d	56.7d	56.7d	6	18.6q	18.6q	18.7q	18.6q
15	32.3t	32.2t	32.6t	32.4t	Glc_{26}				
16	81.2d	81.1d	81.3d	81.4d	1			105.0d	105.0d
17	63.0d	62.9d	63.9d	63.0d	2			75.3d	75.2d
18	16.4q	16.4q	16.6q	16.3q	3			78.6d	78.7d
19	19.4q	19.4q	19.6q	19.4q	4			71.8d	71.9d
20	42.0d	42.0d	40.9d	40.8d	5			78.5d	78.4d
21	15.1q	15.1q	16.6q	16.2q	6			62.9t	62.9t
22	109.3s	109.3s	110.9s	112.7s	Palmitoxyl				
23	31.8t	31.8t	37.3t	37.6t	OC=O	173.5s			
24	29.3t	29.3t	28.5t	28.2t	$\underline{C}H_2COO$	34.5t			
25	30.6d	30.6d	34.4d	34.3d	$\underline{C}H_2CH_2CO$	25.5			
26	66.9t	66.9t	75.3t	75.2t	CH_2	29.5–30.1t			
27	17.4q	17.4q	17.6q	17.2q	$\underline{C}H_2CH_2CH_3$	32.4t			
22-OMe				47.4q	$\underline{C}H_2$–CH_3	23.0t			
					CH_3	14.3q			

720, 671, 642, 579 cm^{-1}. FAB-MS (MNBA): *m/z* (%) 1114 (65) [M+H+MNBA]$^-$, 960 (100) [M]$^-$, 721 (10) [M-239 (palmityl)]$^-$, 255 (87) [palmitatyl]$^-$. HRESIMS: *m/z* 960.6547 [M($C_{55}H_{92}O_{13}$)]$^-$ (calcd. for 960.6538). ^{1}H NMR (400 MHz, pyridine-d_5): δ 0.68 (d, J = 5.3 Hz, 3H, H-27), 0.87 (s, 3H, H-18), 0.89 (t, J = 7.3 Hz, 3H, palmitate CH_3-16), 0.94 (m, 1H, H-9), 1.07 (s, 3H, H-19), 1.11 (m, 1H, H-14), 1.14 (d, J = 7.0 Hz, 3H, H-21), 1.24–1.27 (overlap, 26H, palmitatyl CH_2 × 13), 1.66 (d, J = 6.1 Hz, 3H, Rha CH_3-6), 1.82 (dd, J = 7.8, 7.0 Hz, H, H-17), 1.96 (m, 1H, H-20), 2.37 (t, J = 7.6 Hz, 2H, palmitatyl CH_2-2), 3.50 (t, J = 10.3 Hz, 1H, H-26α), 3.58 (d, J = 11.9 Hz, 1H, H-26β), 3.95 (m, 1H, H-3), 4.57 (m, 1H, H-16), 4.70 (dd, J = 4.6, 7.6 Hz, 1H, Gal H-6β), 4.89 (dd, J = 7.6, 4.6 Hz, 1H, Gal H-6α), 4.95 (d, J = 8.0 Hz, 1H, Gal H-1), 5.32 (brs, 1H, H-6), 6.27 (brs, 1H, Rha H-1)ppm. ^{13}C NMR (400 MHz, pyridine-d_5), see Table 1.

2.3.5. *Smilacinoside C (3)*

White amorphous powder, m.p. 201–204 °C; $C_{45}H_{74}O_{18}$; $\alpha_D^{29.2}$ − 77.11° (*c* 0.15, C_5H_5N). IR ν_{max} (KBr) 3423 (OH), 2936, 1636, 1453, 1379, 1256, 1129, 1073, 1051, 913, 894, 838 cm^{-1}. FAB-MS (Gly): *m/z* (%) 976 (25) [M+Gly−H_2O], 901 (100) [M−H]$^-$, 755 (10) [M−H-146 (Rha)]$^-$, 593 (3) [M−H-146 (Rha)-162 (Gal)]$^-$. HRESIMS: *m/z* 901.4771 [M($C_{45}H_{74}O_{18}$)−H]$^-$ (calcd. for 901.4765). ^{1}H NMR (500 MHz, pyridine-d_5): δ 0.88 (s, 3H, H-18), 0.97 (d, J = 6.7 Hz, 3H, H-27), 1.05 (s, 3H, H-19), 1.33 (d, J = 6.2 Hz, 3H, H-21), 1.67 (d, J = 6.2 Hz, 3H, Rha CH_3-6), 3.60 (dd, J = 6.0, 11.2 Hz, 2H, H-26), 3.92 (1H, m, H-3), 5.31 (brs, 1H, H-6), 4.62 (m, 1H, H-16), 4.79 (d, J = 7.8 Hz, 1H, 26-*O*-Glc H-1), 4.95 (d, J = 7.6 Hz, 1H, Gal H-1), 6.27 (s, 1H, Rha H-1) ppm. ^{13}C NMR (500 MHz, pyridine-d_5), see Table 1.

2.3.6. *Enzymatic hydrolysis of 3*

Compound 3 (10 mg) was solved in H_2O (5 mL) with β-glucosidase (3 mg), and was hydrolyzed at room temperature for 48 h. The reaction mixture was checked directly by silica gel TLC using $CHCl_3$–MeOH–H_2O (70:30:1) as development and 10% vanillin–H_2SO_4 as detection, comparing with smilacinoside A (**1**). The result showed smilacinoside A (**1**) was existed in the reaction mixture (R_f 0.62).

2.3.7. *Smilacinoside D (4)*

White amorphous powder; m.p. 200–204 °C (MeOH); $C_{46}H_{76}O_{18}$; $\alpha_D^{28.8}$ − 77.42° (*c* 0.310, pyridine). IR ν_{max} (KBr) 3425 (OH), 938, 1632, 1451, 1382, 1225, 1064, 1018, 915, 839, 814, 766 cm^{-1}. FAB-MS: *m/z* (%) 915 (100) [M−H]$^-$, 769 (5) [M−H-146 (Rha)]$^-$. HRFABMS: *m/z* 915.4067 [M($C_{46}H_{76}O_{18}$)−H]$^-$ (calcd. for 915.4032). ^{1}H NMR (400 MHz, pyridine-d_5): δ 0.80 (s, 3H, H-18), 0.99 (d, J = 6.4 Hz, 3H, H-27), 1.04 (s, 3H, H-19), 1.18 (d, J = 6.7 Hz, 3H, H-21), 1.68 (d, J = 6.0 Hz, 3H, Rha H-6), 3.25 (s, 3H, 22-OCH_3), 3.58 (brs, 2H, H-26), 3.95 (m, 1H, H-3), 4.65 (m, 1H, H-16), 4.84 (d, J = 7.7 Hz, 1H, 26-*O*-Glc H-1), 4.98 (d, J = 7.4 Hz, 1H, Gal H-1), 5.31 (brs, H, H-6), 6.30 (s, 1H, Rha H-1) ppm. ^{13}C NMR (400 MHz, pyridine-d_5), see Table 1.

2.3.8. *In vitro cytotoxicity bioassay*

Growth inhibition of samples on K562 tumor cells was measured by microculture tetrazolium (MTT) assay [8,9] with minor modification [10]. The assay is established on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to yield a blue formazan product that can be measured spectrophotometrically. Briefly, adherent K562 tumor

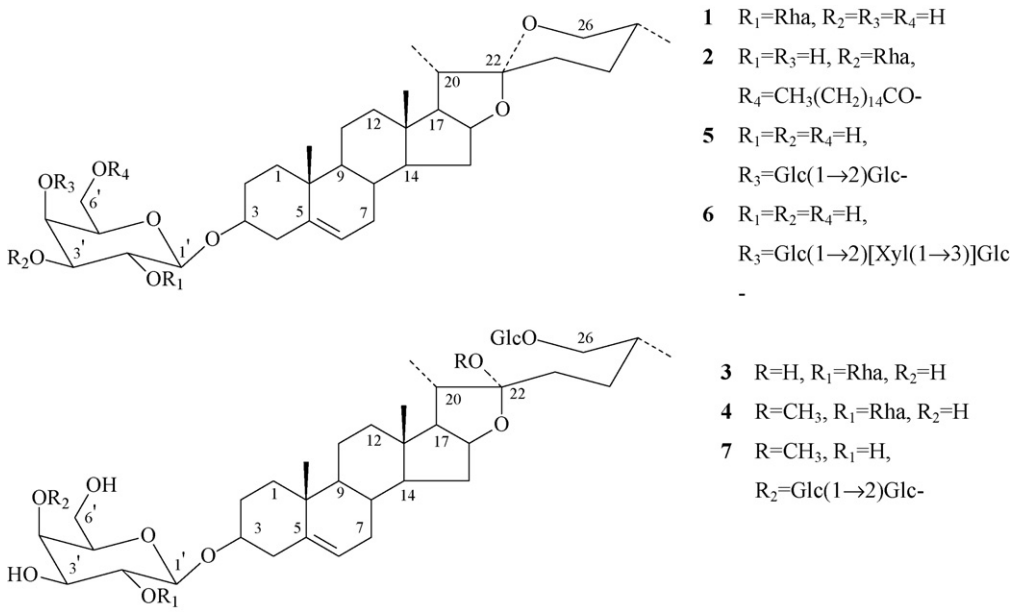

Fig. 1 – Steroidal saponins (1–7) isolated from *Smilacina atropurpurea*.

cells were seeded into 96-well microculture plates and allowed to adhere for 24 h before drug addition, while suspended cells were seeded just before drug addition. K562 tumor cell line was exposed to samples at 0.01, 0.1, 1, 10 and 100 μg/mL concentrations for different periods (adherent cells for 72 h, suspended cells for 48 h) and each concentration was tested in triplicate. At the end of exposure, 20 μL of 5 g/L MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide, Sigma Chemical Co.] was added to each well and the plates were incubated for 4 h at 37 °C, then "triplex solution (10% SDS–5% isobutanol–0.012 M HCl)" was added and the plates were incubated for 12–20 h at 37 °C. The optical density (OD) was read on a plate reader on an ELISA reader (Bioteck EL-340, USA) at a wavelength of 570 nm. DMSO solvent control wells were included in all the experiments. And cis-platin was used as the positive control substance with concentration of 0.1, 1 and 10 μg/mL. The cytotoxicity of samples on K562 tumor cells was expressed as IC_{50} values calculated by LOGIT method.

3. Results and discussion

The methanolic extract of the air-dried tender aerial parts of *S. atropurpurea* was repeatedly chromatographed on silica gel and reversed phase silica gel (RP-18) columns to afford four new steroidal saponins (**1–4**) and three known compounds funkioside D (**5**) [11], aspidistrin (**6**) [12] and 26-*O*-β-D-glucopyranosyl-22-OCH$_3$-(25*R*)-furost-5-en-3β,26-diol 3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (**7**) [13], which were identified by comparison of their spectroscopic data with the reported literature values (Fig. 1).

Compound **1** was isolated as a colorless needle crystal and positive to anisaldehyde–sulphuric acid reagent and negative to Ehrich reagent [11]. The molecular formula was assigned as $C_{39}H_{62}O_{12}$ on the basis of the ^{13}C NMR data (Table 1) and positive ion HRFABMS ([M−H]$^+$-1, *m*/*z* 721.4177). The positive FABMS showed characteristic fragment ion peak at *m*/*z* 577 [M+H-146]$^+$, 415 [M+H-146-162]$^+$, suggesting the existence of a terminal deoxyhexosyl unit and a hexosyl unit in the molecule. The absorption signals at 981, 919, 899 (intensity: 899 > 919) and 865 cm^{-1} in the IR spectrum implied **1** was a (25*R*)-methyl spirostanol aglycone. The ^1H and DEPT NMR spectra of **1** displayed it was composed of an aglycone of diosgenin and two glycoside [δ_H 4.98 (d, *J* = 7.7 Hz, 1H), 6.30 (brs, 1H), 4.41 (d, *J* = 6.4 Hz, 2H),1.69 (d, *J* = 6.1 Hz, 3H), δ_C 100.7 (CH), 102.1 (CH), 62.4 (CH_2) 18.6 (CH_3)]. Acid hydrolysis of **1** produced diosgenin, and D-galactose and L-rhamnose as sugar residues with the authentic samples by TLC. A broad signal peak of the anomeric proton signal at δ 6.30 (brs) indicated the α-configuration at the anomeric center of L-rhamnose, and the *J* value (7.7 > 7 Hz) of the anomeric proton signal at δ 4.98(d) showed the β-configuration at the anomeric center of D-galactose, respectively. The assignment of ^1H and ^{13}C of **1** were carried out by the HMQC experiment. In the HMBC spectrum of **1**, correlations of δ 4.98 (Gal H-1) with δ 77.8 (C-3), and δ 6.30 (Rha H-1) with δ 76.0 (Gal C-2) were observed. The sequence of the sugars and linkage site to the aglycone of **1** were determined by ROESY and HMQC-TOCSY experiments also. The key correlations are shown in Fig. 2. Therefore, **1** was determined as diosgenin 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-β-D-galactopyranoside, and named smilacinoside A. X-ray crystallographic analysis (Fig. 3) of **1** confirmed the structure deduced above and the relative configuration of **1**.

Compound **2** was obtained as a white amorphous powder and positive to anisaldehyde–sulphuric acid reagent and negative to Ehrlich reagent. The molecular formula was assigned as $C_{55}H_{92}O_{13}$ on the basis of the ^{13}C NMR data (Table 1) and HRESIMS: *m*/*z* 960.6547 [M($C_{55}H_{92}O_{13}$)]$^-$ (calcd. for 960.6538). The ^1H and ^{13}C NMR (Table 1) signals of the aglycone moiety of **2** were identical to those of **1**, and analogical two anomeric proton signals at δ 4.95 (d, *J* = 8.0 Hz, 1H) and 6.27 (brs, 1H)

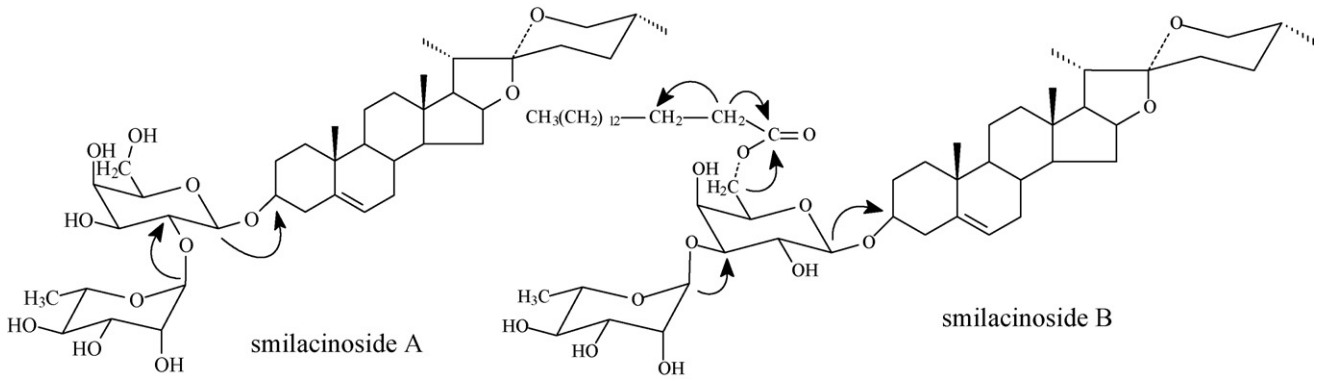

Fig. 2 – Key correlations of smilacinoside A and B.

with one methyl proton signal of deoxyhexopyranose at δ 1.66 (d, J = 6.1 Hz, 3H), and two anomeric carbon signals of sugar at δ 102.1 (CH) and 101.0 (CH) with one methyl carbon signal of deoxyhexopyranose at δ 18.6 (CH_3). On comparison of the whole ^{13}C NMR spectrum with that of **1**, an additional series of signals at δ 173.5 (O–C=O), 14.3 (CH_3) and 19.5–30.1 (a set of methylene carbon) were observed. Considering the fragments at *m*/*z* 721 [M-239(palmityl)]$^-$ and 255 [palmitoyl]$^-$ in the FAB-MS spectrum, the additional signals were assigned to the group of palmitoyl. The assignment of ^{1}H and ^{13}C of **2** was carried out by the HMQC experiment. The sequence of the sugars and palmitoyl group, and the linkage site to the aglycone of **2** were determined by HMBC and ^{1}H–^{1}H COSY experiments. In the HMBC experiment of **2**, correlations of δ 4.95 (Gal H-1) with δ 78.3 (C-3), and δ 6.27 (Rha H-1) with δ 75.7 (Gal C-3), δ 4.70 (Gal H-6β) and 4.89 (Gal H-6α) with δ 173.5 (C=O) were observed. The palmitoyl was proved to be linked to galactose at δ 64.5 (Gal C-6) positions and the rhamnose was confirmed to be linked to galactose at δ 75.7 (Gal C-3), and the galactose linked to aglycone (diosgenin) at δ 78.3 (C-3) (Fig. 2). Thus, compound **2** was identified as diosgenin 3-*O*-α-L-rhamnopyranosyl-(1 → 3)-[6-*O*-palmitoyl]-*O*-β-D-galactopyranoside, and named as smilacinoside B.

Compound **3** showed positive reaction to anisaldehyde–sulphuric acid reagent and Ehrich reagent [11]. The IR spectrum of **3** does not exhibit the characteristic absorption signals of spirostanol steroidal skeleton. The molecular formula was assigned as $C_{45}H_{74}O_{18}$ on the basis of the ^{13}C NMR data (Table 1) and HRESIMS: *m*/*z* 901.4771 [M ($C_{45}H_{74}O_{18}$)−H]$^-$ (calcd. for 901.4765). The ^{1}H and ^{13}C NMR spectrum suggesting that three anomeric proton signals of sugar at δ 4.79 (d, J = 7.8 Hz, 1H), 4.95 (d, J = 7.6 Hz) and 6.27 (s, 1H), and three anomeric carbon signals of sugar at δ 100.9 (CH), 102.2 (CH) and 105.0 (CH) in the molecule. On comparison of the whole ^{1}H and ^{13}C NMR spectrum with that of **1**, an additional sugar of signals at δ 105.0 (CH), 75.3 (CH), 78.6 (CH), 71.8 CH), 78.5 (CH) and 62.9 (CH_2) were observed, suggesting that one glucose unit existed in the molecule. The enzymatic hydrolysis of **3** yielded D-glucose as the residue and the corresponding spirostanol glycoside which was identified as **1** by TLC method. These results suggested that **3** were 26-*O*-D-glucopyranosyl furostanol glycoside. Therefore, the structure of **3** was established as to be 26-*O*-β-D-glucopyranosyl-(25R)-furost-5-en-3β,22ξ, 26-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranoside, and identified by ROESY and HMBC experiment, and named as smilacinoside C.

Compound **4** showed positive reaction to anisaldehyde–sulphuric acid reagent and Ehrich reagent. The molecular formula was assigned as $C_{46}H_{76}O_{18}$ on the basis of the ^{13}C NMR data (Table 1) and positive HRFABMS: *m*/*z* 915.4067 [M ($C_{46}H_{76}O_{18}$)−H]$^+$ (calcd. for 915.4032). The ^{1}H and ^{13}C NMR spectra of **4** were closely related to those of **3**, except for a methoxyl signal at δ 47.4 (CH_3) and δ 3.25(s, 3H). The appearance of this methoxyl

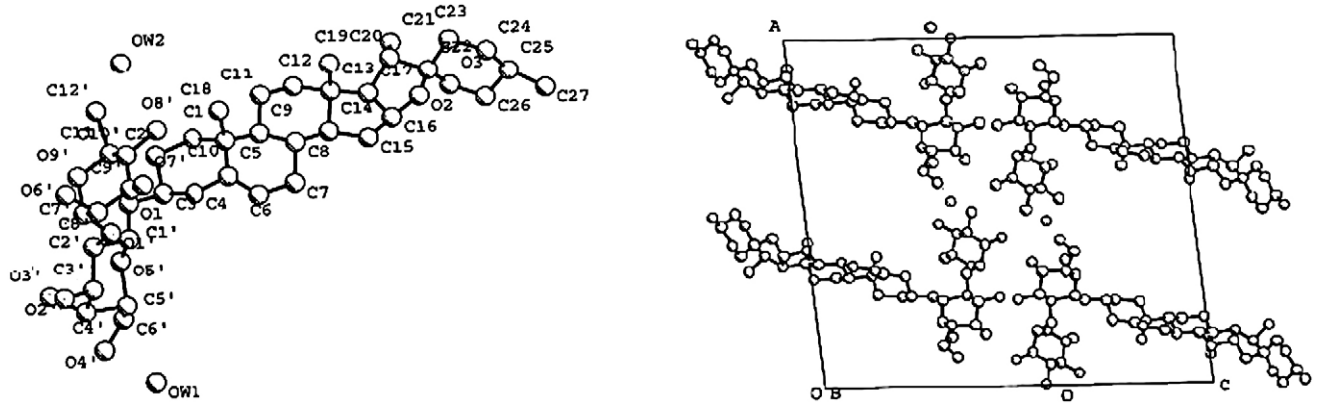

Fig. 3 – X-ray crystallographic structure of compound 1.

Table 2 – Cytoxicity of saponins 1–6 and MeOH extract on K562 tumor cells

Compound	IC_{50} (μg/mL)	Compound	IC_{50} (μg/mL)
1	1.09	**4**	NA
2	NA	**5**	2.93
3	NA	**6**	0.47
MeOH extract	NA	DDP	2.02

IC_{50}: 50% inhibitory concentration, K562: human leukemia cell line, NA: no activity, DDP: cis-platin.

signal and the downfield shift of C-22 to δ 112.7 suggested that the 22-OH was methylated [14]. Therefore, compound **4** was determined as 26-O-β-D-glucopyranosyl-22-OCH_3-(25*R*)-furost-5-en-3β,26-diol 3-O-α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranoside, and named as smilacinoside D.

Saponins **1–6** and MeOH extract were examined for their cytoxicity against K562 human tumor cells *in vitro*, using cis-platin as a positive control. The result of their cytoxicity as shown in Table 2, smilacinoside A (**1**), aspidistrin (**6**) and funkioside D (**5**) exhibited significant *in vitro* cytotoxicity against K562 tumor cell line with IC_{50} values of 1.09, 0.47 and 2.93 μg/mL, respectively. It appears that the cytoxicity against K562 human tumor cells of these steroidal saponins has a correlation with their structure.

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