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Cytotoxic steroidal glycosides from *Paris rugosa* rhizomes

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ABSTRACT

Two previously undescribed steroidal saponins, rugosarosides A (**1**) and B (**2**), as well as 16 known compounds (**3–18**), were isolated from the rhizomes of *Paris rugosa* H. Li & S. Kurita (Melanthiaceae). Their chemical structures were elucidated based on extensive analyses of NMR and MS data and acidic hydrolyses. These isolates were evaluated for their cytotoxicity to five human cancer cell lines (HL-60, SW480, MDA-MB-231, A549, and A549/Taxol) and the normal human bronchial epithelial cell line BEAS-2B using the MTS test. Spirostanol saponins **6–12** and furostanol saponin **16** showed cytotoxic activity, with IC_{50} values ranging from 0.13 to 3.88 μM. The furostanol saponins **14**, **15**, and **17** selectively inhibited HL-60, A549, and A549/Taxol cells (IC_{50}: 3.45–9.51 μM), with no cytotoxicity to SW480 and MDA-MB-231 cells ($IC_{50} > 40$ μM).

1. Introduction

The genus *Paris* (Melanthiaceae) contains 26 species, some of which are commonly used medicinally in China and neighboring countries and which exhibit varied medicinal values (Dao et al., 2003; Ji, 2021). *Paris rugosa* H. Li & S. Kurita is widely distributed and cultivated in northwestern Yunnan. The Nu people in northwestern Yunnan use this plant to treat innominate swelling and pains, snake bites, and mumps (Wang et al., 2015; Yang et al., 2017).

The genus *Paris* contains mainly steroidal saponins. The structural characteristics of spirostanols and furostanols are mainly different in terms of whether ring F is opened, spirostanol steroidal saponins have strong cytotoxic activity, while furostanol steroidal saponins have low cytotoxic activity (Jia et al., 2024; Rawat et al., 2023). The chemical constituents of *P. rugosa* have been previously reported. Eleven undescribed steroidal saponins and ten known saponins were isolated and identified, and evaluated for their cytotoxicity to several human cancer cell lines (Yu et al., 2022). In our previous study, one new compound, pariposide G, and eight known compounds were isolated from *P. rugosa*. The antibacterial and antifungal activities of the compounds were evaluated, and the results showed that ophiopogonin C′ had strong inhibitory effects on *Candida albicans* ($MIC_{90} = 4.68 \pm 0.01$ μM) and on the fluconazole-resistant strain of *C. albicans* ($MIC_{90} = 4.66 \pm 0.02$ μM) (Duan et al., 2023).

To further explore the anticancer active ingredients of *P. rugosa*, we isolated 18 compounds from the plant's rhizomes including two new steroidal saponins (**1** and **2**, Fig. 1), 15 known steroidal saponins and one known phytosterol (**3–18**, Fig. S1), Their chemical structures were elucidated using spectroscopic methods, and their cytotoxic effects on six human cell lines—the leukemia cell line HL-60, the breast cancer cell line MDA-MB-231, the colon cancer cell line SW480, the lung adenocarcinoma cell line A549, the paclitaxel-resistant A549 (A549/Taxol) cell line, and the normal human bronchial epithelial cell line BEAS-2B—were evaluated. The compounds with high toxicity to cancer cells were screened out. The structural elucidation and bioassay results are reported.

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2. Results and discussion

2.1. Determining the structures of the isolated compounds

The *n*-BuOH-soluble fraction from the 70 % EtOH extract of *P. rugosa* rhizomes was chromatographed repeatedly on columns using silica gel G, reversed-phase (RP) C_{18} silica gel, and Sephadex LH-20, followed by semipreparative high-performance liquid chromatography (HPLC) purification, which isolated two undescribed steroidal saponins, named rugosaroside A (**1**) and rugosaroside B (**2**), and sixteen known compounds (**3–18**). The known compounds were identified as pariposide G (Duan et al., 2023) (**3**), parisyunnanoside G (Kang et al., 2012) (**4**), parisyunnanoside I (Kang et al., 2012) (**5**), ophiopogonin C′ (Hu et al., 1996) (**6**), polyphyllin I (Nohara et al., 1973) (**7**), formosanin C (Munday et al., 1993) (**8**), paris H (Miyamura et al., 1982) (**9**), paris saponin VII (Yang et al., 2003) (**10**), gracillin (Duan et al., 2023) (**11**), 17-hydroxygracillin (Chen and Zhou, 1984) (**12**), dianchonglouoside A (Wen et al., 2015) (**13**), methyl protogracillin (Hu et al., 1997) (**14**), protogracillin (Hu et al., 1997) (**15**), methyl protopolyphyllin I (Miyamura et al., 1982) (**16**), methyl protobioside (Ali et al., 2013) (**17**), and crustecdysone (Suksamrarn et al., 2002) (**18**) by comparing and matching their spectral data with published data.

^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) are all nuclear magnetic resonance (NMR) spectroscopy techniques used in the field of organic chemistry for the structural elucidation of organic compounds. These techniques allow chemists to determine the connectivity between different proton or carbon nuclei within a molecule, which is crucial for determining the structure of an unknown compound.

Rugosaroside A (**1**) was assigned the molecular formula $C_{45}H_{68}O_{19}$, with 12 degrees of unsaturation, as determined using ^{13}C NMR data (Table 1) and a positive ion at *m/z* 935.4245 $[M + Na]^+$ (calcd for $C_{45}H_{68}NaO_{19}$, 935.4247) in high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). The ^{1}H and ^{13}C NMR data indicated the presence of five methyl groups [δ_H 1.73 (3 H, d, J = 6.2 Hz) (belonging to rhamnose); 1.21 (3 H, s), 1.05 (d, J = 6.8 Hz), 0.89 (3 H, s), and 0.67 (d, J = 5.4 Hz) (belonging to aglycone)], one disubstituted double bond [δ_H 6.67 (1 H, d, J = 8.5 Hz) and 6.33 (1 H, d, J = 8.5 Hz); δ_C 136.6 (CH) and 130.9 (CH)], one trisubstituted double bond [δ_H 5.48 (1 H, dd, J = 5.3, 1.7 Hz); δ_C 144.0 (C) and 119.4 (CH)], and three sugars [δ_H 6.39 (1 H, br s), 5.09 (1 H, d, J = 7.6 Hz), and 4.80 (1 H, d, J = 7.5 Hz); δ_C 104.5 (CH), 102.1 (CH), and 100.6 (CH)]. After acidic hydrolysis of **1**, D-glucopyranose and L-rhamnopyranose were detected. Combined with the NMR data, two *β*-D-glucopyranosyl moieties and one *α*-L-

Table 1
^{1}H NMR (500 MHz) and ^{13}C NMR (126 MHz) data for compound **1** in pyridine-d_5 (δ in ppm, J in Hz).

no.	δ_H	δ_C	no.	δ_H	δ_C
1*α*	2.25, m	33.1, CH_2	glc′		
1*β*	1.66, m				
2*α*	2.20, m	29.9, CH_2	1′	4.80, d (7.5)	100.6, CH
2*β*	1.96, m				
3	4.45, m	73.6, CH	2′	4.18, m	76.7, CH
4*α*	2.70, dd (14.0, 4.2)	33.3, CH_2	3′	4.12, m	89.4, CH
4*β*	2.50, dd (14.0, 11.8)				
5		82.9, C	4′	4.11, m	69.3, CH
6	6.33, d (8.5)	136.6, CH	5′	3.66, m	77.7, CH
7	6.67, d (8.5)	130.9, CH	6′	4.30, m	62.1, CH_2
				4.23, m	
8		77.8, C	rha″		
9		144.0, C	1″	6.39, br s	102.1, CH
10		38.7, C	2″	4.86, m	72.4, CH
11	5.48, dd (5.3, 1.7)	119.4, CH	3″	4.55, m	72.8, CH
12*α*	1.98, m	41.5, CH_2	4″	4.30, m	74.2, CH
12*β*	2.03, m				
13		41.6, C	5″	4.89, m	69.6, CH
14	2.08, dd (13.9, 5.8)	48.6, CH	6″	1.73, d (6.2)	18.7, CH_3
15*α*	2.19, m	29.4, CH_2	glc‴		
15*β*	1.94, m				
16	4.51, m	80.8, CH	1‴	5.09, d (7.6)	104.5, CH
17	2.00, m	61.6, CH	2‴	4.01, m	75.0, CH
18	0.89, s	17.2, CH_3	3‴	4.02, m	78.8, CH
19	1.21, s	25.5, CH_3	4‴	4.10, m	71.5, CH
20	1.94, m	42.4, CH	5‴	4.18, m	78.6, CH
21	1.05, d (6.8)	14.6, CH_3	6‴	4.55, m	62.4, CH_2
				4.25, m	
22		109.5, C			
23	1.66, m	31.7, CH_2			
24	1.56, m	29.2, CH_2			
25	1.55, m	30.6, CH			
26	3.54, dd (10.6, 2.6)	67.0, CH_2			
	3.41, dd (10.9, 10.6)				
27	0.67, d (5.4)	17.3, CH_3			

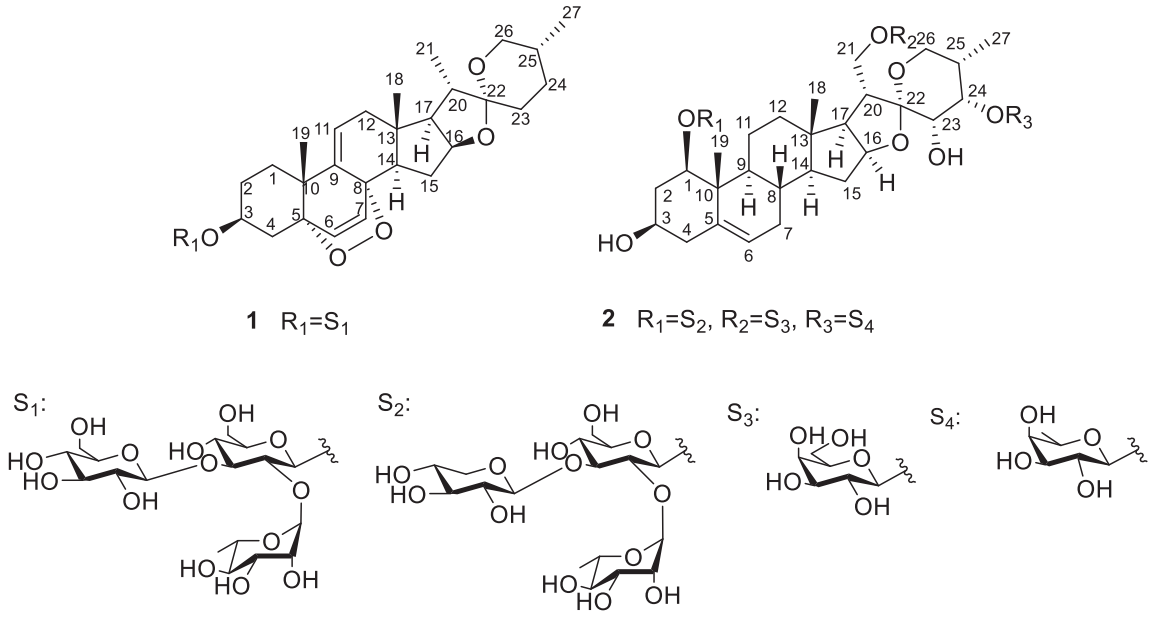

Fig. 1. Chemical structures of saponins **1** and **2** from *Paris rugosa* rhizomes.

rhamnopyranosyl moiety in the structure of **1** were confirmed.

By comparison of its NMR data with those of parisvanioside A (Yan et al., 2022), compound **1** was elucidated to be a steroidal saponin with the same aglycone of (25*R*)-spirost-6,11-diene-5α,8α-epidioxy-3β-ol, confirmed using the ^{1}H-^{1}H COSY correlations (Fig. 2; fragments **a**–**e**); with the HMBC correlations of H_3-19 with C-1, C-5, C-9, and C-10; H_2-4 with C-10; H-6 with C-4 and C-10; H-7 with C-9; H_2-15 with C-8; H_3-18 with C-12, C-13, C-14, and C-17; and H_3-21 and H_2-26 with C-22 (Fig. 2); and with the ROESY correlations of H-1α/H-3, H-1β/H_3-19, H_3-19/H-6, H-7/H_3-18, H_3-18/H-20, H-20/H_2-23, H-14/H-16, H-14/H-17, and H_3-27/H_2-26 (Fig. 2). The sugar chain was determined to be an α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl moiety located at the 3-OH of the aglycone using the HMBC correlations of H-3 with C-1′, H-1′ with C-3, H-2′ with C-1″, H-1″ with C-2′, H-3′ with C-1‴, and H-1‴ with C-3′. Therefore, the chemical structure of rugosaroside A (**1**) was determined to be (25 *R*)-spirost-6,11-diene-5α,8α-epidioxy-3β-ol 3-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside.

The biosynthetic pathway for rugosaroside A is proposed as shown in Fig. 3. Mevalonate (MVA) is an important biosynthetic pathway for the synthesis of steroids. MVA is a metabolic pathway for the synthesis of isoprene pyrophosphate and dimethylallyl pyrophosphate from acetyl-CoA. Squalene synthase (SQS) could catalyze the first dedicated step, converting two molecules of farnesyl diphosphate (FPP) into squalene, a key intermediate in the biosynthetic pathway of steroidal saponins. Squalene may be converted to 2,3-oxidosualene by squalene epoxidase (SQE). 2,3-Oxyoxidosualene could undergo a series of oxidations, cycloartenol synthase, reduction, and other modifications to form cholesterol. Finally, diosgenin may be generated under the action of the CYP450 (PpCYP90G4, PpCYP94D108, PpCYP94D109, and PpCYP72A616) enzyme system (Gao et al., 2021). Diosgenin may be transformed into compound **A** under the action of dehydrogenase. Compound **A** may undergo a [4 + 2] reaction to form compound **B** under the action of oxidase (Ning et al., 2021), compound **B** may be converted into compound **C** under the action of dehydrogenase (Wang and Abe, 2024), and then compound **C** may be converted into rugosaroside A under the action of glycosyltransferases.

Rugosaroside B (**2**) has the molecular formula $C_{56}H_{90}O_{29}$ based on its ^{13}C NMR data (Table 2) and positive ion at *m/z* 1225.5497 $[M - H]^-$ (calcd for $C_{56}H_{89}O_{29}$, 1225.5495) in HR-ESI-MS. The ^{1}H NMR spectrum shows resonances for five methyl groups at δ_H 1.71 (3 H, d, J = 6.1 Hz), 1.48 (3 H, d, J = 6.4 Hz), 1.35 (3 H, s), 1.08 (3 H, s), and 1.00 (3 H, d, J = 6.8 Hz); five anomeric protons at δ_H 6.36 (1 H, br s), 5.08 (1 H, d, J = 7.9 Hz), 4.90 (1 H, d, J = 7.6 Hz), 4.88 (1 H, d, J = 7.6 Hz), and 4.74 (1 H, d, J = 7.6 Hz); and one trisubstituted double bond at δ_H 5.54 (1 H, br d, J = 5.8 Hz) (Table 2). Based on the spectra of sugars obtained after acidic hydrolysis of **2** and its NMR data, β-D-glucopyranosyl, α-L-rhamnopyranosyl, β-D-xylopyranosyl, β-D-galactopyranosyl, and β-D-fucopyranosyl groups in the structure of **2** were confirmed. The ^{13}C NMR spectrum showed resonances for 56 carbon atoms, including one double bond (δ_C 139.5 and 124.8), five methyl groups (δ_C 19.3, 17.3, 16.9, 15.1, and 13.2), 11 sp^3 methylenes (five oxygenated, δ_C 70.3, 67.3, 63.2, 62.4, and 61.5), 35 sp^3 methines (29 oxygenated), and three sp^3 quaternary carbons (one oxygenated, δ_C 111.3). These NMR data were very similar to those of a known polyhydroxylated steroidal glycoside, padelaoside B (Zhang et al., 2009), with the exception of signals for one more hexose observed in **2**.

According to the ^{1}H-^{1}H COSY correlations in the 2D spectra of **2** (Fig. 2), eight connections (**a**–**h**) were confirmed. The planar structure of the sapogenin was further deduced as spirost-5-ene-1,3,21,23,24-pentol

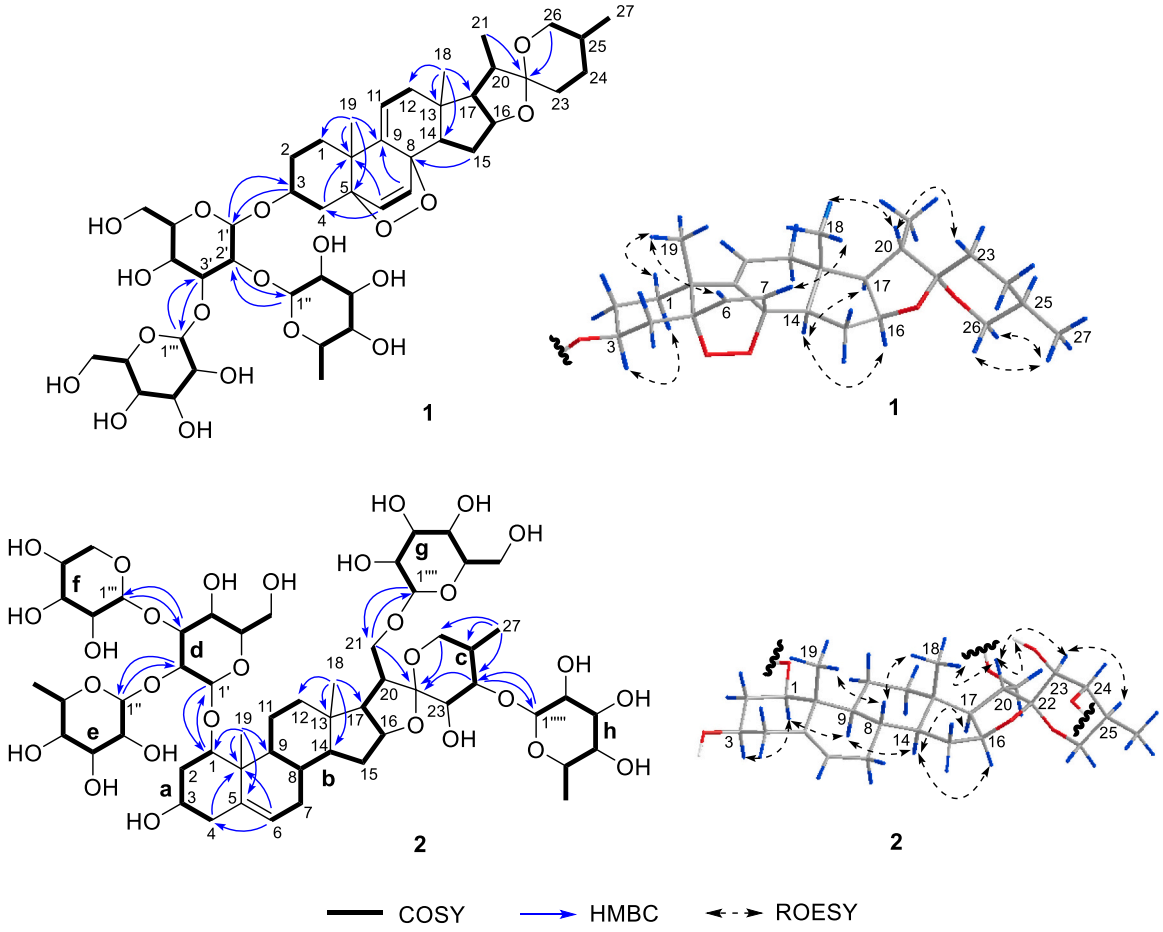

Fig. 2. Key 2D NMR correlations for compounds **1** and **2**.

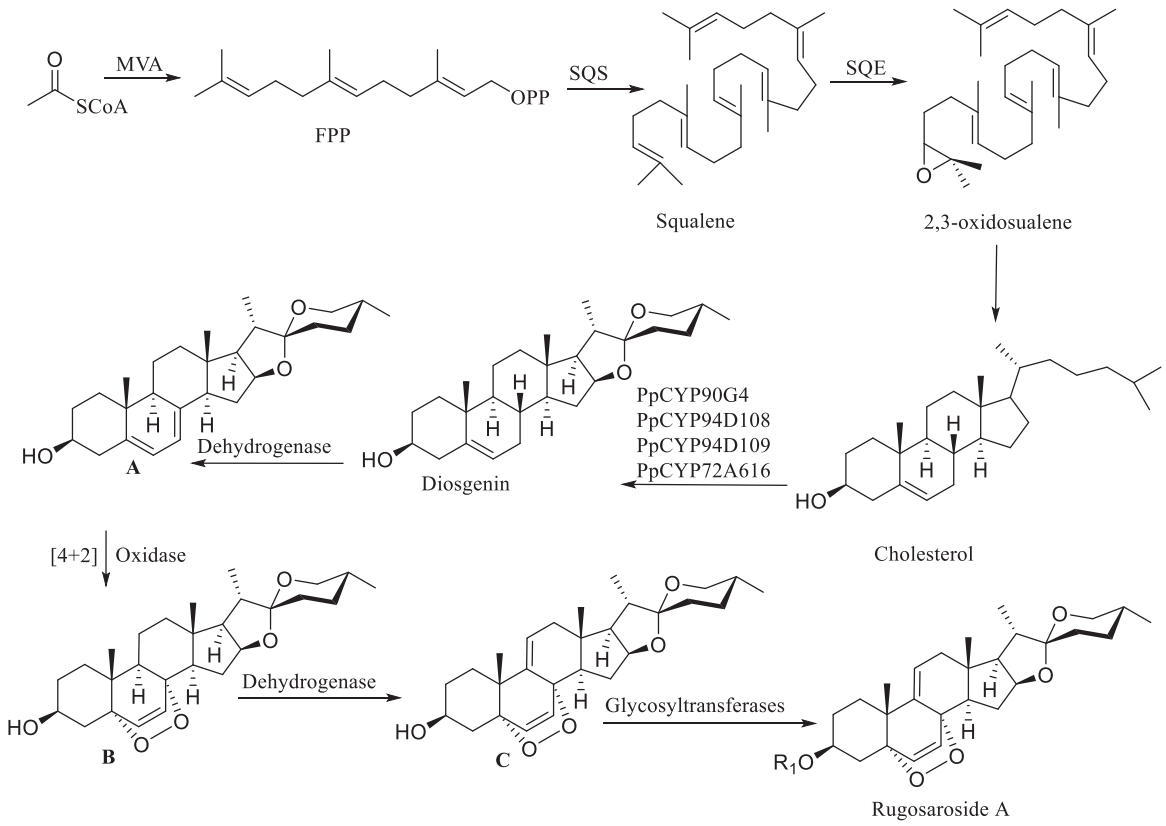

Fig. 3. Speculated biosynthetic pathway for rugosaroside A.

by the HMBC correlations from H_3-19 to C-1, C-5, C-9, and C-10; from H_2-4 to C-10; from H-6 to C-4 and C-10; from H_3-18 to C-12, C-13, C-14, and C-17; and from H_2-21, H-24, and H_2-26 to C-22. Based on the HMBC correlations of H-1 with C-1′, H-1′ with C-1, H-2′ with C-1″, H-1″ with C-2′, H-3′ with C-1‴, H-1‴ with C-3′, H_2-21 with C-1⁗, H-1⁗ with C-21, H-24 with C-1⁗′, and H-1⁗′ with C-24, the *β*-D-glucopyranose, *α*-L-rhamnopyranose, *β*-D-xylopyranose, *β*-D-galactopyranose, and *β*-D-fucopyranose were located at C-1, C-2′, C-3′, C-21, and C-24, respectively. The 1-OH and 3-OH configurations of the aglycone were assigned as 1*β*,3*β* based on the key ROESY correlations of H-1/H-3 and H-1/H-9 (Fig. 2). The C-23 and C-25 configurations were assigned as *S* by the key ROESY correlations of 23-OH/H-20 and H-23/H-25, which indicated that both H-23 and H-25 were in the axial orientation in ring F. The small coupling constant ($J_{23,24}$ = 2.7 Hz) between H-23 and H-24 indicated that H-24 was in the equatorial conformation, implying that C-24 was in the *S* configuration. Accordingly, the chemical structure of **2** (rugosaroside B) was determined to be (1*β*,3*β*,23 *S*,24 *S*,25 *S*)-21-(*β*-D-galactopyranosyloxy)-24-(*β*-D-fucopyranosyloxy)-3,23-dihydroxyspirost-5-en-1-yl *O*-*α*-L-rhamnopyranosyl-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranoside.

2.2. Cytotoxicity of the compounds toward cancer cell lines

The steroidal saponins from the genus *Paris* have good anticancer activity. Therefore, in this study, five human tumor cell lines, HL-60, MDA-MB-231, SW480, A549, and A549/Taxol cells, as well as normal BEAS-2B cells, were used to evaluate the *in vitro* cytotoxic activity of these 18 isolated compounds compared with the positive control drugs cisplatin and paclitaxel. The results of their cytotoxicity are shown in Table 3. Spirostanol saponins **6**–**12** and furostanol saponin **16** showed significant cytotoxic activity, with IC_{50} values ranging from 0.13 to 3.88 *μ*M. Furostanol saponins **14**, **15**, and **17** selectively inhibited HL-60, A549, and A549/Taxol cells (IC_{50}: 3.45–9.51 *μ*M) but had no cytotoxic effect on SW480 or MDA-MB-231 cells (IC_{50} > 40 *μ*M). 5,8-Epidioxy (**1** and **3**) and polyhydroxylated spirostanol saponins (**2**, **4**, **5**, and **13**) were inactive (IC_{50} > 40 *μ*M). Compared with 17-hydroxygracillin (**12**), the activity of dianchonglouoside A (**13**) decreased, implying that 27-OH substitution has a disadvantage for activity. Compared with methyl protopolyphyllin I (**16**), methyl protobioside (**17**) has lower cytotoxic activity because of the loss of an *α*-L-arabinofuranose.

3. Conclusions

In conclusion, we identified 18 compounds from *P. rugosa*, with two undescribed steroidal saponins, rugosarosides A (**1**) and B (**2**). The spirostanol saponins ophiopogonin C′ (**6**), polyphyllin D (**7**), formosanin C (**8**), paris H (**9**), paris saponin VII (**10**), gracillin (**11**), and 17-hydroxygracillin (**12**), and the furostanol saponin methyl protopolyphyllin I (**16**) showed significant cytotoxic activity. Furostanol saponins, methyl protogracillin (**14**), protogracillin (**15**), and methyl protobioside (**17**) selectively inhibited HL-60, A549, and A549/Taxol cells. The cytotoxicity of steroidal saponins is influenced by both the variety of aglycones and the sugar chains. The results of this study could promote the development and utilization of this plant.

4. Experimental

4.1. General experimental procedures

Optical rotations were recorded using a JASCO P-1020 polarimeter (Jasco Corp., Tokyo, Japan). Ultraviolet (UV) spectra were recorded on a Shimadzu UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan). Electronic circular dichroism (ECD) spectra were recorded using a Chirascan CD spectrometer (Applied Photophysics Ltd., Leatherhead, UK). ^{1}H and ^{13}C NMR spectra were collected on Bruker DRX-500, Avance III-600, and Ascend™ 800 MHz NMR spectrometers (Bruker

Table 2
^{1}H (500 MHz) and ^{13}C (126 MHz) NMR data for compound **2** in pyridine-d_5 (δ in ppm, J in Hz).

no.	δ_H	δ_C	no.	δ_H	δ_C
1	3.79, dd (12.1, 4.1)	84.6, CH	glc′		
2α	2.63, m	38.1, CH_2	1′	4.74, d (7.6)	100.2, CH
2β	2.40, m				
3	3.74, m	68.1, CH	2′	4.09, m	76.4, CH
4α	2.63, m	43.8, CH_2	3′	4.01, m	88.3, CH
4β	2.66, m				
5		139.5, C	4′	3.83, t (9.3)	70.0, CH
6	5.54, br d (5.8)	124.8, CH	5′	3.75, m	77.7, CH
7α	1.41, m	31.8, CH_2	6′	4.45, m	63.2, CH_2
7β	1.69, m			4.22, m	
8	1.45, m	33.1, CH	rha″		
9	1.58, m	50.3, CH	1″	6.36, br s	101.8, CH
10		42.8, C	2″	4.76, m	72.5, CH
11α	2.77, m	24.0, CH_2	3″	4.57, m	72.5, CH
11β	1.49, m				
12α	1.84, m	40.1, CH_2	4″	4.29, m	74.2, CH
12β	1.42, m				
13		41.1, C	5″	4.79, m	69.6, CH
14	1.12, m	57.0, CH	6″	1.71, d (6.1)	19.3, CH_3
15α	1.78, m	32.4, CH_2	xyl‴		
15β	1.45, m				
16	4.51, m	83.1, CH	1‴	4.90, d (7.6)	105.2, CH
17	1.88, m	58.3, CH	2‴	3.93, m	74.7, CH
18	1.08, s	16.9, CH_3	3‴	4.06, m	78.4, CH
19	1.35, s	15.1, CH_3	4‴	4.10, m	70.7, CH
20	3.42, m	43.5, CH	5‴	4.24, m	67.3, CH_2
				3.66, m	
21	4.51, m	70.3, CH_2	gal⁗		
	3.94, m				
22		111.3, C	1⁗	4.88, d (7.6)	105.4, CH
23	4.26, dd (10.5, 2.7)	70.7, CH	2⁗	4.48, m	72.6, CH
24	4.01, m	81.5, CH	3⁗	4.15, m	75.5, CH
25	1.87, m	35.1, CH	4⁗	4.55, m	70.3, CH
26	3.90, m	61.5, CH_2	5⁗	4.09, m	77.1, CH
	3.32, dd (10.7, 4.5)				
27	1.00, d (6.8)	13.2, CH_3	6⁗	4.43, m	62.4, CH_2
23-OH	5.86, d (10.5)		fuc⁗′		
4′-OH	5.39, br s		1⁗′	5.08, d (7.9)	106.0, CH
6′-OH	5.72, t (5.3)		2⁗′	4.40, m	73.4, CH
6⁗-OH	6.49, t (5.4)		3⁗′	4.04, m	75.4, CH
4⁗′-OH	6.10, d (4.3)		4⁗′	3.94, m	72.9, CH
			5⁗′	3.69, m	71.5, CH
			6⁗′	1.48, d (6.4)	17.3, CH_3

Corporation, Karlsruhe, Germany). ESI-MS and HR-ESI-MS analyses were performed on HRESIMS data obtained using a Shimadzu LC/MS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan). Gas chromatography (GC) was performed on an Agilent 8890 N apparatus with an HP-5 capillary column (30 mm × 0.32 mm × 0.25 μm) for the trimethylsilylated hydrolysates analyses. Silica gel G (80–100 and 300–400 mesh, Qingdao Meigao Chemical Co., Ltd., Qingdao, China), RP C_{18} silica gel (40–75 μm, Fuji Silysia Chemical Ltd., Aichi, Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were selected for column chromatography. Thin-layer chromatography spots were visualized under UV light at 254 nm and by dipping in 5 % H_2SO_4 in alcohol followed by heating. Semipreparative HPLC was performed on an Agilent 1200 series pump (Agilent Technologies, Santa Clara, USA) equipped with a diode array detector, an Agilent Zorbax RX-C_8 column (5 μm, ϕ 9.4 × 250 mm), and an Agilent Zorbax SB-C_{18} column (5.0 μm, ϕ 9.4 × 250 mm). The absorbance from the MTS assay was measured with a Thermo Multiskan FC microplate reader (Waltham, MA, USA).

4.2. Plant material

The plant material was collected from local cultivation in Fugong County, Yunnan Province, China, in September 2020. The plant samples were identified as *Paris rugosa* H. Li & Kurita by Jun Yang, a technician at Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (no. YJ2020007) was deposited at the Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany, Chinese Academy of Sciences.

Table 3
Cytotoxicity of steroidal saponins from *Paris rugosa* rhizomes.

Compound	HL-60	SW480	MDA-MB-231	A549	A549/Taxol	BEAS-2B
IC_{50} (μM)						
6	1.97 ± 0.04	3.48 ± 0.10	3.34 ± 0.06	3.09 ± 0.05	3.26 ± 0.13	2.14 ± 0.14
7	1.04 ± 0.01	1.15 ± 0.05	1.26 ± 0.08	0.51 ± 0.04	0.61 ± 0.02	0.64 ± 0.01
8	0.23 ± 0.01	0.17 ± 0.01	0.26 ± 0.01	0.19 ± 0.00	0.13 ± 0.00	0.14 ± 0.00
9	3.28 ± 0.14	2.42 ± 0.12	1.69 ± 0.10	0.82 ± 0.03	0.84 ± 0.04	1.12 ± 0.06
10	2.54 ± 0.11	0.94 ± 0.01	2.68 ± 0.16	0.69± 0.04	0.75 ± 0.04	0.61 ± 0.03
11	2.05 ± 0.02	0.9 ± 0.06	1.85 ± 0.06	0.70 ± 0.02	0.66 ± 0.00	0.67 ± 0.01
12	3.23 ± 0.09	3.88 ± 0.08	3.48 ± 0.04	2.93 ± 0.17	3.19 ± 0.03	3.48 ± 0.12
14	7.06 ± 0.42	> 40	> 40	3.68 ± 0.08	3.70 ± 0.06	26.45 ± 0.48
15	9.51 ± 0.78	> 40	> 40	3.45 ± 0.08	3.76 ± 0.07	> 40
16	2.10 ± 0.06	3.49 ± 0.11	0.97 ± 0.02	0.59 ± 0.01	0.67 ± 0.02	3.32 ± 0.19
17	4.69 ± 0.18	> 40	> 40	3.57 ± 0.07	3.58 ± 0.20	> 40
Cisplatin[a]	15.06 ± 0.30	22.67 ± 0.73	23.34 ± 0.46	20.07 ± 0.30	19.76 ± 0.45	> 40
Paclitaxel[a]	< 0.008	< 0.008	< 0.008	< 0.008	0.73 ± 0.05	0.71 ± 0.16

[a] Positive control
[b] The inhibition rates of compounds **1–5**, **13**, and **18** were less than 50 % at 40 μM.

4.3. Extraction and isolation

The air-dried and powdered rhizomes of *P. rugosa* (4.1 kg) were extracted with 70 % EtOH (50 L each) at 80 °C three times (4 h, 3 h, and 3 h each). The combined extraction mixture was evaporated to yield the crude extract (1.4 kg), which was subsequently suspended in 16 L of water and then successively extracted with EtOAc (16 L × 3) and *n*-BuOH (16 L × 4). The organic layers were evaporated under reduced pressure to yield the EtOAc-soluble fraction (123.4 g) and the *n*-BuOH-soluble fraction (219.3 g).

The *n*-BuOH-soluble fraction (209.2 g) was applied to a silica gel column using CH_2Cl_2-MeOH (10:1–1:1) to yield seven additional fractions (Fr. I–Fr. VII). Fr. VII (162.0 g) was applied to a silica gel column using EtOAc-MeOH (10:1–1:1) to yield four fractions (Fr. VII-1–Fr. VII-4). Fr. VII-2 (6.1 g) was subjected to RP C_{18} chromatography (MeOH-H_2O, 10:90–100:0) to yield three fractions, namely, Fr. VII-2–1 (146.7 mg), Fr. VII-2–2 (84.9 mg), and Fr. VII-2–3 (334.0 mg). Fr. VII-2–1 (146.7 mg) was separated and purified on a silica gel column using CH_2Cl_2-MeOH (15:1–1:1) to yield **18** (35.0 mg). Fr. VII-2–2 (84.9 mg) was separated and purified on a silica gel column using CH_2Cl_2-MeOH (12:1–1:1) to yield **6** (12.0 mg). Fr. VII-2–3 (334.0 mg) was purified on a Sephadex LH-20 column (CH_2Cl_2-H_2O, 1:1) to yield **7** (150.0 mg).

Fr. VII-4 (112.0 g) was subjected to RP C_{18} chromatography (MeOH-H_2O, 10:90–100:0) to yield four fractions Fr. VII-4–1 (0.8 g), Fr. VII-4–2 (18.6 g), Fr. VII-4–3 (13.3 g), and Fr. VII-4–4 (56.8 g). Fr. VII-4–1 (0.8 g) was isolated using Sephadex LH-20 column chromatography (MeOH) and then semipreparative HPLC (Agilent Zorbax RX-C_8 column, 5.0 μm, ϕ 9.4 × 250 mm; v = 2 mL/min, MeCN-H_2O, 20:80) to yield **4** (29.3 mg, t_R = 11.5 min) and Fr. VII-4–1–1 (9.1 mg, t_R = 9.3 min). VII-4–1–1 was subjected to semipreparative HPLC (Agilent Zorbax RX-C_8 column, 5.0 μm, ϕ 9.4 × 250 mm; v = 2 mL/min, MeCN-H_2O, 16:84) to yield **2**

(4.1 mg, t_R = 43.1 min) and **5** (4.3 mg, t_R = 45.2 min). Fr. VII-4–3 (13.3 g) was applied to a silica gel column using CH_2Cl_2-MeOH (8:1–1:1) to yield two fractions, Fr. VII-4–3–1 (8.3 g) and Fr. VII-4–3–2 (0.3 g). Fr. VII-4–3–1 (100 mg) was isolated by Sephadex LH-20 column chromatography (MeOH) and then by semipreparative HPLC (Agilent Zorbax SB-C_{18} column, 5.0 μm, ϕ 9.4 × 250 mm; v = 2 mL/min, MeOH-H_2O, 80:20) to yield **15** (5.1 mg, t_R = 9.7 min) and **17** (9.3 mg, t_R = 20.6 min). Fr. VII-4–3–2 (0.3 g) was isolated using Sephadex LH-20 column chromatography (MeOH) and then by semipreparative HPLC (Agilent Zorbax SB-C_{18} column, 5.0 μm, ϕ 9.4 × 250 mm; v = 2 mL/min, MeOH-H_2O, 70:30) to yield **16** (18.7 mg, t_R = 17.5 min) and **14** (23.3 mg, t_R = 21.0 min).

Fr. VII-3 (22.2 g) was subjected to RP C_{18} chromatography (MeOH-H_2O, 10:90–100:0) to yield four fractions Fr. VII-3–1 (63.4 mg), Fr. VII-3–2 (312.3 mg), Fr. VII-3–3 (334.0 mg), and Fr. VII-3–4 (56.0 mg). VII-3–1 was subjected to Sephadex LH-20 column chromatography (MeOH) followed semipreparative HPLC (Agilent Zorbax SB-C_{18} column, 5.0 μm, ϕ 9.4 × 250 mm; v = 2 mL/min, MeOH-H_2O, 63:37) to yield **13** (4.5 mg, t_R = 21.9 min). Fr. VII-3–2 (312.3 mg) was subjected to Sephadex LH-20 column chromatography (CH_2Cl_2-MeOH, 1:1) and then semipreparative HPLC (Agilent Zorbax SB-C_{18} column, 5.0 μm, ϕ 9.4 × 250 mm; v = 2 mL/min, MeCN-H_2O, 43:57) to yield **3** (154.5 mg, t_R = 24.7 min). Fr. VII-3–3 (334.0 mg) was subjected to Sephadex LH-20 column chromatography (CH_2Cl_2-MeOH, 1:1) followed by semipreparative HPLC (Agilent Zorbax SB-C_{18} column, 5.0 μm, ϕ 9.4 × 250 mm; v = 2 mL/min, MeCN-H_2O, 43:57) to yield **9** (2.1 mg, t_R = 17.5 min), **12** (7.3 mg, t_R = 16.2 min), **10** (5.0 mg, t_R = 13.8 min), and **1** (4.5 mg, t_R = 19.4 min). Fr. VII-3–4 (56.0 mg) was purified by Sephadex LH-20 column chromatography (CH_2Cl_2-MeOH, 1:1) to yield compound **8** (5.2 mg) and Fr. VII-3–4–1 (16.0 mg). Fr. VII-3–4–1 (16.0 mg) was subjected to semi-preparative HPLC (Agilent Zorbax SB-C_{18} column, 5.0 μm, ϕ 9.4 × 250 mm; v = 2 mL/min, MeCN-H_2O, 55:45) to yield compound **11** (3.1 mg, t_R = 12.8 min).

*Rugosaroside A (**1**)*: White amorphous powder, $[\alpha]_D^{25}$ –28 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 323 (2.03), 274 (2.51), 216 (3.54); ECD (*c* 0.08, MeOH) λ_{max} ($\Delta\varepsilon$) 217 (+10.04) nm; ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS *m/z* 935 $[M + Na]^+$; HR-ESI-MS *m/z* 935.4245 $[M + Na]^+$ (calcd for $C_{45}H_{68}NaO_{19}$, 935.4247).

*Rugosaroside B (**2**)*: White amorphous powder, $[\alpha]_D^{25}$ –43 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 279 (2.19), 253 (2.58); ^{1}H and ^{13}C NMR data, see Table 2; ESI-MS *m/z* 1249 $[M + Na]^+$; HR-ESI-MS *m/z* 1225.5497 $[M - H]^-$ (calcd for $C_{56}H_{89}O_{29}$, 1225.5495).

4.4. Acid hydrolysis and GC analysis of the sugar moieties in the new compounds

Compounds **1** and **2** (each 2 mg) were heated with 2 M HCl (2 mL) at 90 °C for 5 h. The reaction mixture was partitioned with EtOAc (2 mL × 3). The aqueous layer was concentrated and dissolved in 1 mL of anhydrous pyridine and treated with 1 mg of L-cysteine methyl ester hydrochloride. The mixture was subsequently stirred at 60 °C for 1 h. After the reaction mixture was cooled, 1.0 mL of trimethylsilyl imidazole was added, and the mixture was heated at 60 °C for 1 h. The upper layer was analyzed using GC with an initial column temperature of 180 °C, after which the temperature was programmed to 275 °C at a rate of 3 °C/min. The carrier gas was N_2 (1 mL/min), and the flame ionization detector (FID) was operated at 250 °C. The retention times for authentic sugars after derivatization were 14.79 min (L-xylopyranose), 15.66 min (L-rhamnopyranose), 15.93 (D-rhamnopyranose), 15.94 min (D-fucopyranose), 16.73 min (L-fucopyranose), 19.0 min (D-xylopyranose), 19.03 min (D-glucopyranose), 19.5 min (L-glucopyranose), 19.62 min (D-galactopyranose), and 20.30 min (L-galactopyranose). The sugars of the new compounds were identified according to their retention times. By comparison, the sugars of compound **2** were L-rhamnopyranose, D-galactopyranose, D-fucopyranose, D-glucopyranose, and D-xylopyranose; the sugars of compound **1** were L-rhamnopyranose and D-glucopyranose.

4.5. MTS assay for cytotoxicity

The cytotoxic effects of compounds **1**–**18** on the proliferation of HL-60, SW-480, MDA-MB-231, A549, A549/Taxol, and BEAS-2B cells *in vitro* were tested using a 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2*H*-tetrazolium (MTS; Promega, Beijing, China) assay. These cell lines were obtained from the ATCC (Manassas, VA, USA).

MTS experiments were performed as described in previous studies (Mosmann, 1983). In general, a single-cell suspension was prepared with culture medium containing 10 % fetal bovine serum (DMEM), and 3 000–15 000 cells per well were inoculated into 96-well plates at a volume of 100 μL per well. The cells were inoculated and cultured for 12–24 hours. After incubation at 37 °C for 12 h, the sample to be tested was added. The cancer cell lines were exposed to five different concentrations of test extracts or compounds, each at a concentration of three portions. After incubation at 37 °C for 48 h, MTS (20 μL) solution and DMEM (100 μL) were added. The cells were continually incubated for 2–4 h. The absorbance was measured at a detection wavelength of 492 nm, and the cytotoxicity was expressed as the IC_{50} (Reed and Muench, 1938).

CRediT authorship contribution statement

Jian-Ke Jia: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Jun Yang:** Resources, Investigation, Formal analysis. **Xing-Zhi Yang:** Software, Methodology, Investigation. **Xiao-Yan Duan:** Resources, Methodology. **Ying-Li Yang**: Resources. **Ji-Feng Luo**: Resources. **Jin-Fu Wan:** Visualization, Validation, Supervision. **Yue-Hu Wang:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation.

Declaration of Competing Interest

The authors have no conflicts of interest associated with this study to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phytol.2024.07.011.

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