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New triterpenoid saponins from the steaming treated roots of *Panax notoginseng*

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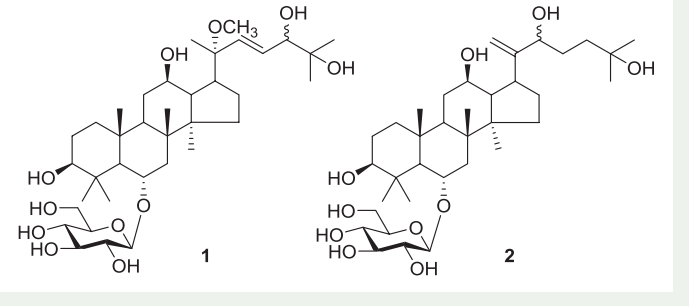
ABSTRACT

Further phytochemical investigation of the steaming treated roots of *Panax notoginseng* (Araliaceae) led to the identification of two new dammarane-type triterpenoid saponins, notoginsenoside SP20 (**1**) and SP21 (**2**). In addition, a pair of new phenolic glycosides (**3a** and **3b**) was also isolated together with two known compounds. Their structures were elucidated by HRESIMS, 1D- and 2D-NMR spectra. Compounds **1** and **2** showed no *in vitro* cytotoxicity against five human cancer cell lines (HL-60, SMMC-7712, A-549, MCF-9 and SW480).



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Panax notoginseng; Araliaceae; triterpenoid saponins

1. Introduction

The roots of *Panax notoginseng* (Burk.) F. H. Chen (Araliaceae), known as a famous traditional Chinese medicine, have been used widely in both raw and processed forms. The raw one is traditionally for injuries from falls and removing blood stasis, while in recent years, it is focused on the cardio- and cerebro-vascular diseases, central nervous system and endocrine system (Jiang and Qin 1995; Yuan et al. 1997; Li and Chu 1999; White et al. 2000). The processed root, used as a tonic, is to nourish blood and increase the production of various blood cells in anaemic conditions (State Administration of Traditional Chinese Medicine 1996). As

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the major bioactive components of *P. notoginseng*, a series of triterpenoid saponins have been reported (Xiong and Sun 1989; Wu et al. 1995; Li et al. 2004; Wang et al. 2008). A novel strategy for detection of main saponins in *P. notoginseng*, *P. ginseng* and *P. quinquefolium* has also been established (Xu et al. 2015). Over 70 saponins including 30 new ones with promoting effects on the differentiation of PC12 cells have been isolated from the steamed roots of *P. notoginseng* (Gu et al. 2015a, 2015b). From which, notoginsenoside ST-4 was found to be a promising anti-HSV agent (Pei et al. 2011). Saponins produced by two novel endophytic fungi from *P. notoginseng* showed potent antimicrobial activity (Lin et al. 2017). The chemical constituents and bioactivities of raw and processed roots of *P. notoginseng* were quite different (Wang et al. 2012).

As a continuing study of the steamed roots of *P. notoginseng*, two new saponins were isolated, together with a pair of new phenolic glycosides and two known compounds. Their structures were elucidated by detailed spectroscopic analysis, and the cytotoxicity against five human cancer cell lines was also evaluated.

2. Results and discussion

The 80% MeOH extract of the steaming treated roots of *P. notoginseng* was subjected to column chromatography over D-101 macro porous resin, silica gel and RP-18, followed with semi-preparation HPLC, to afford two new dammarane-type triterpenoid saponins **1** and **2**, together with a pair of new phenolic glycosides (**3**). Two known compounds were also identified as ecdysterone (**4**) (Zhang et al. 2013) and *p*-ethylphenyl-1-*O*-*β*-D-glucopyranoside (**5**) (Xu et al. 2013) (Figure 1), on the basis of their spectroscopic data.

Compound **1** had a molecular formula of $C_{37}H_{64}O_{11}$, as deduced by the HRESIMS (*m/z* 707.4345 $[M + Na]^+$). The IR spectrum indicated the presence of hydroxy group (3425 cm^{-1})

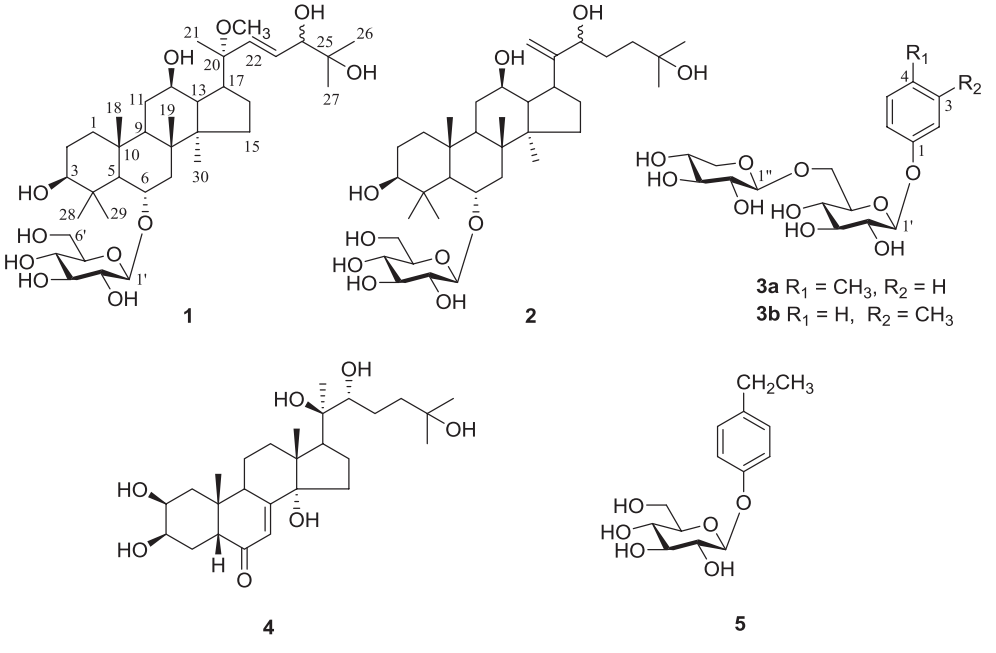

Figure 1. The structures of compounds **1**–**5**.

and double bond (1631 cm^{-1}). The ^{13}C NMR and DEPT spectra indicated 37 carbon resonances. In 1D NMR of **1**, one anomeric proton at δ_H 5.02 (1H, d, J = 7.8 Hz) and six carbon signals at δ_C 105.9, 75.7, 80.0, 72.0, 78.4 and 63.3, suggested the presence of one *β*-glucosyl unit, which was determined to be D configuration by acidic hydrolysis followed with GC analysis of its corresponding trimethylsilylated L-cysteine adduct. The left 31 carbon signals attributed to eight *tert*-methyls, six methylenes, 10 methines with four oxy (δ_C 78.8, 80.5, 71.0, 79.5) and two olefinic (δ_C 131.0, 136.3) carbons, six quaternary carbons including two oxygen-bearing ones (δ_C 81.8, 73.0), and one methoxy (δ_C 50.1). In the ^1H NMR spectrum, eight singlet methyls (δ_H 1.34, 1.02, 1.27, 1.61, 1.56, 2.10, 1.64 and 0.78), and two *trans* coupled olefinic protons [δ_H 6.32 (1H, d, J = 16.2 Hz) and 6.24 (1H, dd, J = 5.4, 16.2 Hz)] were observed. The above NMR data were similar to those of notoginsenoside T4, a protopanaxatriol triterpenoid saponin (Teng et al. 2004). However, compound **1** has one more methoxy group (δ_C 50.1, δ_H 3.17) attached to C-20 of the side chain, which was confirmed by HMBC correlation of the methoxy proton (δ_H 3.17) with C-20 (δ_C 81.8). Moreover, HMBC correlation of H-1′ (δ_H 5.02) with C-6 (δ_C 80.5) revealed the glucosyl unit on C-6 of the aglycon. The large coupling constant of $J_{22,23}$ (16.2 Hz) suggested the *E* form of C-22/23 double bond The chemical shifts of C-21 (δ_C 21.7) and C-17 (δ_C 52.6) revealed the 20*R* configuration in **1**, comparing to the 20*S* configuration [C-21(δ_C 27.1) and C-17 (δ_C 54.8)] (Teng et al. 2002). Therefore, compound **1** was identified to be (3*β*,6α,12*β*, 20*R*,24)-3,6,12,24,25-pentahydroxy-20-methoxydmmar-22(23)(*E*)-ene-6-*O*-*β*-D-gluco-pyranoside.

Compound **2** had a molecular formula $C_{36}H_{62}O_{10}$, as determined by the HRESIMS (*m/z* 677.4237 $[M + Na]^+$). The ^1H and ^{13}CNMR data of **2** were similar to those of 25-hydroxyginsenoside Rk_3 (Wu et al. 2011), except for the signals arising from the side chain. Compound **2** had one more hydroxy group on the side chain. In the ^1H-^1H COSY spectrum, H_2-23 (δ_H 2.32) was coupled with both H_2-24 (δ_H 1.77) and an oxymethine at δ_H 4.49 (H-22). The HMBC correlations of H-22 (δ_H 4.49) with C-17 (δ_C 39.3), C-20 (δ_C 160.2) and C-21 (δ_C 110.6) indicated that the additional hydroxyl group was located on C-22. Therefore, compound **2** was determined to be (3*β*,6α,12*β*,22)-3,6,12,22,25-penta-hydroxydmmar-20(21)-ene-6-*O*-*β*-D-glucopyranoside.

The molecular formula of **3** was deduced to be $C_{18}H_{26}O_{10}$, by the HRESIMS (*m/z* 425.1419 $[M + Na]^+$). The ^{13}C NMR spectrum showed the presence of two methyls (δ_C 21.7, 20.8), four sugars [anomeric C at δ_C 105.6, 105.4, 102.4, 102.2] and two benzene rings. Among which, the sugar and methyl signals appeared in pairs. The carbon numbers from NMR spectrum were two times of the HRESIMS result, revealing **3** was a mixture of two phenolic glycosides (**3a** and **3b)**, with a ratio of 1:1 through analysis of the ^1H NMR spectrum. The sugar units in **3** were determined to be D-glucose and D-xylose by acidic hydrolysis followed with GC analysis. In the ^1H NMR of **3**, eight aromatic proton signals were observed. Two two-proton-doublets at δ_H 7.01, 7.09 (each 2H, d, J = 7.8 Hz) revealed the presence of one *para*-substituted symmetric [δ_C 157.1, 132.9, 131.0 (x 2),117.8 (x 2)] (**3a**), while the other four including one singlet at δ_H 6.93 (1H, s), two one-proton-doublets at δ_H 6.83, 6.91 (each 1H, d, J = 7.8 Hz) and one triplet at δ_H 7.16 (1H, t, J = 7.8 Hz) showed the presence of one *meta*-substituted (δ_C 159.2, 118.5, 140.7, 124.3, 130.4, 114.8) (**3b**) benzene rings. Compound **3** is a mixture of *p*- (**3a**) and *m*- (**3b**) methyl phenyl glycosides.

In the HMBC spectrum of **3**, correlations of glucosyl H-1′ [δ_H 4.83 (**3a**), 4.87 (**3b**)] with C-1[δ_C 157.1 (**3a**), 159.2 (**3b**)], and xylosyl H-1″ [δ_H 4.31 (**3a**, **3b**)] with the glucosyl C-6′ [δ_C 69.9 (**3a**), 69.8 (**3b**)] indicated that the terminal xylosyl was linked to the inner glucosyl C-6′

in both **3a** and **3b**. Furthermore, HMBC correlations of the methyl protons at δ_H 2.27 (**3a**) with C-4 (δ_C 132.9, **3a**), and δ_H 2.32 (**3b**) with C-3 (δ_C 140.7, **3b**), confirmed the location of methyl group on *para* (**3a**) and *meta* (**3b**) sites of the glycosylation position, respectively. Therefore, compound **3** was determined to be *p/m*-methyl phenyl-1-*O*-*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranoside (**3a**/**3b**).

Compounds **1** and **2** displayed no *in vitro* cytotoxicities on HL-60, SMMC-7712, A-549, MCF-7 and SW480 cell lines at a concentration of 40 μM.

3. Experimental

3.1. General

Optical rotations and IR were performed on a P-1020 polarimeter (JASCO, Tokyo, Japan) and a Bruker Tensor 27 spectrometer with KBr pellets, respectively. ESIMS and HRESIMS were measured at Bruker HCT/Esquire and Agilent G6230. 1D and 2D NMR were run on AVANCE III-600, at 600 MHz for ^{1}H and 150 MHz for ^{13}C, respectively. Coupling constants are expressed in Hertz and chemical shifts are given on ppm scale with solvents as internal standard. The apparatus of HPLC was an Agilent 1260 with DAD detector. Semi-preparative HPLC was performed on an Agilent 1260 liquid chromatography with a 5 μm Thermo BDS HYPERSIL-C_{18} column (10 × 250 mm) column. Column chromatography (CC) was performed with D101 macro porous resin (Shandong Lukang Pharmaceutical Co., Ltd., China), silica gel (200–300 mesh) (Qingdao Marine Chemical and Industrial Factory, China), RP-18 gel (40–60 μm) (Merck, Darmstadt, Germany). Fractions were monitored by TLC and spots were visualised by heating the silica gel plates sprayed with 10% H_2SO_4 ethanol solution.

3.2. Plant material

Air-dried roots of three-year-cultivated *P. notoginseng* were collected from Wenshan County, Yunnan Province, China, on April 2011, and identified by one of the authors (C.-R. Yang). A voucher specimen (KIB-00336) has been deposited in Kunming Institute of Botany, Chinese Academy of Sciences.

3.3. Extraction and isolation

The air-dried roots of *P. notoginseng* were crushed into small grains and then steamed at 120 °C with a pressure of 0.12 MPa for 12 h. The yielded product (15.0 kg) was refluxed with 80% aqueous methanol three times, each time 3 h. The MeOH extract (3 kg) was subjected to D101macro porous resin (250 × 30 cm), eluting with water to remove saccharides, and then methanol to afford the total saponin fraction (2 kg), which were applied to silica gel CC (250 × 30 cm), eluting with $CHCl_3$-MeOH-H_2O (85:15:1–75: 25: 2) to afford eight fractions (Fr. A-H).

Fr. F (40 g) and Fr. H (10 g) were separately applied to RP-18 CC, eluting with MeOH-H_2O (4:6 to 9:1) to yield the subfractions, F1-F5 and H1-H4, respectively. Fr. F5 (51 mg) was purified by semi-preparative HPLC with CH_3CN-H_2O (25:75 - 32:68) to afford **1** (7 mg). Fr. H2 (133 mg) and Fr. H3 (112 mg) were separately purified by semi-preparative HPLC (CH_3CN-H_2O, 19:81–22:78 for Fr. H2; 22:78- 27:73 for Fr. H3) to afford **2** (6 mg), and **3** (6 mg), **4** (23 mg) and **5** (11 mg), respectively.

3.4. Spectroscopic data

Notoginsenoside SP20 (**1**): white amorphous powder; $[\alpha]_D^{21}$ + 49.7 (*c* = 0.55, MeOH); IR (KBr) ν_{max} cm^{-1}: 3425, 2966, 2933, 2876, 1631, 1463, 1426, 1383, 1158, 1077, 1031; ^{1}H NMR (600 MHz, Pyridine-d_5): δ_H 6.32 (1H, d, *J* = 16.2 Hz, H-22), 6.24 (1H, dd, *J* = 16.2,5.4 Hz, H-23), 5.02 (1H, d, *J* = 7.8 Hz, H-1′), 4.52 (1H, dd, *J* = 11.4, 2.4 Hz, H-6′b),4.52 (1H, d, *J* = 5.4 Hz, H-24), 4.37 (1H, dd, *J* = 11.4, 5.4 Hz, H-6′a), 4.13 (1H, t, *J* = 7.8 Hz, H-2′), 3.55 (1H, dd, *J* = 4.8, 11.4 Hz, H-3), 2.46 (1H, dd, *J* = 3.0, 12.6 Hz, H-7b), 1.45 (1H, d, *J* = 10.8 Hz, H-5), 4.43, 4.28, 4.26, 3.95, 3.84, 2.21, 2.14, 1.95, 1.94, 1.92, 1.87, 1.70, 1.67, 1.57, 1.56, 1.45, 1.33, 1.05, 1.04 (each 1H, m, H-6, 3′, 4′, 5′, 12, 17, 11b, 13, 2b, 7a, 2a, 1b, 16b, 15b, 9, 11a, 16a, 1a, 15a), 2.10, 1.64, 1.61, 1.56, 1.34, 1.27, 1.02, 0.78 (each 3H, s, H-28, 29, 26, 27, 18, 21, 19, 30); ^{13}C NMR (150 MHz, pyridine-d_5): δ_C 136.3 (C-23), 131.0 (C-22), 105.9 (C-1′), 81.8 (C-20), 80.5 (C-6), 80.0 (C-3′), 79.5 (C-24), 78.8 (C-3), 78.4 (C-5′), 75.7 (C-2′), 72.0 (C-4′), 73.0 (C-25), 71.0 (C-12), 63.3 (C-6′), 61.6 (C-5), 52.6 (C-17), 52.2 (C-14), 50.1 (C-9), 49.9 (C-13), 45.3 (C-7), 41.8 (C-8), 40.8 (C-4), 40.0 (C-1), 39.8 (C-10), 32.2 (C-28), 31.6 (C-11), 31.4 (C-15), 28.4 (C-2), 27.0 (C-16), 26.8 (C-26), 26.6 (C-27), 21.7 (C-21), 18.2 (C-19), 17.7 (C-18), 17.0 (C-30), 16.3 (C-29); ESIMS: *m/z* 707[M + Na]$^+$; HRESIMS: *m/z* 707.4345 [M + Na]$^+$ (Calcd for $C_{37}H_{64}O_{11}Na$, 707.4341).

Notoginsenoside SP21 (**2**): white amorphous powder; $[\alpha]_D^{21}$ + 11.2 (*c* = 0.85, MeOH); IR (KBr) ν_{max} cm^{-1}: 3407, 2963, 2934, 2934, 2875, 1639, 1463, 1383, 1366, 1153, 1075, 1030; ^{1}H NMR (600 MHz, pyridine-d_5): δ_H 5.21, 5.17 (each 1H, s, H-21a, 21b), 5.06 (1H, d, *J* = 7.8 Hz, H-1′), 4.57 (1H, dd, *J* = 11.4, 2.4 Hz, H-6′b), 4.49 (1H, t, *J* = 6.6 Hz, H-22), 4.40 (1H, dd, *J* = 11.4, 5.4 Hz, H-6′a), 4.31 (1H, t, *J* = 8.4 Hz, H-3′), 4.26 (1H, t, *J* = 9.0 Hz, H-4′), 4.13 (1H, t, *J* = 7.8 Hz, H-2′), 3.55 (1H, dd, *J* = 4.2, 11.4 Hz, H-3), 2.56 (1H, dd, *J* = 3.0, 12.6 Hz, H-7b), 1.44 (1H, d, *J* = 10.8 Hz, H-5), 4.46, 3.99, 3.98, 3.04, 2.32, 2.16, 2.13, 2.06, 2.05, 1.97, 1.96, 1.93, 1.91, 1.86, 1.77, 1.67, 1.59, 1.57, 1.49, 1.23, 1.01 (each 1H, m, H-6,5′, 12, 17, 23b, 16b, 13, 23a, 11b, 24b, 7a, 2b, 15b, 2a, 24a, 1a, 16a, 9, 11a, 15a, 1b), 2.10, 1.63, 1.25, 1.03, 0.83 (each 3H, s, H-28, 29, 18, 19, 30), 1.44 (6H, s, H-26, H-27); ^{13}C NMR (150 MHz, pyridine-d_5): δ_C 160.2 (C-20), 110.6 (C-21), 106.0 (C-1′), 80.1 (C-6), 79.7 (C-3′), 78.3 (C-5′), 77.6 (C-22), 75.5 (C-2′), 72.7 (C-12), 71.9 (C-4′), 69.4 (C-25), 63.1 (C-6′), 78.6 (C-3), 61.4 (C-5), 58.8 (C-13), 51.7 (C-14), 50.6 (C-9), 45.4 (C-7), 41.2 (C-8), 41.1 (C-24), 40.4 (C-4), 39.7 (C-1), 39.5 (C-10), 39.3 (C-17), 34.5 (C-16), 33.0 (C-15), 32.2 (C-11), 31.8 (C-23), 31.7 (C-28), 30.3 (C-26), 29.8 (C-27), 27.9 (C-2), 17.7 (C-19), 17.3 (C-18), 16.7 (C-30), 16.3 (C-29); ESIMS: *m/z* 677 [M + Na]$^+$; HRESIMS: *m/z* 677.4237 [M + Na]$^+$ (Calcd for $C_{36}H_{62}O_{10}Na$, 677.4235).

p/*m*-Methyl phenyl-1-*O*-*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranoside (**3a/3b**): white amorphous powder; $[\alpha]_D^{21}$ − 79.2 (*c* = 1.28, MeOH); IR (KBr) ν_{max} cm^{-1}:3423, 2922, 1613, 1589, 1510, 1489, 1459, 1256, 1231, 1073, 1044, 534; ^{1}H NMR (600 MHz, CD_3OD): δ_H **3a**: 7.09, 7.01 (each 2H, d, *J* = 7.8 Hz, H-3, 5, 2, 6), **3b**: 6.93(1H, s, H-2), 6.83 (1H, d, *J* = 7.8 Hz, H-4), 7.16 (1H, t, *J* = 7.8 Hz, H-5), 6.91 (1H, d, *J* = 7.8 Hz, H-6), **3a**/**3b**: 4.83/4.87 (1H, d, *J* = 7.8, H-1′), 4.31/4.31 (1H, d, *J* = 7.2, H-1″), 4.10/4.10, 3.83/3.83, 3.77/3.77, 3.64/3.64, 3.46/3.46, 3.37/3.37, 3.28/3.28, 3.20/3.20, 3.12/3.12 (each 1H, m, H-6′a, 5″a, 6′b, 5′, 4″, 4′, 3″, 2″, 5″b), 3.44/3.44 (2H, m, H-2′, 3′), 2.27/2.32 (3H, s, CH_3); ^{13}C NMR (150 MHz, CD_3OD): δ_C 157.1/159.2 (C-1), 117.8/118.5 (C-2), 131.0/140.7 (C-3), 132.9/124.3 (C-4), 131.0/130.4 (C-5), 117.8/114.8 (C-6), 102.4/102.2 (C-1′), 74.8/74.8 (C-2′), 77.9/77.9 (C-3′), 71.4/71.4 (C-4′), 77.3/77.3 (C-5′), 69.9/69.8 (C-6′), 105.4/105.6 (C-1″), 74.9/74.9 (C-2″), 77.7/77.7 (C-3″), 71.2/71.2 (C-4″), 66.8/66.8 (C-5″), 20.8/21.7 (CH_3); ESIMS: *m/z* 425 [M + Na]$^+$; HRESIMS: *m/z* 425.1419 [M + Na]$^+$ (Calcd for $C_{18}H_{26}O_{10}Na$, 425.1418).

3.5. Acidic hydrolysis of compounds 1–3

Compounds **1**–**3** (each 5 mg) were hydrolysed as previously reported (Gu et al. 2015a, 2015b). The monosaccharides were identified as glucose in **1** and **2**, and glucose and xylose in **3**, by co-TLC with authentic sugars, eluting with chloroform/*n*-butanol/ methanol/acetic acid/ water 17:10:6:2:3 (R_f = 0.35 for glucose, 0.46 for xylose). The configurations of glucose in **1**–**3**, and xylose in **3** were determined to be all D form.

3.6. Cytotoxic bioassay

Human myeloid leukaemia HL-60, hepatocellular carcinoma SMMC-7721, lung cancer A-549, breast cancer MCF-7 and colon cancer SW-480 cell lines were used in the cytotoxic assay. The assay was performed by means of MTT [3-(4,5-dimethylthiazo-2- yll)-2,5-diphenly-tetrazolium bromide] (Sigma, St. Louis, USA) method in 96-well microplates (Mosmann 1983). All the cells were cultured in RMPI 1640 or DMEM medium (Hyclone, USA), supplemented with 10% foetal bovine serum (Hyclone, USA) in 5% CO_2 at 37 °C. About 100 µL suspension was added to each well to seed cells in 96-well microplates, in which the tested samples were added with varied concentrations. After 48 h incubation, MTT solution [5 mg/mL in phosphate buffered saline (PBS)] was added (20 µL/well), and the incubation continued for another 4 h to give the formation product. In cell well, 100 µL 20% SDS was added after 100 µL medium was removed, and it was then incubated over night to make the formazan product dissolve completely. The absorbance of the solution was measured at 595 nm in Bio-Rad 680. Concentration of a compound inhibiting 50% of cell growth (IC_{50}) was calculated by the Reed and Muench method.

4. Conclusions

In conclusion, two new saponins, **1** and **2**, were isolated from the steaming treated roots of *P. notoginseng*, together with a pair of new phenolic glycosides (**3a** and **3b**) and two known compounds. These new saponins could be formed through dehydration and oxidation from the main saponins of the plant during the steaming process with higher temperature and moisture. Though **1** and **2** displayed no *in vitro* cytotoxicities on HL-60, SMMC-7712, A-549, MCF-7 and SW480 cell lines at a concentration of 40 µM, the result enriched the chemical diversity of notoginsenosides for further pharmaceutical research.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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