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A NEW *ent*-LABDANE DITERPENE SAPONIN FROM THE FRUITS OF *Rubus chingii*

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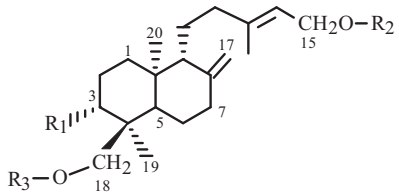
UDC 547.918

A new ent-*labdane diterpene saponin, named goshonoside-G (**1**), together with thirteen known compounds were isolated from the fruits of* Rubus chingii *Hu. The chemical structure of the new compound was elucidated on the basis of spectral evidence. The new compound exhibited anti-inflammatory activity in the nitrite assay using LPS-induced RAW 264.7 cells.*

Keywords: *Rubus chingii*, Rosaceae, *ent*-labdane diterpene saponin, anti-inflammatory.

The unripe fruits of *Rubus chingii* Hu (Rosaceae), referred to as "Fu-pen-zi" in Chinese, have long been used traditionally as a food and tonic for aged people. It is widely distributed in the southeast part of China. In recent decades modern pharmacological experiments have revealed that *R. chingii* has antioxidative, hepatoprotective [1], and immunomodulatory [2] properties and has effects on the hypothalamus–pituitary–sex gland axis [3]. Previous phytochemistry of this genus resulted in the isolation of various compounds classified as flavonoids [4], triterpenoids [5], and organic acids [6]. In the paper, we report our work on the isolation and characterization of a novel compound from *R. chingii*, together with thirteen known compounds. Their structures have been established with the aid of extensive NMR spectroscopic studies and mass spectrometry data. The anti-inflammatory activity of the new compound was tested as well.



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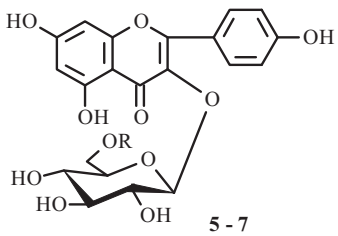

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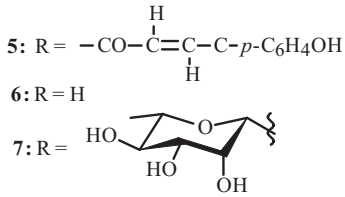

1: R_1 = OH, R_2 = *β*-D-Glc*p*, R_3 = *β*-D-Glc*p*-(6←1)-*α*-L-Ara*f*
2: R_1 = OH, R_2 = H, R_3 = *β*-D-Glc*p*; **3:** R_1 = H, R_2 = R_3 = *β*-D-Glc*p*
4: R_1 = OH, R_2 = R_3 = *β*-D-Glc*p*

Compound **1** was obtained as a white powder, with $[\alpha]_D^{20}$–38.6° (*c* 0.8, MeOH). The ESI mass spectrum in positive mode showed quasimolecular ion peaks at *m/z* 801 $[M + Na]^+$, indicating a molecular mass (M 778) corresponding to the formula $C_{37}H_{62}O_{17}$. The IR spectrum showed bands at 3423 (OH), 1641 and 888 (C=CH_2), and 1075 and 1040 (C=C *trans*). The ^{1}H NMR spectrum of **1** showed signals due to three tertiary methyl groups at δ 0.80, 0.97, and 1.70 (each s), eight methylene proton resonances at δ 3.63, 4.40 (each 1H, d, J = 10.0 Hz), 4.48 (2H, d, J = 6.0 Hz), 2.02, 2.40 (each 1H, m), 1.80 (2H, m), 1.60 (2H, m), 4.39, 4.46 (overlap at with other signals), 4.21, 4.80 (overlap with other signals), and 4.39, 4.63 (overlap with other signals), an exo-methylene at δ 4.63, 4.95 (each br.s), a trisubstituted double bond at δ 5.67 (t, J = 6.0 Hz), and three anomeric protons at δ 4.88 (1H, d, J = 7.5 Hz), 5.01 (1H, d, J = 8.0 Hz), and 5.77 (1H, d, J = 2.0 Hz).

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TABLE 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data of Compound **1** and Goshonoside-F6 (C_5D_5N, δ, ppm, J/Hz)

C atom	**1**		Goshonoside-F6	
	δ_H	δ_C	δ_H	δ_C
1	1.10 (1H, m), 1.70 (1H, m)	37.3		38.1
2	1.72 (m), 1.48 (m)	28.0	1.74 (m), 1.47 (m)	27.8
3	4.30 (1H, m)	71.9	4.23 (1H, m)	71.8
4		43.6		43.4
5	1.95 (1H, m)	47.1		46.9
6	1.91 (1H, m), 1.45 (1H, m)	24.4		24.3
7	2.02 (m), 2.40 (m)	38.3		37.1
8		149.0		148.9
9	1.54 (1H, m)	56.5		56.5
10		39.7		39.5
11	1.60 (2H, m)	22.5		22.5
12	1.80 (2H, m)	38.9		38.9
13		140.7		137.6
14	5.67 (1H, t, J = 6.0)	121.2	5.77 (1H, t, J = 6.3)	125.8
15	4.48 (2H, d, J = 6.0)	66.2	4.47 (2H, d, J = 6.3)	59.0
16	1.70 (3H, s)	16.8	1.66 (3H, s)	16.5
17	4.63 (1H, s), 4.95 (1H, s)	106.7	4.57 (1H, s), 4.88 (1H, s)	106.5
18	3.63, 4.40 (each 1H, d, J = 10.0)	74.4	3.53, 4.36 (each 1H, d, J = 9.8)	74.2
19	0.97 (3H, s)	13.0	0.88 (3H, s)	12.8
20	0.80 (3H, s)	15.4	0.71 (3H, s)	15.3
15-*O*-Glc*p*				
1′	5.01 (1H, d, J = 8.0)	103.8		
2′	4.12a	75.0		
3′	4.26a	78.7		
4′	4.14a	72.1		
5′	4.05a	78.6		
6′	4.39a, 4.46a	63.0		
18-*O*-Glc*p*				
1″	4.88 (1H, d, J = 7.5)	105.5	4.84 (1H, d, J = 7.8)	105.5
2″	4.12a	75.0		74.9
3″	4.05a	78.6		78.4
4″	4.10a	72.4		72.3
5″	4.20a	76.8		76.7
6″	4.21a, 4.80a	68.7		68.9
18-*O*-Ara*f*				
1‴	5.77 (1H, d, J = 2.0)	110.3	5.69 (1H, d, J = 2.0)	110.2
2‴	4.95a	83.3		83.2
3‴	4.26a	78.7		78.5
4‴	4.83a	86.2		86.0
5‴	4.29a, 4.63a	62.9		62.7

a: Overlap with other signals.

The ^{13}C NMR spectrum and DEPT spectrum exhibited three methyls at (δ 13.0, 15.4, 16.8), six methylenes (δ 22.5, 24.4, 27.9, 37.3, 38.3, 38.9), two oxygenated methylenes (δ 66.2, 74.4), an exo-methylene at δ 106.7, two methines (δ 47.1, 56.5), an oxygenated methine at δ 71.9, a trisubstituted double bond at δ 121.2, and four quaternary carbons (δ 39.7, 43.6, 140.7, 149.0) in addition to three anomeric carbons (δ 103.8, 105.5, 110.3) for the sugar part. The NMR spectral data indicated the presence of twenty carbon signals for the aglycone, which suggested that compound 1 was an *ent*-labdane diterpene, together with seventeen signals ascribable to a terminal α-arabinofuranoside unit and two β-glucopyranoside units.

On acid hydrolysis with 2 M trifluoroacetic acid (TFA), **1** afforded sugar moieties identified as D-glucose and L-arabinose based on TLC analysis with standard sugars. In the HMBC spectrum, one anomeric proton signal at δ_H 5.01 (H-1′) correlated with the carbon signals at δ_C 66.2 (C-15), suggesting that a glycosyl was located at C-15. Another anomeric proton signal at δ_H 4.88 (H-1″) correlated with the carbon signals at δ_C 74.4 (C-18), suggesting that another glycosyl was located at C-18.

TABLE 2. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data of Compounds **2–4** (CD_3OD, δ, ppm, J/Hz)

C atom	**2**		**3**		**4**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.21 (m), 1.81 (m)	38.3	2.07 (m), 2.31 (m)	39.3	1.20 (m), 1.77 (m)	38.4
2	1.67 (m), 1.72 (m)	28.2	1.52 (m), 1.61 (m)	20.1	1.64 (m)	28.2
3	3.77 (1H, t, J = 8.0)	73.2	1.32 (m), 1.60 (m)	37.4	3.75 (1H, t, J = 8.0)	73.3
4		44.1		39.0		44.2
5	1.74 (m)	47.8	1.68 (m)	49.8	1.85 (m)	48.0
6	1.38 (m), 1.74 (m)	25.2	1.46 (m), 1.65 (m)	23.1	1.35 (m), 1.70 (m)	25.3
7	2.10 (m), 2.37 (m)	39.1	2.05 (m), 1.76 (m)	40.1	2.07 (m), 2.33 (m)	39.2
8		150.0		150.5		150.0
9	1.68 (m)	57.1	1.73 (m)	57.2	1.82 (m)	57.1
10		40.4		40.8		40.5
11	1.50 (m), 1.67 (m)	23.3	1.15 (m), 1.31 (m)	25.8	1.46 (m), 1.62 (m)	23.2
12	1.87 (m), 2.17 (m)	39.7	1.88 (m), 2.15 (m)	39.7	1.88 (m), 2.15 (m)	39.7
13		140.4		142.9		142.8
14	5.34 (1H, t, J = 7.0)	124.9	5.31 (1H, t, J = 7.0)	121.7	5.32 (1H, t, J = 7.0)	121.8
15	4.10 (2H, d, J = 7.0)	59.7	4.30 (2H, d, J = 7.0)	66.5	4.31 (2H, d, J = 7.0)	66.6
16	1.68 (3H, s)	16.6	1.68 (3H, s)	16.7	1.68 (3H, s)	16.8
17	4.56 (1H, s), 4.83 (1H, s)	107.2	4.50 (1H, s), 4.80 (1H, s)	107.0	4.52 (1H, s), 4.82 (1H, s)	107.2
18	3.28, 3.80 (each 1H, d, J = 9.5)	74.1	3.23, 3.48 (1H, d, J = 9.5)	80.4	3.24, 3.80 (1H, d, J = 9.5)	74.4
19	0.70 (3H, s)	13.0	0.79 (3H, s)	18.6	0.73 (3H, s)	13.0
20	0.77 (3H, s)	15.8	0.72 (3H, s)	15.8	0.68 (3H, s)	15.8
15-*O*-Glc*p*						
1′			4.29 (1H, d, J = 7.5)	102.8	4.29 (1H, d, J = 8.0)	102.9
2′			3.20 (m)	75.3	3.22 (m)	75.2
3′			3.23 (m)	78.1	3.26 (m)	78.1
4′			3.28 (m)	71.9	3.30 (m)	71.9
5′			3.31 (m)	78.3	3.35 (m)	78.4
6′			3.65 (m), 3.85 (m)	63.0	3.64 (m), 3.84 (m)	63.1
18-*O*-Glc*p*						
1″	4.25 (1H, d, J = 7.5)	104.9	4.19 (1H, d, J = 7.5)	105.5	4.22 (1H, d, J = 8.0)	105.0
2″	3.24 (m)	75.2	3.20 (m)	75.3	3.18 (m)	75.3
3″	3.30 (m)	78.0	3.23 (m)	78.4	3.27 (m)	78.3
4″	3.30 (m)	72.1	3.28 (m)	71.9	3.26 (m)	72.1
5″	3.37 (m)	78.5	3.31 (m)	78.5	3.35 (m)	78.5
6″	3.60 (m), 3.90 (m)	63.1	3.65 (m), 3.85 (m)	63.0	3.64 (m), 3.84 (m)	63.2

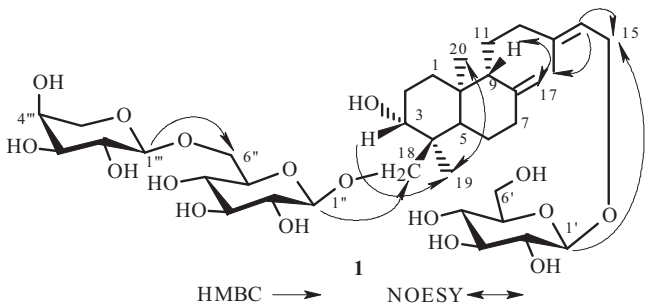

Fig. 1. Significant HMBC and NOESY correlations for compound **1**.

The third anomeric proton signal at δ_H 5.77 (H-1‴) correlated with the carbon signals at δ_C 68.7 (C-6″), suggesting that an arabinofuranoside was located at C-6″. This was similar to the known compound goshonoside-F6 (*α*-L-arabinofuranosyl-(1→6)-*β*-D-glucopyranoside of 13 (*E*)-*ent*-labda-8(17),13-diene-3*β*,15,18-triol) [7]. This indicated that **1** must be a 15-*O*-glucose of goshonoside-F6, which had the same aglycone of **2** and **4**. The NMR spectral data of compound **1** and goshonoside-F6 are listed in Table 1. Complete assignment was achieved by studying the results of HMQC, HMBC, and ^{1}H–^{1}H COSY experiments. The relative stereochemistry of **1** was established with the aid of a NOESY experiment (Fig. 1). Therefore, the structure of **1** was determined and named goshonoside-G by the authors.

The new compound was detected for its ability to inhibit nitric oxide (NO) production by LPS-induced RAW 264.7 cells. The inorganic free radical NO, synthesized by a family of enzymes termed NO-synthase (NOS), acts as a host defense mechanism by damaging pathogenic DNA and also acts as a regulatory molecule with homeostatic activities [8]. However, excess production of NO due to the reaction with superoxide in biological systems gives rise to various diseases such as inflammation, carcinogenesis, and atherosclerosis [9]. Therefore, down-regulation of NO production may be of therapeutic benefit in various diseases induced by pathological levels of NO. In the present study, it was found that the new compound inhibited nitric oxide production compared with the positive control indomethacin. The IC_{50} of compound **1** and indomethacin were 54.98 and 49.76, respectively. Cell viability was also determined by application of the MTT method in order to evaluate whether inhibition of NO production was due to the cytotoxicity of these tested compounds. It was found that none of the concentrations used in the experiment was cytotoxic (cell viability > 85%). As a result, compound **1** has the ability to inhibit NO production by LPS-induced RAW 264.7 cells.

EXPERIMENTAL

General. The IR spectra were obtained on a Nicolet 470 spectrometer. ESI-MS was measured on a Varian MAT-212 mass spectrometer. Silica gel (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, P. R. China) and ODS (50 mesh, AA12S50, YMC) were used. The ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, and HMBC spectra were recorded on a Bruker DMX-400 NMR spectrometer with tetramethylsilane (TMS) as an internal standard.

Plant Material. The unripe fruits were collected from Zhejiang Province, China, in September 2009, and authenticated by Prof. Mei-Li Guo, the Second Military Medical University. A voucher specimen (PT. 20090910) is deposited at the Laboratory of Pharmacognosy, Pharmaceutical College, the Second Military Medical University, China.

Extraction and Isolation. Dry fruits of *R. chingii* (50 kg) were extracted with 70% ethanol six times. The solvent was evaporated under reduced pressure to give an ethanol extract (6.5 kg). This residue was suspended in H_2O and then partitioned with petroleum ether, dichlormethane, and *n*-BuOH successively. The *n*-BuOH extract (1780 g) was subjected to column chromatography packed with D101 resin and eluted with water and 30%, 60%, and 95% ethanol. The 60% ethanol extract (326 g) was further fractionated by silica gel column chromatography using a stepwise gradient of EtOAc–EtOH (50:1 to 1:1) to give 10 fractions (Fr.1–Fr.10). Fraction 3 was repeatedly chromatographed over Sephadex LH-20 eluting with MeOH to yield **5** (900 mg), and then separated by preparative TLC to yield **6** (23 mg). Fraction 4 was subjected to reverse phase CC (MeOH–H_2O, 7:3) to yield **2** (123 mg). Fraction 5 was subjected by repeated column chromatography (silica gel, EtOAc–EtOH 5:1; reverse phase CC MeOH–H_2O 1:1) to yield **3** (123 mg) and **4** (97 mg). Fraction 7 was subjected to repeated Sephadex LH-20 column chromatography eluting with MeOH to yield **7** (900 mg), **8** (31 mg). Fraction 8 was subjected to repeated column chromatography (reverse phase CC, MeOH–H_2O 1:1; Sephadex LH-20, MeOH–H_2O 1:1) to yield **1** (27 mg). The 95% EtOH fraction (47 g) was chromatographed on a silica gel column eluted with a gradient of CH_2Cl_2–MeOH (50:1 to 1:1) to afford eight fractions, and fraction 3 was futher purified on a silica gel column (CH_2Cl_2–MeOH, 25:1) to yield **11** (80 mg), **12** (20 mg), and **13** (256 mg). Fraction 4 was subjected to Sephadex LH-20 column chromatography eluting with MeOH to yield **9** (72 mg). Fraction 5 was subjected to reverse phase CC eluting with MeOH to yield **14** (13 mg). The 30% EtOH fraction (178 g) was subjected to Sephadex LH-20 column chromatography eluting with MeOH to yield **10** (72 mg).

Goshonoside-G (1). White amorphous powder, $[\alpha]_D^{20}$–38.6° (*c* 0.8, MeOH). UV (MeOH, λ_{max}, nm): 210. HR-ESI-MS *m/z* 801.3889 $[M + Na]^+$ (calcd 801.3885). For ^{1}H NMR and ^{13}C NMR, see Table 1.

Goshonoside-F2 (2). White amorphous powder. ESI-MS *m/z* 507 $[M + Na]^+$. For ^{1}H and ^{13}C NMR, see Table 2 [10].

Goshonoside-F4 (3). White amorphous powder. ESI-MS *m/z* 653 $[M + Na]^+$. For ^{1}H and ^{13}C NMR, see Table 2 [10].

Goshonoside-F5 (4). White amorphous powder. ESI-MS *m/z* 669 $[M + Na]^+$. For ^{1}H and ^{13}C NMR, see Table 2 [10].

Tiliroside (5). Yellow powder. ESI-MS *m/z* 595 $[M + H]^+$. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 8.00 (2H, d, J = 8.5, H-2′, 6′), 7.34 (1H, d, J = 16.0, H-7‴), 7.38 (2H, d, J = 8.5, H-2‴, 6‴), 6.87 (2H, d, J = 8.5, H-3‴, 5‴), 6.80 (2H, d, J = 8.5, H-3′, 5′), 6.40 (1H, d, J = 2.0, H-8), 6.16 (1H, d, J = 2.0, H-6), 6.11 (1H, d, J = 16.0, H-8‴), 5.46 (1H, d, J = 7.5, H-l″), 4.31–3.30 (6H, m, H-2″-6″). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 177.8 (C-4), 166.6 (C-1‴), 164.6 (C-7), 161.6 (C-5), 160.4 (C-7‴), 160.2 (C-4′), 156.9 (C-2), 156.8 (C-9), 145.0 (C-3‴), 133.5 (C-3), 131.2 (C-5‴, C-9‴), 130.6 (C-6′, C-2′), 125.3 (C-4‴), 121.2 (C-1′), 116.2 (C-6‴, C-8‴), 115.5 (C-5′, C-3′), 114.1 (C-2‴), 104.3 (C-10), 101.4 (C-1″), 98.7 (C-6), 94.1 (C-8), 76.6 (C-2″), 74.7 (C-5″), 74.6 (C-3″), 70.4 (C-4″), 63.4 (C-6″) [11].

Astragalin (6). Yellow powder. ESI-MS *m/z* 447 $[M - H]^+$. ^1H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 8.03 (2H, d, J = 8.5, H-2′, 6′), 6.91 (2H, d, J = 8.5, H-3′, 5′), 6.49 (1H, d, J = 2.0, H-8), 6.25 (1H, d, J = 2.0, H-6), 5.47 (1H, d, J = 7.0, H-1″), 4.29–3.09 (6H, m, H-2″-6″). ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 177.4 (C-4), 164.3 (C-7), 161.1 (C-5), 160.0 (C-4′), 156.3 (C-9), 156.2 (C-2), 133.1 (C-3), 130.8 (C-2′), 130.8 (C-6′), 120.8 (C-1′), 115.1 (C-3′), 115.1 (C-5′), 103.8 (C-10), 100.9 (C-1″), 98.7 (C-6), 93.6 (C-8), 77.4 (C-5″), 76.4 (C-3″), 74.2 (C-2″), 69.8 (C-4″), 60.7 (C-6″) [12].

Kaempferol-3-*O*-*β*-D-rutinoside (7). Yellow powder. ESI-MS *m/z* 595 $[M + H]^+$. ^1H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.99 (2H, d, J = 8.5, H-2′, 6′), 6.83 (2H, d, J = 8.5, H-3′, 5′), 6.32 (1H, d, J = 1.7, H-8), 6.13 (1H, d, J = 1.7, H-6), 5.06 (1H, d, J = 6.5, Glc H-1), 4.47 (1H, d, J = 1.2, Rha H-1), 3.04–3.68 (10H, m, Glc H-2–H-6 Rha H-2–H-5), 0.96 (3H, d, J = 6.1, Rha H-6). ^{13}C NMR (125 MHz, CD_3OD, δ, ppm): 179.6 (C-4), 166.3 (C-7), 163.2 (C-5), 161.7 (C-4′), 159.7 (C-9), 158.7 (C-2), 135.8 (C-3), 132.6 (C-2′, 6′), 122.9 (C-1′), 116.4 (C-3′, 5′), 105.9 (C-10), 104.9 (C-1″), 102.6 (C-1‴), 100.3 (C-6), 95.2 (C-8), 78.4 (C-3″), 76.0 (C-2″), 77.4 (C-5″), 74.2 (C-4‴), 72.6 (C-2‴), 72.3 (C-3‴), 71.7 (C-4″), 69.9 (C-5‴), 68.8 (C-6″), 18.2 (C-6‴) [13].

Isoquercitrin (8). Yellow powder. ESI-MS *m/z* 463 $[M - H]^+$ [13].

Kaempferol (9). Yellow powder. ESI-MS *m/z* 285 $[M - H]^+$ [13].

Quercetin (10). Yellow powder. EI-MS *m/z* 302 $[M]^+$ [14].

***β*-Sitosterol (11)**. White amorphous powder [15].

Stigmast-5-en-3-ol, Oleate (12). White amorphous powder [16].

Lacceroic Acid (13). White amorphous powder [17].

Hexacosanol (14). White amorphous powder [18].

Inhibition Ability against LPS-Induced NO Production and Cell Viability. RAW 264.7 macrophages were seeded at 1×10^6/mL in 96-well plates. The cells were co-incubated with the compounds under investigation and LPS (3 μg/mL) for 24 h. The amount of NO was assessed by determining the nitrite concentration in the cultured RAW 264.7 macrophage supernatants with Griess reagent. Aliquots of supernatants (100 μL) were incubated in sequence with 50 μL of 1% sulfanilamide and 50 μL of 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution. The absorbance at 540 nm was read using a microplate reader (POLAR star). Cell viability was determined using the mitochondrial respiration-dependent MTT reduction method. After transferring the required supernatant to another plate for the Griess assay, the remaining supernatant was aspirated from the 96-well plates, and 100 μL of fresh medium containing 2 mg/mL of MTT was added to each well. The cells were then incubated at 37°C in a humidified atmosphere containing 5% CO_2. After incubating for 4 h, the medium was removed, and the violet crystals of formazan in viable cells were dissolved in dimethyl sulfoxide. Absorbance at 570 nm was measured using a microplate reader.

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