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Protective effects of dammarane-type triterpenes from hydrolyzate of *Gynostemma pentaphyllum* against H_2O_2-induced injury and anti-hepatic fibrosis activities

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ABSTRACT

Gynostemma pentaphyllum (Thunb.) Makino is a kind of health food, and it is also used as a traditional medicine in Asia. In this study, phytochemical investigation of hydrolyzate of total *G. pentaphyllum* saponins led to the isolation of three previously undescribed triterpenes, Gypensapogenin R (**1**), Gypensapogenin S (**2**), and Gypensapogenin T (**3**). Their structures were mainly identified by 1D, 2D-NMR and HR-ESI–MS evidences. Anti-hepatic fibrosis activity was determined and the data showed that **2** and **3** exhibited more potent inhibitory effects (IC_{50} values 14.93 and 29.19, respectively) on the growth of t-HSC/Cl-6 cells than Silymarin (positive control), while having much weaker effect in the growth of normal cell. Additionally, all the compounds exhibited excellent increased the ratio of viability of H9c2 induced by H_2O_2. Here in we report isolation, structure elucidation, as well as the evaluation of the anti-hepatic fibrosis and cardiomyocytes oxidative injury activities of these three compounds.

1. Introduction

Recently, *Gynostemma pentaphyllum,* which is an edible functional food have been attracted extensive research and public attention because of their potential beneficial effects on human health. In China, *G. pentaphyllum* were also used as a traditional Chinese medicine for alleviating various diseases and conditions, including liver protection, enhance immunity, and lower cholesterol levels (Circosta et al., 2005; Megalli et al., 2006; Suntararuks et al., 2008; Yeo et al., 2008). Previous phytochemical investigations of *G. pentaphyllum,* various structure similar dammarane gypenosides were discovered. Moreover, the structure of these gypenosides was similar to the ginseng saponins. Because of this similarity structure, people claimed that drinking a tea or eat some food made of *G. pentaphyllum* could longevity, more energy and fewer illnesses (Lv et al., 2009). Pharmacology has demonstrated that gypenosides isolated from *G. pentaphyllum* have various bioactivities, including hepatoprotective, anti-inflammatory, cardiovascular and antioxidant effects (Xie et al., 2010). Since 1990s, an extensive range of health-food and beverages based on *G. pentaphyllum* have been developed and sold in China markets, such as total Jiaogulan saponin tablets, Jiaogulan tea and Jiaogulan oral liquid (Lu et al., 2013). To search for diversity of new bioactive from Jiaogulan, a continuation work on phytochemical investigation of the hydrolyzed saponins of *G. pentaphyllum*, three previously undescribed triterpenes namely gypensapogenin R (**1**), gypensapogenin S (**2**), and gypensapogenin T (**3**) were obtained and identified by HR-ESI–MS, 1D and 2D NMR (Fig. 1). Herein, the purification procedure and structure elucidation of these compounds were described. Meanwhile, all the obtained compounds were tested protective effects against cardiomyocytes injury induced by H_2O_2, and their cytotoxic activity against t-HSC/Cl-6 cells and normal cell lines.

2. Results and discussion

2.1. Structure determination

Acid hydrolysis of the crude saponins of *G. pentaphyllumwith* a MeOH solution of HCl provided crude hydrolysates. The hydrolysates were subjected to silica gel, Sephadex LH-20, and prep-HPLC chromatograpic methods to afford three new compounds **1**-**3**.

Gypensapogenin R (**1**) was isolated as a white amorphous solid from the hydrolyzed saponins of *G. pentaphyllum*. The HR-ESI mass spectrum

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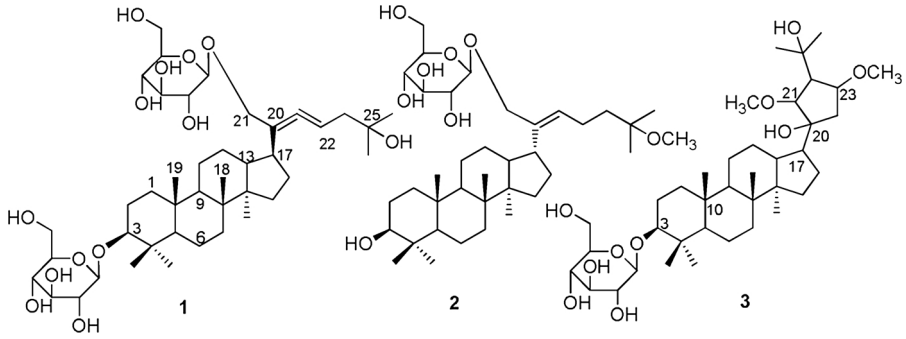

Fig. 1. Structures of Compounds **1-3**.

of compound **1** showed $[M + Na]^+$ at *m/z* 807.4817 (calcd. for $C_{42}H_{72}O_{13}Na$, 807.4865). The ^1H NMR spectrum included signals due to seven methyl groups (δ 0.76, 0.90, 0.98, 1.00, 1.32, 1.40 and 1.41), an olefinic proton at δ 5.70 (1H, t, J = 7.4 Hz), three oxygen-bonded methine and methylene groups (δ 3.38, 4.52 and 4.72) and two anomeric proton at δ 4.91 (1H, d, J = 7.7 Hz) and δ 4.96 (1H, d, J = 7.8 Hz) (Table 1). Meanwhile, from the ^{13}C NMR spectrum, 42 carbon were observed including that for two olefinic carbon at δ 131.6 and 138.8, two glucopyranosyl subunits at δ 103.7 (C-1′), 75.6 (C-2′), 79.0 (C-3′), 72.4 (C-4′), 78.7 (C-5′), 63.5 (C-6′) and δ 107.3 (C-1′), 76.2 (C-2′), 79.1 (C-3′), 72.3 (C-4′), 78.7 (C-5′), 63.3 (C-6′). The skeleton of compound **1** suggested by the ^1H and ^{13}C NMR spectrum data that the basic mother nucleus of **1** was similar to that of 3-*O*-*β*-D-glucopyranosyl-gypensapogenin D (Li et al., 2012a,b), including a similar side chain of gypensapogenin L (Zhang et al., 2015). Meanwhile, the observed long-range correlations from *δ* 4.96 (H-1′) to δ 89.2 (C-3); from δ 4.91 to δ 66.2 (C-21), indicated that glycosidation of the alcoholic function at C-3 and C21 was informed. Acid hydrolysis of **1** gave a D-glucose, which was clarified by GC analysis (Li et al., 2012b). Its *β*-glycosidic linkages were evident from the J values in the ^1H NMR spectrum ($J_{1'2'}$ = 7.8 Hz and $J_{1''2''}$ = 7.7). Based on HRESIMS, ^1H NMR, ^{13}C NMR, HSQC, HMBC and NOESY spectra, the structure of compound **1** was identified to be gypensapogenin R (Fig. 1).

Gypensapogenin S (**2**) was isolated as a white amorphous solid from the hydrolyzed saponins of *G. pentaphyllum*. The HR-ESI mass spectrum of compound **2** showed $[M + Na]^+$ at *m/z* 659.4493 (calcd. for $C_{37}H_{64}O_8Na$, 659.4493). The NMR data of **2** was similar to that of **1** except for inexistence of glucopyranosyl moiety and highfield of C-3 and presence of one methoxy group. On the basis of ^1H NMR, ^{13}C NMR, HSQC and HMBC spectra the structure of **2** was identified and named gypensapogenin S (Fig. 1).

Gypensapogenin T (**3**) was isolated as a white amorphous solid from the hydrolyzed saponins of *G. pentaphyllum*. The HR-ESI mass spectrum of compound **1** showed $[M + Na]^+$ at *m/z* 705.4481 (calcd. for $C_{38}H_{66}O_{10}Na$, 705.4548). The ^1H NMR spectrum included signals due to seven methyl groups (δ 0.75, 0.89, 0.95, 0.98, 1.29, 1.31 and 1.40), two methoxy group (δ 3.22 and 3.28), three oxygen protons (δ 3.37, 3.84, and 4.00) and an anomeric proton at δ 4.94 (1H, d, J = 7.8 Hz) (Table 1). Meanwhile, from the ^{13}C NMR spectrum, 36 carbon were observed including that for one glucopyranosyl subunit at δ 107.3 (C-1′), 76.1 (C-2′), 79.1 (C-3′), 72.2 (C-4′), 78.7 (C-5′), 63.4 (C-6′). The observed long-range correlations from δ 4.94 (H-1′) to δ 89.2 (C-3), indicated that glycosidation of the alcoholic function at C-3 was informed. The HMBC correlations from the proton signals at δ 3.22 (-OCH_3) to 76.1 (C-23), *δ* 3.28 (-OCH_3) to 81.2 (C-21). On the basis of ^1H NMR, ^{13}C NMR, HSQC and HMBC spectra the structure of **3** was identified and named gypensapogenin T (Fig. 1).

2.2. Anti-hepatic fibrosis activity

The obtained compounds **1-3** were assessed for the anti-hepatic fibrosis activity by examining the inhibitory activity against t-HSC/Cl-6 cells (Table 2). In particular, our results showed that all the tested compounds exhibited inhibitory activity against t-HSC/Cl-6 cells. Of them, compounds **2** and **3** displayed potent inhibitory activity with IC_{50} values ranging from 14.93 ± 1.69 and 29.19 ± 2.33 μM, whereas compounds **1** showed lower inhibitory activity. Impressively, all the tested compounds exhibited more significant that the positive control Silymarin with IC_{50} values of 225.27 ± 2.73 and without any toxicity to normal cells (Fig. 2).

2.3. Protective effects of the compounds against H_2O_2-induced myocardial cell injury

Compounds **1-3** were tested for their protective effects against H_2O_2-induced myocardial cell injury (Table 3). The activity was evaluated on various concentrations. Three compounds were thought to increase the rate of viability of H9c2 induced by H_2O_2-induced myocardial cell injury in a dose-dependent relationship. Notably, the effect of low dose was also outstanding, which closed to positive control Vitamin E, while no observable toxicity. Of them, compound **2** were even more significant than Vitamin E. From the data, we speculated that this result was related to the number of sugar and the site of methoxy group.

Three previously undescribed compounds were isolated and characterized from hydrolyzate of total *Gynostemma pentaphyllum* saponins. Results of bioassay showed that all the obtained compounds exhibited protective effects against H_2O_2-induced myocardial cell injury and anti-hepatic fibrosis activity. Further studies are warranted to clear their potential mechanism and to reveal their other bio-activities.

3. Experimental

3.1. General experimental procedures

Saponins of *G. pentaphyllum* (> 80%) were purchased from Hu Nan Province Jiuhui modern Chinese materia medica Co. Ltd. Optical rotations: Perkin-Elm erpolarimeter. UV absorption spectra were measured on Unico UV-2800AH UV–vis Spectrophotometer (Unico Instruments Ltd., Shanghai). IR absorption spectra were recorded on an IR S-55 Infrared spectropho-tometer (Bruker, Germany) with KBr pellets. HR-ESI–MS spectra were recorded using an Agilent 1100 LC–MSD time-of-flight system. NMR spectra were recorded on Bruker AV-600 spectrometer with TMS as internal standard, J in Hz. Column chromatography (cc): silica gel (SiO_2: 200–300 mesh, Qingdao Marine Chemical Group, Co. Ltd., Shandong, China); Sephadex LH-20 (pharmacia, Co.). Prep. HPLC (Beijing CXTH3000 system): P3000 pump, UV3000 spectrophotometric detector at 210 nm, YMC C_{18} reversed-phase column (5 μm, 10 × 250 nm; flow rate 3.0 mL/min). All chemicals and solvents were of analytical or HPLC grade. FBS (fetal bovine serum), RPMI 1640 medium and DMEM medium were bought from Thermo Fisher Scientific Co., Ltd. MTT reagent was acquired from Sigma Aldrich (St. Louis, MO, USA). Silimarin was purchased from

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of **1-3** in C_5D_5N.

No.	**1**		**2**		**3**	
	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)	δ_C	δ_H, mult. (*J* in Hz)
1	40.0	0.78, m 1.47, m	40.0	0.94, m 1.62, m	39.6	0.75, m 1.44, m
2	28.5	1.29, m 1.83, m	28.7	1.32, m 1.86, m	29.0	1.30, m 1. 85, m
3	89.2	3.38, dd, 11.8, 4.4	78.4	3.45, m	89.2	3.37, dd, 11.8, 4.3
4	40.1	–	40.0	–	40.1	–
5	56.8	0.73, m	56.8	0.82, m	56.8	0.73, m
6	18.8	1.48, m 1.66, m	19.1	1.46, m 1.57, m	18.8	1.49, m 1.65, m
7	36.2	1.21, m 1.53, m	36.3	1.24, m 1.58, m	36.1	1.21, m 1.50, m
8	41.1	–	41.2	–	41.0	–
9	51.6	1.26, m	51.7	1.32, m	51.5	1.27, m
10	37.4	–	37.9	–	37.7	–
11	22.0	1.45, m 1.51, m	22.1	1.80, m 1.94, m	22.2	1.18, m 1.41, m
12	25.7	1.14, m 1.78, m	26.0	1.16, m 1.43, m	27.1	1.83, m 2.23, m
13	46.8	2.31, m	45.9	1.96, m	47.1	2.14, m
14	49.9	–	50.0	–	50.7	–
15	32.2	1.11, m 1.20, m	32.5	1.24, m 1.57, m	32.0	1.06, m 1.54, m
16	29.3	1.22, m 1.82, m	27.8	1.87, m 1.93, m	28.0	1.82, m 1.53, m
17	48.1	2.54, m	41.3	2.93, m	44.3	1.89, m
18	16.2	0.98, s	16.4	1.01, s	16.1	0.95, s
19	16.3	0.90, s	16.9	0.83, s	16.8	0.89, s
20	138.8	–	138.0	–	83.8	–
21	66.2	4.52, m 4.72, m	71.4	4.01, m 4.39, m	81.2	3.84, m
22	131.6	5.70, t, 7,4	130.7	5.94, t, 7.4	37.4	2.16, m 2.20, m
23	24.0	2.66, m	22.9	2.28, m 2.30, m	76.1	4.00, m
24	45.3	1.82, m 1.84, m	41.0	1.56, m 1.56, m	62.2	2.51, m
25	70.0	–	74.6	–	72.1	–
26	30.1	1.40, s	25.5	1.15, s	23.6	1.40, s
27	30.4	1.41, s	25.4	1.15, s	23.6	1.31, s
28	29.0	1.32, s	29.0	1.23, s	28.5	1.29, s
29	17.2	1.00, s	16.7	1.04, s	17.2	0.98, s
30	16.8	0.76, s	16.2	0.92, s	16.8	0.75, s
C3-1′	107.3	4.96, d, 7.8			107.3	4.94,m
2′	76.2	4.05, m			76.1	4.04, m
3′	79.1	4.26, m			79.1	4.26, m
4′	72.3	4.23, m			72.2	4.20, m
5′	78.7	4.02, m			78.7	4.02, m
6′	63.3	4.39, m 4.60, m			63.4	4.39, m 4.59, m
C21-1”	103.7	4.91, d, 7.7	104.0	5.02, d, 7.8		
2”	75.6	4.04, m	75.7	4.12, m		
3”	79.0	4.26, m	79.1	4.29, m		
4”	72.4	4.18, m	72.4	4.23, m		
5”	78.7	4.02, m	78.8	4.01, m		
6”	63.5	4.39, m 4.60, m	63.4	4.39, m 4.60, m		
C21-OCH_3					56.5	3.28, s
C23-OCH_3					49.5	3.22, s
C25-OCH_3			49.4	3.15, s		

Furui Medical Technology Co., Ltd. (Nei Menggu, China). Vitamin E was purchased from J&K Scientific Ltd. (Beijing, China). All the cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

3.2. Extraction and isolation

The total saponins (1000 g) of *G. pentaphyllum* dissolved in MeOH (1500 mL) were treated with 10% HCl (1000 mL) and heated at 50 °C for 8 h. After 3 days standing, washed with plenty of water, filtration, and then vacuum evaporated to recover the solvent. A 70 g portion of the residue was subjected to silica gel CC (CH_2Cl_2: MeOH 100:0 ~ 0:100), which resulted in eight fractions (Fr.A-H). Fr. F (2.1 g) chromatographed over silica gel eluting with a gradient of EtOAc-MeOH (0:100 ~ 100:0) to obtain five subfractions F1-5. Subfraction F3 (1.6 g) was further separated on a Sephadex LH-20 column (CH_2Cl_2: MeOH, 1:1) to yield four portions (F3a-F3d). Portion F3c was purified by semipreparative HPLC eluting with 87% and 75% MeOH-H_2O to yield **3** (28 mg). Similarly, F3e was purified using Sephadex LH-20 and semipreparative HPLC eluting with 76% MeOH-H_2O to obtain **2** (22 mg). Compound **1** (31 mg) was obtained from Fr. G (1.4 g) by semipreparative HPLC using MeOH-H_2O (77:23).

Table 2
IC_{50} values of the isolated compounds for inhibition t-HSC/Cl-6 cells and normal cells.

Compounds	IC_{50} μM		
	t-HSC/Cl-6	H9c2	gastric mucosa cells (GES-1)
1	108.40 ± 3.48	> 300	> 300
2	14.93 ± 1.69	> 300	> 300
3	29.19 ± 2.33	> 300	> 300
Silymarin[a]	225.27 ± 2.73	> 300	> 300

[a] Silymarin was used as positive control.

3.3. Spectroscopic data of the new compounds

Gypensapogenin R (1): amorphous powder, $[\alpha]_D^{25} - 10°$(c 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 238 (1.04) nm; IR (KBr) νmax 3417, 2925, 2857, 1687, 1587, 1319, 1032, 656; The data of ^{1}H and ^{13}C NMR were shown in Table 1; HR-ESI–MS 807.4817 $[M + Na]^+$ ($C_{42}H_{72}O_{13}Na$, Calcd. 807.4865).

Gypensapogenin S (2): amorphous powder, $[\alpha]_D^{25} - 22°$(c 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 230 (1.20) nm; IR (KBr) νmax 3424, 2943, 2798, 1645, 1569, 1354, 1044, 608; The data of ^{1}H and ^{13}C NMR were shown in Table 1; HR-ESI–MS 659.4493 $[M + Na]^+$ ($C_{37}H_{64}O_8Na$, Calcd. 659.4493).

Gypensapogenin T (3): amorphous powder, $[\alpha]_D^{25} - 17°$(c 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 226 (1.18) nm; IR (KBr) νmax 3427, 2941, 2835, 1623, 1567, 1366, 1039, 647; The data of ^{1}H ^{13}C NMR were shown in Table 1; HR-ESI–MS 705.4481 $[M + Na]^+$ ($C_{38}H_{66}O_{10}Na$, Calcd. 705.4548).

3.4. Acid hydrolysis of compounds 1–3

The acid hydrolysis was used to identify the type and configuration of sugar moiety and aglycone (Li et al., 2012b). Each compound (2 mg) in 2 M HCl/MeOH (4:1) (8 mL) was stirred at 90 °C for 2 h. After cooling, the reaction mixture was diluted to 30 mL with water and then extracted with CH_2Cl_2 (30 mL × 3). The water layer was neutralized with 1 M aqueous KOH.

3.5. Anti-hepatic fibrosis activity

Hepatic stellate cells (t-HSC/Cl-6) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in a Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% heat inactivated fetal bovine serum and antibiotics (100 U/mL penicillin and streptomycin) in moist air containing 5% CO_2 at 37 °C. First, the t-HSC/Cl-6 cells were plated in 96-well plates (4000 cells/well) for 12 h, and then cells were treated by compounds at different concentrations (10 μM, 30 μM, 60 μM, 100 μM) for 48 h. Four replicate wells were used at each point in the experiment with four parallel control wells. 100 μL fresh medium with MTT reagent (5 μg/

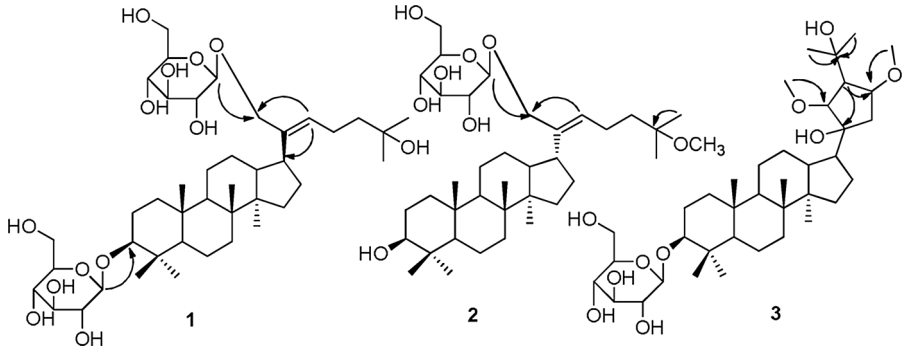

Fig. 2. The key HMBC correlations of **1-3**.

Table 3
Protective effects of compounds **1-3** on H_2O_2-induced H9c2 cell injury.

Compounds	Viabilities of H9c2 cardiomyocyte (%)				
	0 μM	10 μM	30 μM	60 μM	100 μM
1	46.81 ± 1.87	52.89 ± 1.25	80.24 ± 2.19	78.35 ± 3.41	80.71 ± 3.61
2	48.19 ± 2.16	76.68 ± 1.68	87.64 ± 3.16	85.83 ± 2.89	83.57 ± 4.12
3	43.63 ± 1.42	43.30 ± 1.01	61.54 ± 2.55	59.93 ± 3.22	79.98 ± 3.59
Vitamin E[a]	45.75 ± 1.90	52.47 ± 2.06	54.33 ± 1.93	61.79 ± 3.31	75.01 ± 3.20

[a] Positive control.

mL) was added to each well for 4 h of incubation at 37 °C after removing all the left liquid in the wells. After the incubation period, the formazan crystal was dissolved with DMSO, and the reduction of cell viability was determined at 490 nm using a microplate reader (Bio-Rad, USA) (Mosmann, 1983).

3.6. Assay for testing the protective effects against myocardial cell injury induced by H_2O_2

The rat $H9_C2$ cardiomyocytes were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and routinely maintained in DMEM medium supplemented with 10% heat inactivated fetal bovine serum and antibiotics (100 U/mL penicillin and streptomycin) in a humidified atmosphere containing 5% CO_2 at 37 °C, and allowed to adhere for 24 h before the tested compounds (**1-3**). The tested compounds with five different concentrations (10–100 μM) were added and following 12 h of incubation, 200 μM H_2O_2 was added to each well for 24 h (Fearon and Faux, 2009; Yasuoka et al., 2004). The cells in the control groups were treated with the same volume of PBS. Cell viability was evaluated by MTT assay, based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. The reduction of cell viability was determined at 490 nm using a microplate reader (Bio-Rad, USA). All experiments were repeated three times.

3.7. Statistical analysis

All the presented data and results were performed in at least three independent experiments and expressed as the mean ± SD. Statistical comparisons were made by Student's *t*-test and One-way ANOVA method. Statistical calculations were performed using the SPSS 16.0 for Windows software package.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.phytol.2018.03.010.

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