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A new dammarane-type saponin from *Gynostemma pentaphyllum* induces apoptosis in A549 human lung carcinoma cells

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ABSTRACT

Gynostemma pentaphyllum has been widely used as a traditional herb for its antioxidant and immunostimulatory activities. We have previously reported several useful dammarane-type saponins with cytotoxicity against A549 human lung cancer cells from heat-processed *G. pentaphyllum*. In this study, a new dammarane-type saponin, 20(*S*)-2α,3β,12β-tetrahydroxydammar-3-*O*-β-D-glucopyranoside (namely gypenoside Jh1), was isolated from the ethanol extract of heat-processed *G. pentaphyllum* using column chromatography and semi-preparative HPLC. Gypenoside Jh1 exhibited strong cytotoxicity against A549 cells in a concentration-dependent manner, which was associated with apoptotic cell death characterized by morphological changes, Hoechst 33258 nuclear staining, Annexin V and propidium iodide binding and mitochondrial potentials assay. Quantitative analysis using flow cytometry also showed that the proportion of apoptotic cells was increased after gypenoside Jh1 treatment. These findings indicated that gypenoside Jh1 showed antiproliferative effects on A549 cells and mitochondrial-dependent pathway is involved in gypenoside Jh1-induced apoptosis.

Cancer has been a leading cause of global morbidity and mortality due to its rapid progression and poor prognosis.[1,2] For a long time, cancer chemoprevention and chemotherapy have been developed as a major field of scientific research. Although various chemical synthetic drugs have been put into use clinically, overall success in treating the disease is not satisfactory due to lots of serious side effects.[3] Thus, much attention has been focused on anticancer agents from natural resources to change the unsatisfactory situation.[4,5] Indeed, some natural products have been clinically used as anticancer drugs because of their high efficiency and low toxicity.[6,7]

The herb *Gynostemma pentaphyllum* (Thunb.) Makino (Cucurbitaceae) has been used as popular folk medicine in Asia for centuries. It exhibits a variety of biological activities such as immune-stimulation,[8] regulation of liver functions,[9] inhibition of hyperlipoproteinemia,[10] and tumor suppression.[11] There are many dietary products containing *G. pentaphyllum* extract in China, such as Jiaogulan tea and Jiaogulan concentrated juice. *G. pentaphyllum* has been reported to contain saponins,[12] flavonoid,[13] polysaccharides,[14] vitamins and amino acids,[15] among which gypenosides, dammarane-type saponins, are the major active components.[16] Previous studies have shown pharmacological activities of gypenosides against human tongue cancer,[17] leukemia,[18] colon cancer,[19] and cervical cancer.[20] However, studies that examine anticancer effect of individual active components from *G. pentaphyllum* are sparse. In our previous study, we found that total gypenosides from ethanol extract of heat-processed *G. pentaphyllum* exhibited much stronger cytotoxic activity against human lung adenocarcinoma A549 cells than that of the raw plant.[21] Further, four dammarane-type saponins with greatly stronger anticancer activity than total gypenosides have been isolated from heat-processed *G. pentaphyllum*.[22] In the present study, a new compound, gypenoside Jh1 (**1**), was isolated from the ethanol extracts of heat-processed *G. pentaphyllum* (Fig. 1). Potential inhibitory activity of the isolated gypenoside on growth of human lung cancer cells was evaluated. In addition, basic mechanism underlying cytotoxicity of gypenoside Jh1 was also investigated with the aspect of inducing apoptosis.

G. pentaphyllum collected from Fujian Province in September 2011 were purchased from Tong Ren Tang (Beijing, China). Voucher specimen (No. GP2011-01) was deposited at the Isolation and Structure Identification Laboratory in Minzu University of China, China. The air dried whole *G. pentaphyllum* (10 kg) were

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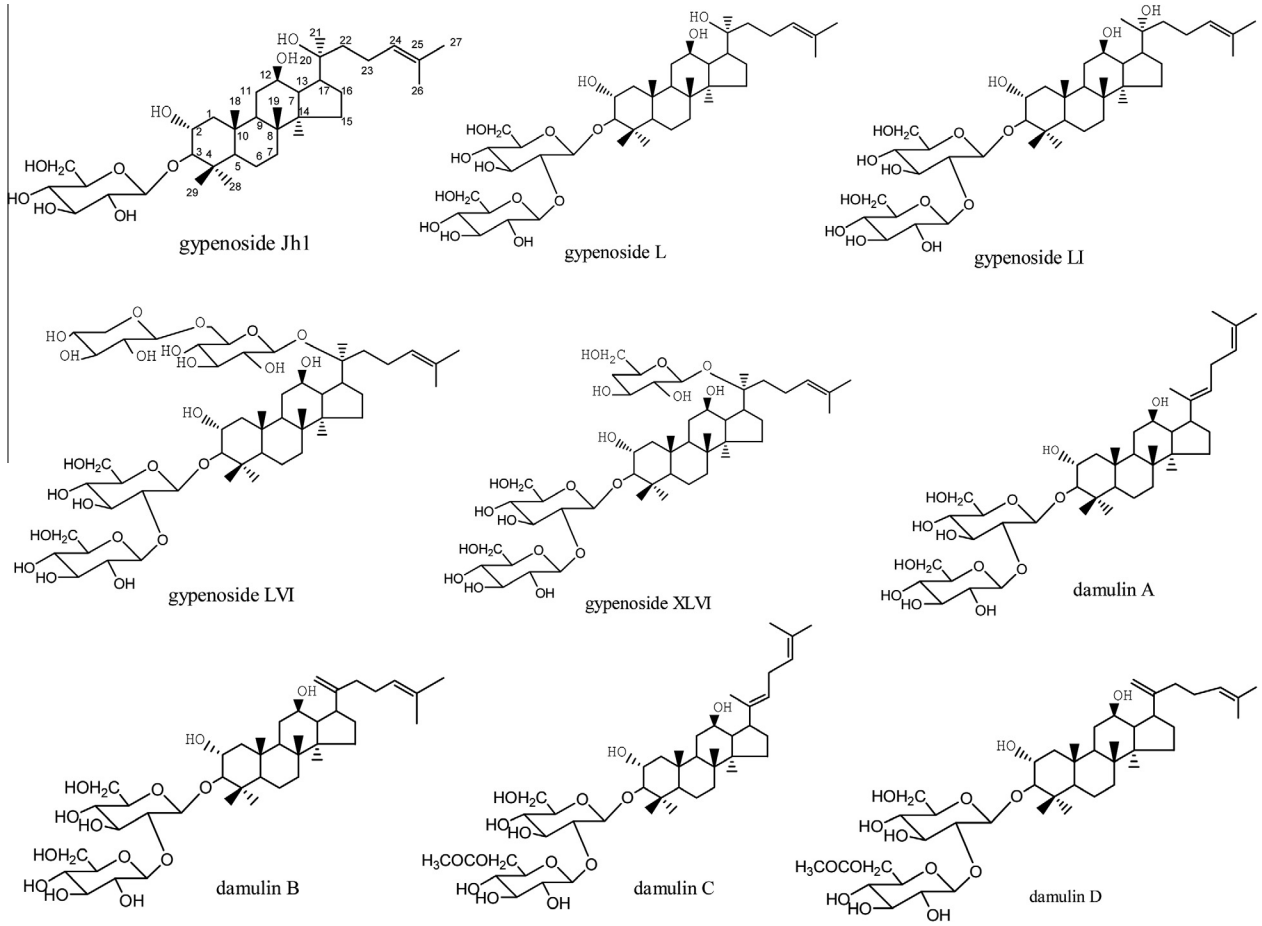

Figure 1. Chemical structures of dammarane-type saponins from raw and heat-processed *G. pentaphyllum*.

cut into pieces and steamed at 125 °C and 0.24 MPa for 3 h. The steamed *G. pentaphyllum* were then refluxed with 80% ethanol three times (2 h, 2 h and 1 h, respectively). The organic solvent was collected and then removed under a vacuum to give ethanol extract (1.35 kg). Using various chromatographic and spectroscopic techniques, compound **1** was isolated and identified from the extract.[23]

Compound **1** was obtained as white powder. In the negative mode ESI-MS of **1**, a quasimolecular ion peak at *m*/*z* 637.4 $[M-H]^-$ was appeared. The HRESIMS analysis indicated a deprotonated ion peak at *m*/*z* 637.4316 (Error: +2.29 ppm) which corresponds to the molecular formula $C_{36}H_{61}O_9$. Under the optimized collision conditions, the $[M-H-162]^-$ ion at *m*/*z* 475.5 clearly resulted from the loss of glucosyl moiety of C3 position. The IR spectrum revealed the presence of hydroxyl functional groups at 3435 cm^-, olefinic C=C double bond at 1631 cm^{-1}, and C—O stretching vibrations at 1082 cm^{-1}. The ^1H NMR (CD_3OD, 600 MHz) spectrum of compound **1** indicated the presence of 8 methyl signals at δ 0.92 (3H, s, H-29), 0.95 (3H, s, H-30), 1.00 (3H, s, H-19), 1.04 (3H, s, H-18), 1.14 (3H, s, H-28), 1.16 (3H, s, H-21), 1.62 (3H, s, H-27) and 1.68 (3H, s, H-26), and an anomeric proton signal at δ 4.34 (1H, d, J = 7.8 Hz, H-1′). According to the anomeric proton coupling constant value of 7.8 Hz, the configuration of the sugar could be identified as β-type (Table 1). The ^{13}C NMR (CD_3OD, 125 MHz), DEPT and HMQC spectra showed 36 carbon signals, among which 30 carbons were assigned to aglycon. The ^1H NMR data and the chemical shift of two olefinic carbon signals at δ 124.8 (C-24) and 130.6 (C-25) supported the fact that compound **1** has a dammarane skeleton. The ^1H and ^{13}C NMR spectra of compound **1** also showed signals for an anomeric proton at δ 4.34 (H-1′) and an anomeric carbon at δ 105.0 (C-1′). These results indicated the presence of one monosaccaride moiety.[24] Combined use of COSY, HSQC, and HMBC experiments allowed the sequential assignments of all resonances for the monosaccharide, starting from the anomeric proton (Table 1). The sequence and linkages between the sugar moiety and the aglycon were revealed by HMBC spectrum. The H-3 of the aglycon (δ 3.02) showed a correlation with the anomeric carbon of glucose (C-1′, δ 105.0). On the basis of the obtained data, the structure of compound **1** was assigned as 20(*S*)-2α,3β,12β-tetrahydroxydammar-3-*O*-β-D-glucopyranoside, namely gypenoside Jh1 (Fig. 1).

In our previous study, we found that gypenoside LVI and gypenoside XLVI (Fig. 1) were main saponins of raw *G. pentaphyllum* and their contents were decreased in the extract from heat-processed *G. pentaphyllum*.[25] A newly isolated gypenoside Jh1 was not detected in raw *G. pentaphyllum* and it can be inferred that gypenoside Jh1 was produced from gypenoside LVI and gypenoside XLVI by the loss of glucose and xylose moiety during heat treatment.

The isolated compound was evaluated for its ability to inhibit growth of A549 cells to search for anticancer agent against lung cancer. To investigate cytotoxicity of gypenoside Jh1 in A549 cells, we employed CCK-8 assay method.[26] In previous study, gypenoside L and gypenoside LI, which are structurally similar to gypenoside Jh1, have been reported to exert cytotoxic effect on A549 cells,[21] thus they were also tested to compare the cytotoxicity. As shown in Figure 2, all tested gypenosides induced a decrease in the viable A549 cells in a concentration-dependent manner. Gypenoside Jh1 showed a significantly stronger inhibitory effect against A549 cells growth than gypenoside L and gypenoside LI over the concentra-

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR chemical shift values of compound **1** isolated from heat-processed *G. pentaphyllum* in CD_3OD

No.	^{1}H	^{13}C	DEPT
1	2.11 (1H, m), 0.96 (1H, m)	46.4	CH_2
2	3.74 (1H, m)	66.8	CH
3	3.02 (1H, d, *J* = 9.0 Hz)	94.6	CH
4		40.2	C
5	0.90 (1H, m)	55.8	CH
6	1.62 (1H, m), 1.53 (1H, m)	17.9	CH_2
7	1.61 (1H, m), 1.36 (1H, m)	34.4	CH_2
8		39.6	C
9	1.53 (1H, m)	49.9	CH
10		37.5	C
11	1.87 (1H, m), 1.32 (1H, m)	30.8	CH_2
12	3.58 (1H, m)	70.6	CH
13	1.75 (1H, m)	47.9	CH
14		51.2	C
15	1.56 (1H, m), 1.08 (1H, m)	30.6	CH_2
16	1.90 (1H, m), 1.35 (1H, m)	25.9	CH_2
17	2.06 (1H, m)	53.7	CH
18	1.04 (3H, s)	14.8	CH_3
19	1.00 (3H, s)	16.5	CH_3
20		73.0	C
21	1.16 (3H, s)	25.1	CH_3
22	1.56 (1H, m), 1.42 (1H, m)	34.9	CH_2
23	2.16 (1H, m), 2.03 (1H, m)	21.9	CH_2
24	5.13 (1H, m)	124.8	CH
25		130.6	C
26	1.68 (3H, s)	24.5	CH_3
27	1.62 (3H, s)	16.3	CH_3
28	1.14 (3H, s)	27.3	CH_3
29	0.92 (3H, s)	16.5	CH_3
30	0.95 (3H, s)	15.7	CH_3
1′	4.34 (1H, d, *J* = 7.8 Hz)	105.0	CH
2′	3.27 (1H, m)	74.1	CH
3′	3.36 (1H, m)	76.8	CH
4′	3.34 (1H, m)	70.0	CH
5′	3.34 (1H, m)	76.7	CH
6′	3.85 (1H, m), 3.66 (1H, m)	61.0	CH_2

tion range from 40 to 80 μg/mL. Treatment of A549 cells with 60 μg/mL gypenoside Jh1 led to 44.7 ± 1.9% decrease in the viable cell numbers, whereas gypenoside LI and gypenoside L resulted in 30.0 ± 0.6% and 37.2 ± 1.6% decrease, respectively.

Morphological changes A549 cell after gypenoside Jh1 treatment for 48 h were observed under an inverted microscope (Olympus CKX41, Japan). Compared with control group, treated group showed a significant increase in detached cells in culture medium. Characteristic changes of morphology were also noted in A549 cells treated with gypenoside Jh1. The cells displayed a round and shrunken shape significantly in contrast to normal cells (Fig. 3A). Morphologic changes in the nucleus representing cellular apoptosis were also assessed by staining with membrane-permeable DNA binding dye Hoechst 33258. A549 cells (6×105/well) were seeded in 6-well plates, and incubated with 50 μg/mL gypenoside Jh1 for 48 h. Cells were fixed and stained with DNA fluorochrome Hoechst 33258 (Beyotime, China) for 5 min. The cells were then washed with PBS and the morphological features were examined with a fluorescence microscope (Olympus IX81, Japan). As shown in Figure 3B, normal cells exhibited regular and round shaped nuclei with a pallid blue. However, A549 cells treated with gypenoside Jh1 showed a bright fluorescence resulting from chromatin condensation, which is one of the prominent features of apoptotic cells.

Apoptosis is an important regulator in developmental processes, maintenance of homeostasis and elimination of the damaged cells. Induction of apoptosis in cancer cells is one of strategy for the development of cancer therapy. To further investigate gypenoside Jh1-induced apoptotic effect, we performed Annexin V-FITC staining assay and quantified apoptotic A549 cells using flow cytometry. Cells were double stained with Annexin V-FITC and PI after treatment with 50 μg/mL gypenoside Jh1 for 48 h. The apoptotic cells were quantitatively measured using flow cytometry (Beckman Coulter Epics XL, USA). The proportion of apoptotic cells was increased from 4.6% to 14.1% (Fig. 4A and B). Annexin V has been used to detect the externalization of membrane phospholipid phosphatidylserine at an early stage of apoptosis and these results showed the translocation of phosphatidylserine in gypenoside Jh1-treated cells.

The mitochondrial potential variation caused by gypenoside Jh1 in A549 cells was evaluated using fluorescent dye JC-1. After treatment with 50 μg/mL gypenoside Jh1 for 48 h, some adherent cells were washed with PBS and then stained with JC-1 staining solution according to manufacturer's protocol (Beyotime, China). The green/red fluorescence intensity indicating mitochondrial depolarization was monitored by fluorescence microscopy. Gypenoside Jh1-treated cells emitted obvious green fluorescence, while untreated cells produced orange–red fluorescence with a little green fluorescence (Fig. 5A). The stronger green fluorescence means the more loss of mitochondrial membrane potentials. Flow cytometry analysis was used to investigate the mitochondrial potential variation caused by gypenoside Jh1. The ratio of red fluorescence to green

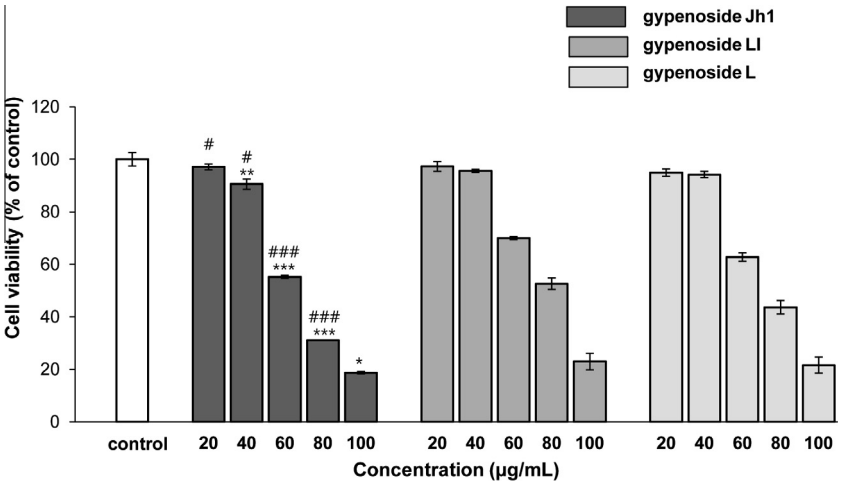

Figure 2. Inhibitory effect of gypenoside Jh1 on A549 cells growth. Data are shown as mean ± SD of three separate experiments and expressed as percentage (%) of control. $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001 compared with gypenoside LI. $^{\#}p$ <0.05, $^{\#\#}p$ <0.01 and $^{\#\#\#}p$ <0.001 compared with gypenoside L.

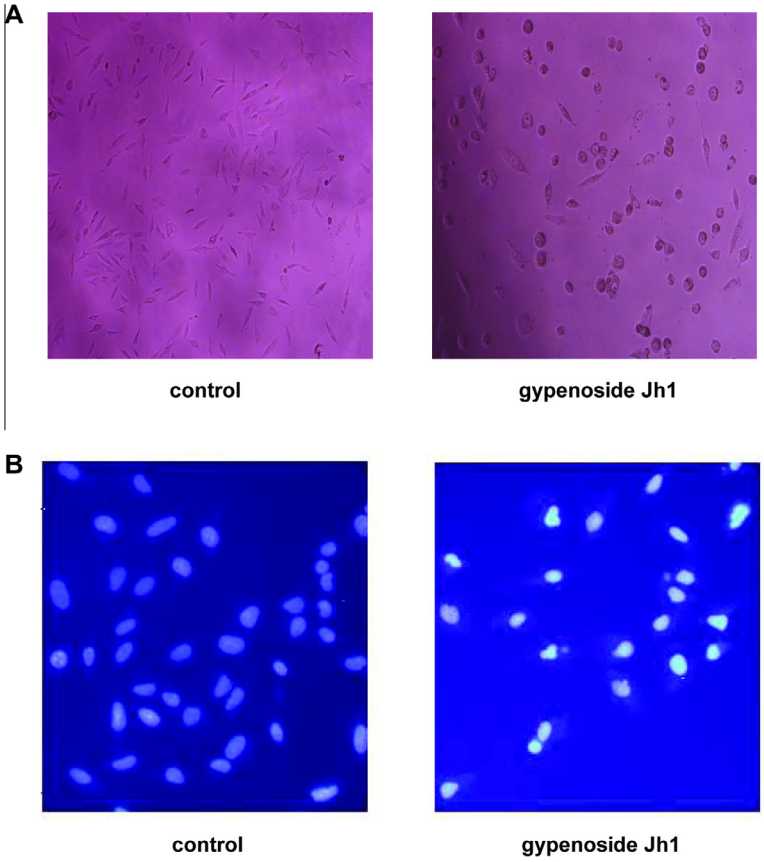

Figure 3. (A) Microscopy images of control A549 cells and cells treated with 50 μg/mL gypenoside Jh1 for 48 h. (B) Morphological changes in the nuclei of A549 cells treated with gypenoside Jh1. A549 cells were treated by 50 μg/mL of gypenoside Jh1 for 48 h and stained with Hoechst 33258.

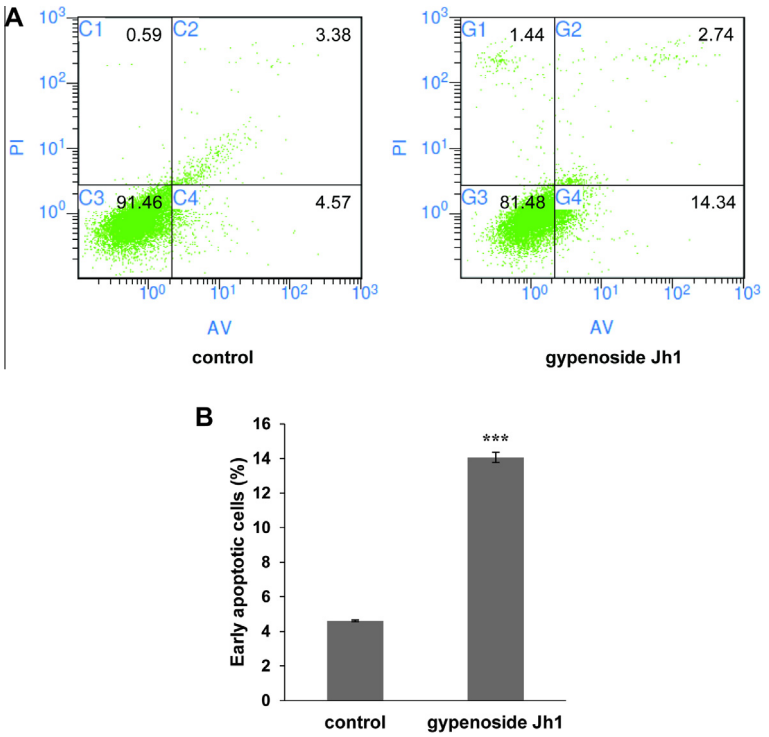

Figure 4. (A) Flow cytometric analysis of apoptosis in A549 cells treated with 50 μg/mL of gypenoside Jh1 for 48 h using Annexin V-FITC and PI staining. Different stage cells were determined by counting the percentage. (B) Quantitative analysis of the number of early apoptotic cells. Data were shown as mean ± SD from three separate experiments. $^{***}p$ <0.001 compared with control cells.

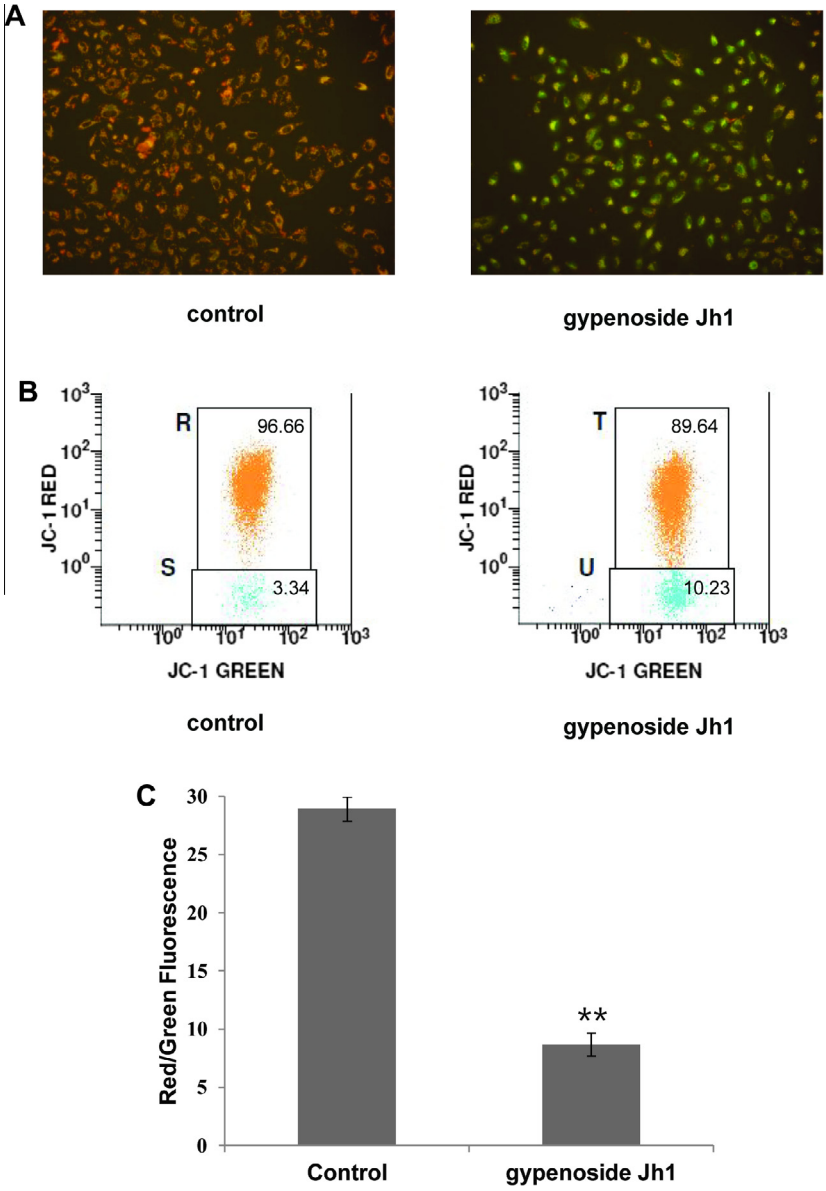

Figure 5. The effect of gypenoside Jh1 on the mitochondrial membrane potential of A549 cells. (A) A549 cells stained with JC-1 probe were imaged with a fluorescence microscope. (B) Apoptotic cells were analyzed by flow cytometry after JC-1 staining. Different group cells were determined by counting the percentage. (C) Quantitative analysis of the shift of mitochondrial orange–red fluorescence to green fluorescence among groups. Data were shown as mean ± SD from three separate experiments. $^{**}p$ <0.01 compared with control cells.

fluorescence was decreased from 28.9 to 8.7 (Fig. 5B and C). These findings revealed that gypenoside Jh1 caused a loss of mitochondrial membrane potential. Generally, apoptosis occurs by cell death receptor-induced extrinsic pathway or mitochondria-mediated intrinsic pathway. And the majority of chemotherapeutic drugs have been reported to be typical activators of the intrinsic mitochondrial pathway of apoptosis.[27,28] In our results, variation from orange–red fluorescence to obvious green fluorescence in JC-1 staining experiment and the increase of green fluorescence intensity ratio suggested that gypenoside Jh1 induced apoptosis, at least in part, through the mitochondria-mediated intrinsic pathway.

In our previous studies, we have isolated some dammarane saponins from raw and heat-processed *G. pentaphyllum* and have evaluated their bioactivities (Fig. 1).[21,22,29,30] To examine a possible structure–function relationship between structurally related saponins, we listed their IC_{50} values against A549 cells (Table 2).

Table 2
A549 cell inhibitory activities of dammarane saponins isolated from raw and heat-processed *G. pentaphyllum*

Compound	IC_{50} (μg/mL)
Gypenoside Jh1	74.3 ± 1.9
Gypenoside L	78.6 ± 0.4
Gypenoside LI	91.0 ± 0.6
Gypenoside XLVI	111.6 ± 10.8
Gypenoside LVI	167.8 ± 0.1
Damulin A	29.0 ± 2.2
Damulin B	23.8 ± 3.0
Damulin C	61.2 ± 0.8
Damulin D	40.4 ± 1.3

Comparing the structures of tested compounds with their cytotoxic activities, several features can be drawn as follows: (1) The loss of sugar may be related to enhanced inhibition of cancer cell

proliferation, which was consistent with Chen et al.[29] and Popovich and Kitts.[31] As expected, the sugar numbers of gypenoside LVI, gypenoside XLVI, gypenoside L and gypenoside Jh1 decreased in succession, while their anti-tumor activities increased. (2) The double bond in C20 position in the dammarane-type saponins may greatly contribute to the cytotoxic activities. The saponins with double bond in C20 (damulin A and damulin B) showed stronger activities than the saponins with hydroxyl group in C20 (gypenoside L and gypenoside LI). (3) Saponins with double bond in C20 (21) exhibited stronger activities than saponins with double bond in C20(22). Damulin B and damulin D showed more cytotoxic than damulin A and damulin C, respectively. (4) Acetylation might reduce cytotoxic activities. Damulin C and damulin D, which were acetylated in their sugar moiety, showed weaker activities than damulin A and damulin D, respectively.

In conclusion, we reported a new dammarane-type saponin, gypenoside Jh1, from *G. pentaphyllum* and presented that gypenoside Jh1-induced A549 cell death is related to mitochondria-mediated apoptosis. This study suggests that gypenoside Jh1 may be a potential chemotherapeutic drug candidate for the treatment of lung carcinoma. Further research is needed to investigate detailed molecular mechanisms underlying the cytotoxicity of gypenoside Jh1.

Acknowledgements

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23. The ethanol extract (500 g) was suspended in water and extracted consecutively with dichloromethane and ethyl acetate three times, respectively. The ethyl acetate soluble portion (9.5 g) was separated by macroporous resin HP-20 in succession with 20%, 50% and 70% ethanol. The 70% ethanol fraction (4.3 g) was further separated by silica gel column eluting with CH_2Cl_2/MeOH gradient (20:1–10:1, v/v) to yield six fractions. And the fractions were divided by silica gel 60 F254 TLC visualized by spraying 10% (v/v) H_2SO_4 in ethanol. The fraction five (1.1 g) was then purified by Shimadzu semi-preparative HPLC using BDS HYPERSIL C18 column (Thermo Scientific, 250 × 10 mm, 5 μm) and acetonitrile/H_2O (53:47, v/v) was used as mobile phase. The flow rate was 3 mL/min and injection volume was 15 μL. Detection was performed at a wavelength of 203 nm. Finally, compound **1** (5.5 mg) was obtained.
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26. A549 cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Stock solutions of the test compounds were prepared in DMSO at a concentration of 1 mg/mL and stored at −20 °C. The cytotoxicity was determined by Cell Counting Kit-8. Briefly, A549 cells were seeded in 96-well plates (5×10^3 cells/well) and grown overnight. Cells were then incubated in DMEM medium containing different amounts of test compounds (0, 20, 40, 60, 80 and 100 μg/mL) for 48 h. The solution was removed from the medium and 100 μL of 10% CCK-8 solution was added to each well. After incubation at 37 °C for 3 h, the absorbance of each well was measured at 450 nm using a Molecular Devices FlexStation 3 microplate reader.
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