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ORIGINAL ARTICLE

Five novel triterpenoid saponins from *Hovenia dulcis* and their Nrf2 inhibitory activities

Wanna Cai [a,b,1], Shengmei Xu [a,c,1], Ting Ma [a,b], Xiaoqi Zhang [d,*], Bo Liu [a,b,*], Fangfang Xu [a,b,*]

[a] *Guangdong Provincial Key Laboratory of Clinical Research on Traditional Chinese Medicine Syndrome, The Second Clinical Medicial College, Guangzhou University of Chinese Medicine, Guangzhou 510006, PR China*
[b] *Guangzhou Key Laboratory of Chirality Research on Active Components of Traditional Chinese Medicine, Guangzhou 510006, PR China*
[c] *Guangdong Provincial Key Laboratory of Research on Emergency in TCM, Guangzhou, PR China*
[d] *Guangdong Provincial Engineering Research Center for Modernization of TCM, College of Pharmacy, Jinan University, Guangzhou 510632, PR China*

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Abstract Five novel triterpenoid saponins, hovenidulciosides C−G (**1**–**5**), and one known saponin hovenidulcioside A_1 (**6**) were isolated from the seeds of *Hovenia dulcis* Thunb. The structures of **1**–**5** were determined through spectroscopic methods such as HR-ESI-MS, 2D-NMR (^{1}H–^{1}H COSY, HSQC, HMBC, ROESY and TOCSY), UV and IR and chemical methods. There were amino acetylated substituent in the sugar resident of hovenidulciosides F−G, which were firstly reported from this genus. Compound **1** supressed ARE-luciferase activity in MDA-MB-231 cells and inhibited the Nrf2 protein levels in A549 cells.

1. Introduction

Hovenia dulcis Thunb. belongs to the genus of Hovenia, family of Rhamnaceae, widely grown in East Asia conutries, such as China, India and Japan, Korea (Sinicae Agendae Academiae Sinicae Edita, 1982). The seeds of *H. dulcis*, named as "Zhijuzi", had been used to relieve the thirst, damp heat and drunk in China with a long history (Hyun et al., 2010; Nanjing University of Chinese Medicine, 2006). Modern pharmacology research showed the crude extracts and pure compounds from *Hovenia* showed hepatoprotective, antiviral, anti-hyperuricemia, anti-alcoholism and anti-tumor activities (Hase et al., 1997; Yu and Xu, 2019; Xu et al.,

* Corresponding authors at: Guangdong Provincial Key Laboratory of Clinical Research on Traditional Chinese Medicine Syndrome, The Second Clinical Medicial College, Guangzhou University of Chinese Medicine, Guangzhou 510006, PR China.
E-mail addresses: xqzhang74@hotmail.com (X. Zhang), doctliu@263.net (B. Liu), xudubfang@163.com (F. Xu).
[1] These authors contributed equally to this work.
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2020; Song et al., 2016; Zhou et al., 2013). Phytochemical works indicated that triterpenoid saponins, flavonoids, polysaccharides and phenylpropanoids were the main types constituents in this plant (Zhou et al., 2013; Xu et al., 2012; Wang et al.,2012; Kang et al.,2017; Xu et al., 2011; Zhang et al., 2012).

The nuclear factor-erythroid 2-related factor (Nrf2) is the transcription factor with redox-sensitive property, that participates the cellular oxidative stress response. The Nrf2 expressed in low levels in normal tissues but in high levels in tumors (Chen et al., 2009). The activated Nrf2 in tumor tissues not only enhances tumor survival but also increases the resistance to anticancer drugs. The natural chemical Nrf2 inhibitors have been reported the anticancer effects through suppressing cancer cells proliferation or sensitizing the cells to chemo- and radiotherapy (Lin et al., 2020). As a part of our ongoing program to discover more good biological antitumor drugs, five novel triterpenoid saponins hovenidulciosides C−G (**1**–**5**), together with one known analogue hovenidulcioside A_1 (**6**) were obtained from the seeds of *H. dulcis*. All structures were elucudated by the comprehensive spectroscopic data analysis and the Nrf2 inhibitory activities were tested by luciferase reporter gene assay and western blotting analysis.

2. Materials and methods

2.1. General

HR-ESI-MS perfotmed on Q Exactive Plus mass spectrometer (Thermo Scientific); Optical rotation were measured by Autopol Ⅰ Polarimeter (Rodolph); The UV were collected by U-2910 (Hitachi); The IR spectra were determined with Spectrum 400 (Perkin Elmer); NMR data were collected on AMX-600 (Bruker); HPLC was conducted by 1260 instrument (Agilent) with a DAD detector. GC was carried out by 7890A (Agilent); Column chromatographies were conducted by D101 macroporous resin (Sinopharm) and silica gel (80–100 mesh, 200–300 mesh, Yantai Huanghai). TLC was performed on GF_{254} silica gel plates (Yantai Huanghai). Luciferase Reporter Assay was measured on gloMax milti plus E9032 detection plate reader (Promega).

2.2. Plant materials

Hovenia dulcis Thunb. seeds were gathered at Yichang City, Hubei Province in fall 2017 and identified by Associate Prof. Jiayun Tong from our university. The voucher number 20,171,102 was authenticated and the materials were deposited at our laboratory in Guangzhou.

2.3. Extractions and isolations

The dried and crudely powdered seeds of *H. dulcis* (23.0 kg) were extracted by mixed ethanol-H_2O solvent (65:35, V/V) by refluxing (2 h) for thrice. The crude extracts were got through concentrated process in vacuo, and then suspended in H_2O to afford the semi-solid crude. The D101 macroporous resin column was used to isolate the extraction and eluting with gradient ethanol–H_2O solvent. The 50% ethanol-H_2O eluting fraction (52.6 g) was firstly separated on silica gel column eluting with dichloromethane–methanol (1:0, 9:1, 8:2, 6:4, 1:1, 0:1) to get 18 subfractions (A1–A18) via TLC profile. A4 (3.2 g) was fractionated via Sephadex LH-20 column [MeOH–water (8:4, v/v)] and got 21 subfractions (A4–F1–A4–F21). The fraction A4–F12 (21 mg) was then purified by semiprepare HPLC using MeOH–water (6 mL/min, 60/40, v/v) as mobile phase to afford **3** (3.5 mg, t_R 12.3 min). Fraction A6 (2.1 g) was preliminary isolated by Sephadex LH-20 column [MeOH–water (6:4, v/v)] and got 26 subfractions (A6–F1–A6–F26). Further purification of subfraction A6–F21 (28 mg) by semipreparative HPLC [MeOH–water (6 mL/min, 60/40, v/v)] afforded compound **4** (8.2 mg, t_R 16.8 min) and compound **5** (1.8 mg, t_R 22.4 min). Compound **1** (35.2 mg) and **2** (20.5 mg) were purified on Sephadex LH-20 column [MeOH–water (8:2, v/v)] from fractions A14 (42 mg) and A10 (39 mg), respectively.

2.3.1. Hovenidulcioside C (*1*)

White powder, $[\alpha]_D^{25}$ − 51.55 (*c* 0.12, MeOH); IR ν_{max} (film): 3358, 1652, 1454, 1373, 1289, 1077, 1040, 978 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 211 (3.42), 227(1.92); HR-ESI-MS: *m/z* 1075.5319 $[M + CH_2O_2\text{-}H]^-$ ($C_{52}H_{83}O_{23}$, calcd. for 1075.5320), 1029.5261 $[M-H]^-$ ($C_{51}H_{81}O_{21}$, calcd. for 1029.5265); ^1H and ^{13}C NMR see Tables 1–2.

2.3.2. Hovenidulcioside D (*2*)

White powder, $[\alpha]_D^{25}$ − 73.03 (*c* 0.52, MeOH); IR ν_{max} (film): 3367, 1648, 1454, 1374, 1287, 1071, 1042, 980 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 203 (3.41); HR-ESI-MS: *m/z* 1093.5444 $[M + CH_2O_2\text{-}H]^-$ ($C_{52}H_{85}O_{24}$, calcd. for 1093.5436), 1047.5383 $[M-H]^-$ ($C_{51}H_{83}O_{22}$, calcd. for 1047.5384); ^1H and ^{13}C NMR see Tables 1–2.

2.3.3. Hovenidulcioside E(*3*)

White powder, $[\alpha]_D^{25}$ − 36.54 (*c* 0.18, MeOH); IR ν_{max} (film): 3356, 1747, 1651, 1380, 1240, 1086, 976 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 213 (3.16); HR-ESI-MS: *m/z* 913.4405 $[M + CH_2O_2\text{-}H^-$ ($C_{45}H_{69}O_{19}$, calcd. for 913.4428), 867.4343 $[M-H]^-$ ($C_{44}H_{67}O_{17}$, calcd. for 867.4373); ^1H and ^{13}C NMR see Tables 1–2.

2.3.4. Hovenidulcioside F(*4*)

White powder, $[\alpha]_D^{25}$ + 48.67 (*c* 0.10, MeOH); IR ν_{max} (film): 3367, 1754, 1644, 1449, 1376, 1082, 1042 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 220 (3.01); HR-ESI-MS: *m/z* 954.4667 $[M + CH_2O_2\text{-}H]^-$ ($C_{47}H_{72}O_{19}N$, calcd for 954.4693), 908.4617 $[M-H]^-$ ($C_{46}H_{70}O_{17}N$, calcd for 908.4638); ^1H and ^{13}C NMR see Tables 1–2.

2.3.5. Hovenidulcioside G (*5*)

White powder, $[\alpha]_D^{25}$ + 33.70 (*c* 0.18, MeOH); IR ν_{max} (film): 3356, 1747, 1380, 1236, 1082, 1038, 979 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 224 (2.94); HR-ESI-MS: *m/z* 956.4828 $[M + CH_2O_2\text{-}H]^-$ ($C_{47}H_{74}O_{19}N$, calcd for 956.4849), 910.4472 $[M-H]^-$($C_{46}H_{72}O_{17}N$, calcd for 910.4795); ^1H and ^{13}C NMR see Tables 1–2.

2.4. Acid hydrolysis and gas chromatographic analysis

Acid hydrolysis was carried out as described previously (Xu et al., 2012). Compounds **1**–**4** (2.0 mg respectively) were acid

Table 1 ^{1}H NMR data of **1**–**5** (CD_3OD, 600 MHz, [a] 700 MHz, δ in ppm, J in Hz).

position	**1**	**2**[a]	**3**	**4**	**5**[a]
	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
1	0.93, m	0.92, m	1.71, m	1.72, m	1.72, m
	1.69, m	1.68, m	0.98, m	0.98, m	0.98, m
2	1.84, m	1.84, m	1.98, m	1.65, m	1.68, m
	1.70, m	1.69, m	1.70, m	1.25, m	1.28, m
3	3.05, dd (11.4, 3.6)	3.06, dd, (10.2, 4.9)	3.20, m	3.20, m	3.20, m
5	0.72, d (11.4)	0.69, d (11.2)	0.80, m	0.80, m	0.79, m
6	1.59, m	1.59, m	1.64, m	1.66, m	1.66, m
	1.49, m	1.53, m	1.50, m	1.50, m	1.51, m
7	1.52, m	1.52, m	1.50, m	1.52, m	1.53, m
	1.47, m	1.17, m	1.39, m	1.39, m	1.42, m
9	0.88, overlaped	0.86, s	0.78, m	0.79, m	0.75, m
11	1.63, m	1.59, m	1.59, m	1.61, m	1.61, m
	1.49, m	1.66, m	1.38, m	1.36, m	1.36, m
12	1.85, m	1.25, m	1.63, m	1.96, m	1.95, m
	1.68, m	1.18, m	1.24, m	1.71, m	1.70, m
13	2.48, m	2.50, m	1.71, m	1.72, m	1.72, m
15	2.06, d (7.8)	2.07, d (9.1)	2.64, m	2.64, m	1.53, m
	1.19, d (7.8)	1.18, d (8.4)	2.43, m	2.42, m	1.42, m
17	1.00, d (7.2)	1.01, d (6.3)	1.73, m	1.72, m	1.96, m
18	1.14, s	1.14, d (4.2)	0.88, s	0.88, d (4.8)	0.87, d (3.5)
19	0.88, s	0.88, s	1.00, s	1.00, s	1.00, s
20			4.68, d (7.8)	4.69, t (7.8)	4.82, m
21	1.14, s	1.14, d (4.2)	0.91, d (7.8)	0.91, d (6.6)	0.92, d (7.7)
22	1.47, m	1.52, m	2.40, m	2.06, m	1.73, m
	1.38, m	1.36, m	1.95, m	1.95, m	
23	4.68, d (7.2)	4.22, t (9.1)	5.07, m	5.07, m	4.59, m
24	5.16, d (7.8)	1.73, m	7.28, t (1.2)	7.28, s	2.18, m
		1.56, m			2.04, m
25					1.86, m
26	1.72, s	1.26, s			
27	1.69, s	1.21, s	1.85, s	1.85, s	1.23, d (7.0)
28	1.04, s	1.04, s	1.08, s	1.10, s	1.10, s
29	0.85, s	0.84, s	0.87, s	0.88, d (4.8)	0.87, d (3.5)
30	4.02, d (7.8)	4.03, d (6.3)	4.46, d (7.2)	4.48, d (10.8)	4.48, d (10.5)
	3.93, m	3.95, m	4.28, d (10.8)	4.28, d (10.8)	4.32, d (10.5)
OAc			2.06, s	2.06, s	2.08, s
NAc				1.99, s	1.99, s
	α-L-Ara	α-L-Ara	β-D-Glu	β-D-Glu	β-D-Glu
1′	4.33, d (7.8)	4.34, d (7.0)	4.43, d (7.2)	4.40, d (7.8)	4.40, d (7.7)
2′	3.96, m	3.93, m	3.56, m	3.44, m	3.44, m
3′	3.87, m	3.88, m	3.52, m	3.25, m	3.24, m
4′	3.99, m	4.00, m	3.95, m	3.26, m	3.25, m
5′	3.82, m	3.83, m	3.35, m	3.45, m	3.59, m
	3.52, m	3.67, m			
6′			3.84, td (12.0, 1.8)	3.83, td (9.0, 1.8)	3.85, td (10.8, 2.1)
			3.61, m	3.57, m	3.56, m
	β-D-Glu	β-D-Glu	β-D-Glu	β-D-Glu-NAc	β-D-Glu-NAc
1″	4.85, overlapped	4.74, d (7.0)	4.67, d (7.8)	4.89, d (8.4)	4.89, d (9.1)
2″	3.14, t (7.8)	3.15, m	3.21, m	3.64, m	3.65, m
3″	3.39, m	3.38, m	3.25, m	3.43, m	3.42, m
4″	3.34, m	3.35, m	3.29, m	3.16, m	3.14, t (9.1)
5″	3.32, m	3.33, m	3.25, m	3.24, m	3.27, m
6″	3.84, m	3.84, m	3.84, td (12.0, 1.8)	3.83, td (9.0, 1.8)	3.85, td (10.8, 2.1)
	3.65, dd (12, 5.4)	3.67, m	3.65, m	3.64, m	3.64, m
	α-L-Ara	α-L-Ara			
1‴	4.84, d (8.4)	4.84, d (8.4)			
2‴	3.39, m	3.38, m			
3‴	3.48, m	3.50, m			
4‴	3.68, m	3.58, m			
5‴	3.77, dd (11.4, 5.4)	3.91, m			
	3.20, m	3.23, m			

(*continued on next page*)

Table 1 (*continued*)

position	**1**	**2**[a]	**3**	**4**	**5**[a]
	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)
	β-D -Xyl	*β*-D -Xyl			
1‴	4.68, d (7.8)	4.70, d (7.0)			
2‴	3.36, m	3.37, m			
3‴	3.60, m	3.62, m			
4‴	3.43, m	3.45, m			
5‴	3.90, m	3.91, m			
	3.22, m	3.19, m			

Table 2 ^{13}C NMR data of **1**–**5** (CD_3OD, 150 MHz, [a] 175 MHz, δ in ppm).

position	**1**	**2**[a]	**3**	**4**	**5**[a]
	δ_C, type	δ_C, type	δ_C, type	δ_C, type	δ_C, type
1	39.8, CH_2	39.7, CH_2	39.5, CH_2	39.5, CH_2	39.1, CH_2
2	27.3, CH_2	27.2, CH_2	27.2, CH_2	27.1, CH_2	27.1, CH_2
3	91.0, CH	90.9, CH	91.1, CH	91.4, CH	91.4, CH
4	40.7, C	40.6, C	40.5, C	40.5, C	40.5, C
5	57.4, CH	57.4, CH	56.4, CH	56.3, CH	56.3, CH
6	19.1, CH_2	19.1, CH_2	18.9, CH_2	19.0, CH_2	19.0, CH_2
7	36.9, CH_2	36.9, CH_2	35.4, CH_2	35.4, CH_2	35.4, CH_2
8	38.5, C	38.4, C	42.3, C	42.3, C	42.3, C
9	54.1, CH	54.1, CH	54.0, CH	54.0, CH	54.0, CH
10	38.3, C	38.2, C	37.9, C	37.9, C	37.9, C
11	22.5, CH_2	22.5, CH_2	21.6, CH_2	21.6, CH_2	21.6, CH_2
12	29.2, CH_2	29.1, CH_2	25.8, CH_2	25.8, CH_2	27.1, CH_2
13	38.0, CH	37.9, CH	39.1, CH	39.1, CH	39.5, CH
14	54.6, C	54.7, C	53.6, C	53.6, C	53.5, C
15	37.1, CH_2	36.9, CH_2	34.8, CH_2	35.0, CH_2	35.0, CH_2
16	111.4, C	111.5, C	180.1, C	180.1, C	182.3, C
17	54.4, CH	54.4, CH	37.0, CH	37.0, CH	37.1, CH
18	19.2, CH_3	19.2, CH_3	16.6, CH_3	16.5, CH_3	16.5, CH_3
19	16.7, CH_3	16.8, CH_3	18.5, CH_3	18.6, CH_3	18.6, CH_3
20	69.5, C	69.5, C	74.8, CH	74.8, CH	76.5, CH
21	29.6, CH_3	29.7, CH_3	11.9, CH_3	11.9, CH_3	12.2, CH_3
22	45.4, CH_2	46.0, CH_2	34.9, CH_2	34.8, CH_2	37.4, CH_2
23	69.7, CH	69.7, CH	80.6, CH	80.6, CH	77.7, CH
24	126.3, CH	49.0, CH_2	151.0, CH	151.0, CH	36.5, CH_2
25	136.7, C	71.5, C	130.4, C	130.4, C	37.1, CH
26	25.8, CH_3	29.7, CH_3	176.2, C	177.3, C	180.1
27	18.4, CH_3	29.8, CH_3	10.5, CH_3	10.5, CH_3	15.9, CH_3
28	28.2, CH_3	28.3, CH_3	28.3, CH_3	28.3, CH_3	28.3, CH_3
29	16.6, CH_3	16.6, CH_3	16.7, CH_3	16.7, CH_3	16.7, CH_3
30	66.9, CH_2	66.6, CH_2	71.8, CH_2	71.8, CH_2	71.8, CH_2
OAc			172.5, C	172.4, C	172.8, C
OAc			21.2, CH_3	21.2, CH_3	21.2, CH_3
NAc				23.0, CH_3	23.0, CH_3
NAc				174.0, C	174.0, C
	α-L-Ara	*α*-L-Ara	*β*-D-Glu	*β*-D-Glu	*β*-D-Glu
1′	106.3, CH	106.2, CH	105.4, CH	105.3, CH	105.3, CH
2′	77.0, CH	76.9, CH	81.1, CH	79.3, CH	78.4, CH
3′	82.0, CH	81.9, CH	78.5, CH	78.4, CH	77.7, CH
4′	70.3, CH	70.0, CH	77.9, CH	71.9, CH	71.9, CH
5′	66.2, CH_2	66.2, CH_2	71.9, CH	79.3, CH	79.3, CH
6′			63.1, CH_2	63.6 CH_2	63.6, CH_2
	β-D-Glu	*β*-D-Glu	*β*-D-Glu	*β*-D-Glu-NAc	*β*-D-Glu-NAc
1″	103.8, CH	103.7, CH	104.5, CH	102.0, CH	102.0, CH
2″	76.2, CH	75.6, CH	76.3, CH	58.1, CH	58.1, CH
3″	78.0, CH	77.9, CH	78.3, CH	76.6, CH	76.6, CH
4″	71.2, CH	70.7, CH	71.6, CH	72.8, CH	72.8, CH

Table 2 (*continued*)

position	**1**	**2**[a]	**3**	**4**	**5**[a]
	δ_C, type	δ_C, type	δ_C, type	δ_C, type	δ_C, type
5″	77.9, CH	77.8, CH	77.7, CH	77.7, CH	78.3, CH
6″	62.5, CH_2	62.4, CH_2	62.8, CH_2	62.8, CH_2	62.8, CH_2
	α-L-Ara	α-L-Ara			
1‴	102.9, CH	102. 8, CH			
2‴	78.0, CH	77.9, CH			
3‴	84.3, CH	84.1, CH			
4‴	70.8, CH	70.2, CH			
5‴	66.7, CH_2	67.2, CH_2			
	β-D -Xyl	β-D -Xyl			
1⁗	106.6, CH	106.4, CH			
2⁗	75.7, CH	76.1, CH			
3⁗	78.2, CH	78.1, CH			
4⁗	71.7, CH	71.0, CH			
5⁗	67.4, CH_2	66.9, CH_2			

hydrolyzed, then derivatized and analyzed by GC method. Comparing the retention times with those of standard sugars, the monosaccharides in **1**–**2** were D-glucose (t_R (min):17.94, reference D-glucose 17.99), L-arabose (t_R (min): 14.07, reference L-arabose 14.10) and D-xylose (t_R (min): 15.10, reference D-xylose 15.11). The monosaccharides in **3**–**4** were D-glucose (t_R (min):17.95, reference D-glucose 17.98).

2.5. Establishment of a reporter cell line and luciferase reporter gene assay

The luciferase reporter assay is commonly used as a tool to study gene expression at the transcriptional level. Luciferase reporter gene is a reporter system that uses luciferin as substrate to detect luciferase activity. In addition, Nrf2 as a transcription factor regulates the antioxidant response by inducing the expression of genes bearing an antioxidant response element (ARE) in their regulatory regions. We used the ARE-luciferase plasmid which was constructed by inserting a 39-bp ARE-containing sequence from the promoter region of the human NAD(P)H quinone oxidoreductase 1 (NQO1) gene into the cloning site of the pGL4.22[luc2CP/Puro] plasmid. Once ARE is activated, the luciferase gene is expressed. Luciferase can catalyze the oxidation of luciferin to oxyluciferin. In the process of luciferin oxidation, bioluminescence is emitted. Then we can determine whether Nrf2 is activated by detecting the intensity of fluorescence. We transfected ARE-luciferase plasmids into MDA-MB-231 cells using lipofectamine 2000 (Invitrogen). At 48 h post-transfection, cells were grown in medium containing 3 μg/mL puromycin for selection. Stable cell lines were continuously grown in the DMEM containing 1 μg/mL puromycin. For the reporter gene assay, the ARE-luciferase stable reporter cells were seeded the day before and treated with compounds **1**–**6** with concentrations ranging from 1 to 80 μM for 24 h. Cells were lysed, and luciferase activities were measured according to the manufacturer's instructions.

2.6. Antibodies and western blotting analysis

Antibodies and Western blotting analysis were conducted by the methods reported previously (Chen et al., 2016). The cells were lysed and then the proteins were separated and transferred to PVDF membrane. The membrane were incubated with antibodies against Nrf2 and GAPDH antibodies (Cell Signaling Technology) and then following incubated with secondary antibodies. The results were measured using the enhanced chemiluminescence (ECL) detection kit (Tanon).

3. Results and discussion

The seeds of *H. dulcis* were extracted with 65% EtOH and then separated by D101 macroporous resin column. The 50% ethanol– H_2O fraction was chromatographed on various column chromatographic separations and afforded 6 triterpenoid saponins including five new ones (**1**–**5**) (seeFig. 1).

Compound **1** was white amorphous powder with formula $C_{51}H_{82}O_{21}$ by the negative HR-ESI-MS peaks $[M + CH_2O_2-H]^-$ at *m/z* 1075.5319 ($C_{52}H_{83}O_{23}$, calcd for 1075.5320) and $[M-H]^-$ at *m/z* 1029.5261 ($C_{51}H_{81}O_{21}$, calcd for 1029.5265). The IR spectrum displayed the presence of the OH (3358 cm^{-1}) and C=C (1635 cm^{-1}). The ^1H NMR spectrum showed signals for seven methyl groups [δ_H 0.85 (3H, s, H_3-21), 0.88 (3H, s, H_3-19), 1.04 (3H, s, H_3-28), 1.14 (6H, s, H_3-18, 21), 1.69 (3H, s, H_3-27), 1.72 (3H, s, H_3-26)], two oxygenated methylene protons [(4.02 (1H, d, *J* = 7.8 Hz), 3.93 (1H, m), H-30], two oxygenated methine protons [δ_H 3.05 (1H, dd, *J* = 11.4, 3.6 Hz, H-3), 4.68 (1H, d, *J* = 7.2 Hz, H-23)], one olefinic proton δ_H 5.16 (1H, d, *J* = 7.8 Hz, H-24) and four anomeric protons [δ_H 4.33 (1H, d, *J* = 7.8 Hz, H-1′ in Ara), 4.85 (1H, m, overlapped, H-1″ in Glc), 4.84 (1H, d, *J* = 8.4 Hz, H-1‴ in Ara), 4.68 (1H, d, *J* = 7.8 Hz, H-1⁗ in Xyl)]. There were 51 carbon signals, including 7 methyls, 13 methylenes, 23 methines, 6 quaternary carbons and 2 olefinic carbon signals according to ^{13}C NMR and DEPT spectra. The hydrogen signals were assigned to the relevant carbon atoms though the HSQC data. All the above data indicated that **1** had a triterpenoid skeleton with four sugar residues. Comparison of the NMR spectral data of **1** with those of the known compounds isolated from this species, revealed that the sapogenin of **1** was jujubogenin (Kimura et al., 1981). ROESY correlations between H-3 (δ_H 3.05) and H-1′ (δ_H 4.33), H-5 (δ_H 0.72), between H-23 (δ_H 4.68) and H_3-18 (δ_H 1.14), indicated the β configuration at C-3 and C-

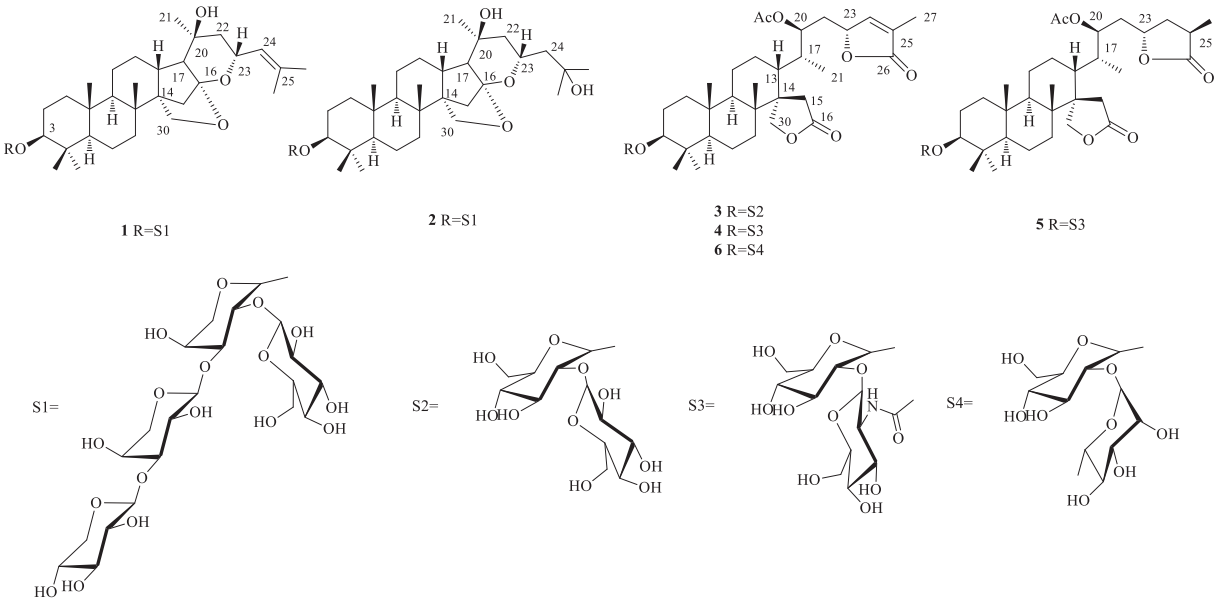

Fig. 1 Structures of compounds **1**–**6.**

23 (Fig. 2). Acid hydrolysis of **1** by 2 N HCl and gas chromatography analysis of the silyl derivatives of sugars obtained D-glucose, L-arabose and L-xyloside. The sugar chain and glycosylation position could be deduced by HMBC correlations between δ_H 4.68 (H-1‴ in Xyl) and δ_C 84.3 (C-3‴ in Ara), between δ_H 4.85 (H-1″ in Glc) and δ_C 77.0 (C-2′ in Ara), between δ_H 4.84 (H-1‴ in Ara) and δ_C 82.0 (C-3′ in Ara), and between δ_H 4.33 (H-1′ in Ara) and δ_C 91.0 (C-3) of aglycone (Fig. 2). The assignment of proton signals in the spin system of each sugar residue was determined by the correlations in TOCSY spectrum. Therefore, **1** was elucidated as 3-*O*-{*β*-D-xylopyranosyl (1 → 3)- α-L-arabinopyranosyl (1 → 3)- [*β*-D-glucopyranosyl (1 → 2)]-α-L-arabinopyranosyl}- jujubogenin, and named as hovenidulcioside C.

Compound **2** was obtained as amorphous powder. The quasimolecular ions $[M + CH_2O_2\text{-}H]^-$ at *m*/*z* 1093.5444 ($C_{52}H_{85}O_{24}$, calcd for 1093.5436) and $[M-H]^-$ at *m*/*z* 1047.5383 ($C_{51}H_{83}O_{22}$, calcd for 1047.5384) suggested the molecular formula was $C_{51}H_{83}O_{22}$. The NMR spectra of **2** showed the similar signal pattern to those of **1**, except for one additional hydroxyl group linked to C-25 and one lost double bond between C-24 and C-25. HMBC correlations from δ_H 1.21 (s, 3H, H_3-27) and 1.26 (s, 3H, H_3-26) to δ_C 49.0 (C-24) and δ_C 71.5 (C-25) (Fig. 2) revealed that the sapogenin of **2** was ziziglaziovigenin (Correa Dos Santos et al., 2019). ROESY correlations between H-3 at δ_H 3.06 and H-1′ at δ_H 4.34, H-5 at δ_H 0.69, between H-23 at δ_H 4.22 and H-18 at δ_H 1.14, indicated the *β* configuration at C-3, 23 (Fig. 2). Compound **2** was identified as 3-*O*-{*β*-D-

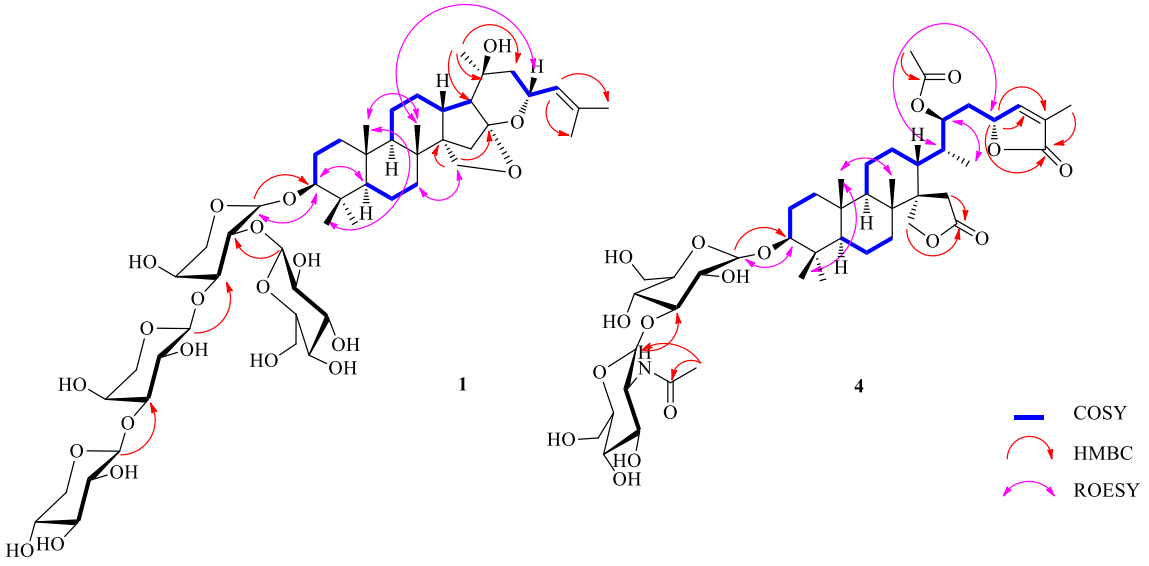

Fig. 2 Key ^1H–^1H COSY, HMBC and ROESY correlations in compounds **1** and **4.**

xylopyranosyl (1 → 3)-α-L- arabinopyranosyl (1 → 3)- [β-D-glucopyranosyl (1 → 2)] -α-L-arabinopyranosyl}- ziziglaziovigenin, and named as hovenidulcioside D.

Compound **3** was obtained as white powder with the formula $C_{44}H_{67}O_{17}$ according to the quasimolecular ions at *m/z* 913.4426 $[M + CH_2O_2\text{-}H]^-$ ($C_{45}H_{69}O_{19}$, calcd for 913.4428) and *m/z* 867.4365 $[M-H]^-$ ($C_{44}H_{67}O_{17}$, calcd for 867.4373). The IR spectrum displayed characteristic absorptions for hydroxyl (3356 cm^{-1}), carbonyl (1747 cm^{-1}) and olefinic (1651 cm^{-1}) groups. The NMR data indicated that **3** was a methyl-migrated 16,17-*seco*-dammarane triterpenoid with two sugar residues. Considering those saponins isolated from this species (Yoshikawa et al., 1996) and our previously studies (Xu et al., 2012; Xu, 2011), it was suggested that the aglycone of **3** was hovenidulcigenin A (Xu et al., 2012). Acid hydrolysis of **3** and GC analysis of the silyl derivatives of sugars afforded D-glucose. The configurations of the D-glucoses were β according to coupling constants of anomeric protons [H-1′ (d, J = 7.2 Hz) and H-1″(d, J = 7.8 Hz)] in ^{1}H NMR. HMBC correlations between δ_H 4.68 (H-1″ in Glc) and δ_C 81.1 (C-2′ in Glc), between δ_H 4.43 (H-1′ in Glc) and δ_C 91.1 (C-3) suggested the sequence and glycosylation position of the sugar. Therefore, **3** was elucidated as 3-*O*-[β-D-glucopyranosyl (1 → 2)-β-D-glucopyranosyl]- hovenidulcigenin A, and named as hovenidulcioside E.

Compound **4** was purified as white powder with the formula of $C_{46}H_{70}O_{17}N$ by the negative HR-ESI-MS data at 954.4667 $[M + CH_2O_2\text{-}H]^-$ ($C_{47}H_{72}O_{19}N$, calcd for 954.4693), 908.4617 $[M-H]^-$ ($C_{46}H_{70}O_{17}N$, calcd for 908.4638). NMR data of **4** were highly consistent with those of **3**, except for the existence of an additional *N*-acetyl amino group, which indicated by the signals at δ_H 1.99 (NHCO$\underline{CH_3}$), δ_C 23.0 (NHCO$\underline{CH_3}$) and 174.0 (NH$\underline{CO}CH_3$), suggesting that **4** was an *N*-acetylated amino derivative of **3** (Zhang et al., 2011). HMBC correlations between δ_H 1.99 (NHCO$\underline{CH_3}$) and δ_C 174.0 (NH$\underline{CO}CH_3$), between δ_H 3.64 (H-2″) and δ_C 174.0 (NH$\underline{CO}CH_3$), between δ_H 3.64 (H-2″) and δ_C 102.0 (C-1″) indicated that the *N*-acetamido group was attached to Glc-C-2″. The carbonal signal at δ_C 58.1 (C-2″) is the typical property for the presence of a 2-deoxy-2-acet-amidoglycosyl unit (Zhang et al., 2011). The β configurations of two sugars were deduced by coupling constants of H-1′ (d, J = 7.8 Hz) and H-1″ (d, J = 8.4 Hz) in ^{1}H NMR spectrum. Furthermore, the HMBC correlations between δ_H 4.89 (H-1″ in Glc-NAc) and δ_C 79.3 (C-2′ in Glc) and between δ_H 4.40 (H-1′ in Glc) and δ_C 91.4 (C-3) suggested the glycosidic chains attched to C-3 (Fig. 2). The ROESY correlations from δ_H 3.20 (H-3) to δ_H 0.80 (H-5), δ_H 0.91 (H-21) and δ_H 1.10 (H-28) indicating the β configuration of C-3. Thus, compound **4** was determined to be 3-*O*-[β-D-2-deoxy-2-acetylaminoglucopyranosyl (1 → 2)-β-D-glucopyranosyl]- hovenidulcigenin A, and named as hovenidulcioside F.

Compound **5** was obtained as white powder with formula of $C_{46}H_{72}O_{17}N$ based on the quasimolecular ions at *m/z* 956.4828 $[M + CH_2O_2\text{-}H]^-$ ($C_{47}H_{74}O_{19}N$, calcd for 956.4849) and *m/z* 910.4472 $[M-H]^-$ ($C_{46}H_{72}O_{17}N$, calcd for 910.4795). The NMR signal pattern of **5** were very similar to those of **4**, except that the vinyl group in **4** was substituted by a methylene and a methine in **5**. This evidence postulated that **5** was the dihydro-analog of **4** on the Δ^{24}-olifinic moiety, which was further confirmed by the correlations between δ_H 4.59 (H-23) and δ_H 2.18/2.04 (H_2-24) and between δ_H 1.86 (H-25) and δ_H 1.23 (H_3-27) in ^{1}H–^{1}H COSY spectrum. The correlations between δ_H 4.59 (H-23) and δ_H 1.23 (H_3-27) in ROESY spectrum indicated the *trans* configurations of C-23 and C-25. The above data revealed that the aglycone of **5** was hovenidulcigenin B, which was previously obtained from the seeds of *H.* dulcis (Xu et al., 2012; Yoshikawa et al., 1996). Thus, compound **5** was determined to be 3-*O*-[β-D-2-deoxy-2-acetylaminoglucopyranosyl(1 → 2)-β-D- glucopyranosyl] - hovenidulcigenin B, and named as hovenidulcioside G.

In this work, compounds **1**–**6** were evaluated the activities on Nrf2 expression using ARE-luciferase reporter assay (Chen et al., 2016). Finally, compound **1** was found to inhibit the ARE-luciferase activity in a dose-dependent manner (Fig. 3A) while **2**–**6** showed neither activatory nor inhibitory activity on ARE-luciferase effect. Then the protein levels of Nrf2 in A549 cells were decreased after **1** treatment (Fig. 3B) as expected. And the cytotoxicity assay showed that the 50% inhibitory concentration of compound **1** was about 95.67 μM within 24 h. Compared with the control group, there was no significant difference to cell viability at concentrations below 40 μM. The results indicated that the compound did not cause cytotoxicity when it acted as an Nrf2 inhibitor (Fig. 3C). The results demonstrated that compound **1** could inhibit the Nrf2 pathway by reducing the Nrf2 protein level. Compounds **1** and **2** had the identical sugar moiety, the Nrf2 inhibitory effect disappeared when the double bond between C-24 and C-25 lost and the hydroxyl group linked to C-25 added in **2**, suggested that importance of the double bond and the hydroxyl group between C-24 and C-25, which maybe was the active crucial group in **1**.

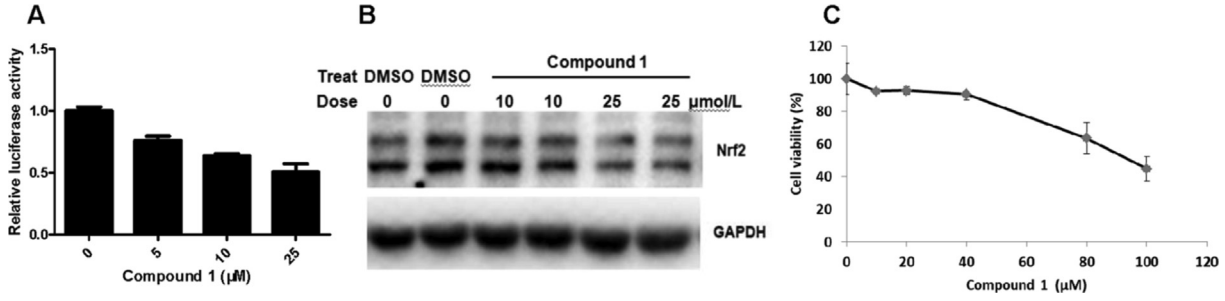

Fig. 3 Compound **1** selectively inhibited the Nrf2 pathway. (A) **1** inhibited ARE-dependent luciferase activity(n = 3). (B) Nrf2 expression in A549 cells, GAPDH was used as control (n = 2). (C) Cell viability of compound **1** in A549 cells (n = 3).

4. Conclusions

In this work, five new triterpenoid saponins hovenidulcioside C–G (**1**–**5**) along with one known analogue were isolated from the seeds of *H. dulcis*. The inhibitory activities on Nrf2 pathway of compound **1** was observed, which had better effect at the dosage of 25 μM. As a cytoprotective transcription factor, Nrf2 plays a systemic role in various cancers and the Nrf2 inhibitor is possible became a favorable strategy for cancer therapy (Lin et al., 2020). This study suggested that the active triterpenoid saponins from *Hovenia* genus may serve as privileged constituents in future anti-tumor drug discovery.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2021.103292.

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