SUPPLEMENTARY MATERIAL

A new cytotoxic diterpenoid glycoside from the leaves of *Blumea lacera* and its effects on apoptosis and cell cycle

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A new diterpenoid glycoside; 6*E*,10*E*,14*Z*-(3*S*)-17-hydroxygeranyllinalool-17-*O*-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (**1**) together with the known diterpenoid glycoside (**2**) and two known flavonoid glycosides (**3**, **4**) were isolated from the methanol extract of *Blumea lacera* leaves. The structures were determined by the interpretation of their spectroscopic data and comparison with the literature. All compounds were isolated for the first time from *Blumea lacera* and evaluated for their cytotoxic activity. Only the new compound (**1**) showed strong cytotoxic activity with the lowest IC₅₀ value (8.3 μM) being displayed against MCF-7 breast cancer cells. In apoptosis and cell cycle analysis **1** revealed strong apoptotic activity against MCF-7 cells (45.5% AV⁺/PT) after 24 h, but showed no arresting of any of the cell cycle phases in MCF-7.

Keywords: *Blumea lacera* leaves; diterpenoid glycoside; flavonoid glycoside; cytotoxicity; apoptosis; cell cycle analysis

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Experimental

General experimental procedures

Optical rotation was measured on a JASCO P-1010 polarimeter. The UV spectra were obtained on a Shimadzu BioSpec-mini UV spectrophotometer. IR spectra were recorded on a Bruker Optics alpha-QuickSnap (A220/D-01) FT-IR spectrophotometer. NMR spectra were recorded on either a Bruker Avance 300 or 600 MHz spectrometer in CD_3OD . The chemical shifts (δ) are given in ppm, with reference to tetramethylsilane (TMS) as internal standard, and coupling constants (J) are in Hz. LR-MS and HR-ESI-MS were obtained on Bruker Daltonics Esquire 3000 and Bruker Daltonics Apex III 4.7e mass spectrometers, respectively. Analytical HPLC was performed on a Varian Prostar instrument with a 335 DAD using a RP (Luna C₁₈, 5 μm , 250 $\times$ 4.6 mm) column. Preparative HPLC was performed on a Waters instrument equipped with a Waters 600E pump, Rheodyne 7725i injector, and Waters 2487 dual-wavelength detector using a RP (Luna C₁₈, 5 μm , 150 $\times$ 21.2 mm) column. SPE cartridges (Alltech, 10 g, RP-C18) were used to fractionate the extract.

Plant materials

The leaves of *Blumera lacera* were collected from the Southern part of Bangladesh during May 2007. The plant material was identified by Dr. Momtaz Mahal Mirza, Principal Scientific Officer, Bangladeshi National Herbarium, Dhaka, and shade dried. A specimen was deposited in the Bangladeshi National Herbarium, Dhaka (Voucher No.: DACB 30550).

Extraction and isolation

The dried and pulverised plant material of *B. lacera* (51.0 g) was extracted by soaking in 500 mL of distilled methanol overnight at room temperature with continuous stirring. The extract was then filtered and the solvent evaporated using a rotary evaporator followed by freeze-

drying to afford 7.86 g (15.4 % w/w) of crude methanol extract. The extract was further fractionated using a C₁₈ SPE column (RP-C18; Alltech, bed weight: 10 g, column size: 60 mL, particle size: 55 µm) and eluted with a methanol/water stepwise gradient (0%, 20%, 40%, 65%, 80% and 100% MeOH in H₂O) to provide six SPE fractions. Cytotoxicity assays (MTT) on each of the SPE fractions was performed and the active fractions, SPE4 and SPE5 were further subjected to purification using semi-preparative RP-HPLC (Luna 5 µm, 150 x 21.2 mm) using H₂O/MeOH (90% water in MeOH with TFA) to solvent B (90% MeOH in water with TFA) for SPE4 and H₂O/ACN (90% water in ACN with TFA) to solvent B (90% ACN in water with TFA) for SPE5 gradient system containing 0.05% TFA at 13 mL/min flow rate. Total run time was 80 and 70 min for SPE4 and SPE5, respectively. Semi-preparative HPLC of SPE5 (0.5 g) yielded **1** (1.5 mg, R_T: Retention time-13.59 min) and **2** (5.0 mg, R_T: Retention time-13.59 min) and SPE4 (1.7 g) yielded **3** (53.3 mg, R_T: Retention time-30.72 min) and **4** (4.9 mg, R_T: Retention time-40.05 min).

6E,10E,14Z-(3*S*)-17-hydroxygeranyllinalool-17-O-β-D-glucopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (**1**): white crystalline solid (MeOH); $[\alpha]_D^{25} = -34.7^\circ$ (c 0.25, MeOH); UV (MeOH, c = 1.93 mM) 201, 275 nm; IR (film) $\nu_{\max}$ 3420, 1630 cm⁻¹; ¹H (600 MHz, CD₃OD) and ¹³C NMR (75 MHz, CD₃OD) data, see Table 1; positive HR-ESI-MS m/z 799.4087 [M+Na]⁺ (calculated for C₃₈H₆₄NaO₁₆, 799.4092 amu).

Cytotoxicity assay (MTT)

All cell lines (VERO, ATCC: CCL-81; NIH 3T3, ATCC: CRL-1658; AGS, ATCC: CRL-1739; HT-29, ATCC: HTB-38; MCF-7, ATCC: HTB-22; and MDA-MB-231, ATCC: HTB-26) were purchased from ATCC, Manassas, VA 20108, USA. Cell lines were cultured in Advanced Dulbecco's modified Eagle's medium supplemented with 10% inactivated newborn calf serum and 5 mM l-glutamine, and grown at 37°C in a humidified atmosphere of 5% CO₂ in air. DMSO, MTT, cycloheximide and paclitaxel were purchased from Sigma Aldrich, Germany. Annexin V-FITC and propidium iodide were purchased from BD Biosciences, NJ, USA.

The cytotoxicity of the isolated compounds was tested against normal mouse fibroblast (NIH3T3) and monkey kidney (VERO) cells, as well as four human cancer cell lines (gastric adenocarcinoma (AGS), colon adenocarcinoma (HT-29), and two breast ductal carcinoma (MCF-7 and MDA-MB-231) cell lines), using the MTT assay. Briefly, cells were seeded in 96-well plates at a density of 1.0×10^4 to 2.0×10^4 cells/well. Following 24 h incubation at 37°C with 5% CO₂, cells were treated with varying concentrations of

1 compounds for 48 h. Following washing and incubation with MTT solution for 2 h, cells
2 were lysed. The absorbance was measured after 45 min using a microplate reader (Wallac
3 1420 Multilevel counter, Perkin-Elmer) at a wavelength of 560 nm. The IC₅₀ values were
4 calculated with probit analysis software (LdP Line Software, Doki, Cairo). Cycloheximide
5 was used as a positive control, generating IC₅₀ values of 24.2, 26.3, 8.5, 11.0, 181.6, and 22.4
6 µM against VERO, NIH3T3, AGS, HT29, MCF-7 and MDA-MB-231 cells, respectively.

Annexin V-FITC apoptosis measurement

The annexin V-FITC apoptosis assay was used to measure apoptosis induction potential of the isolated cytotoxic compound **1** from *B. lacera* against a human breast cancer (MCF-7) cell line. Briefly, cells were seeded in a 6-well plate at a density of 50×10^4 cells/well and incubated at 37°C with 5% CO₂ for 24 h. The following day, cells were treated with 8.3 µM of compound **1** for 24 and 48 h. Cells were rinsed with PBS, trypsinized rapidly, and centrifuged to pellet the cells. The supernatant was removed, and the cells were resuspended in 1× binding buffer (0.1 M Hepes/NaOH, 1.4 M NaCl, 25 mM CaCl₂), and 5 µL aliquots of the staining solutions (FITC annexin V and PI) was added. After 15 min incubation in the dark at room temperature, the cell suspension was diluted with binding buffer and analyzed within 1 h using a CyAnTM ADP flow cytometer (Beckman Coulter, USA) and the data was recorded by using HyperCyt (R) software. The assay was carried out as two separate experiments and each experiment performed in triplicate. Cells with no treatment served as the negative control and paclitaxel (0.023 µM) served as the positive control.

Cell cycle analysis by flow cytometry

Propidium iodide staining cell cycle analysis was used to measure the cell distribution in 3 different phases of the cell cycle of compound **1** against breast carcinoma cells (MCF-7)). Briefly, for cell cycle analysis using flow cytometry, 15×10^4 cells/well were seeded in 12-well plates and incubated at 37°C with 5% CO₂ for 24 h. Following attachment of the cells after 24 h, the cells were treated with 8.3 µM compound **1**, after which the cells were harvested by trypsinization, washed with PBS and fixed with 70% ice cold ethanol. After 15 minutes incubation and following centrifugation, the fixed cells were incubated with 250 µL RNase A solution (0.2 mg/mL RNase and 10% Triton X-100) in PBS and incubated for 40 minutes at 37°C. The cell suspension was then transferred to a falcon polystyrene U bottom tube (5 mL) and resuspended with 10 µL (1 mg/mL) of propidium iodide. Cell cycle distribution was analysed using CyAnTM ADP flow cytometer (Beckman Coulter, USA) and the data was recorded by using HyperCyt (R) software. The results were analyzed using De novo FCS express 4 Flow Cytometry software. Cells with no treatment served as the negative control and paclitaxel (0.023 µM) served as the positive control.

Results

Structure elucidation of compound 1

Table S1. ^1H and ^{13}C NMR data for the new compound **1** (CD_3OD).

Position	δ_{H} (ppm) (multiplicity, J , Hz)	δ_{C} (ppm)
1	5.21 (<i>d</i> , 1H, $J=17.4$ Hz) 5.04 (<i>d</i> , 1H, $J=10.8$ Hz)	112.0
2	5.98 (<i>dd</i> , 1H, $J=17.4, 10.8$ Hz)	144.5
3		73.9
4	1.51 (<i>m</i> , 2H)	43.5
5	2.02 (<i>m</i> , 2H)	23.7
6	5.13 (<i>m</i> , 1H) [#]	125.8
7		135.4
8	2.02 (<i>m</i> , 2H)	40.8
9	2.08 (<i>m</i> , 2H)	27.7
10	5.13 (<i>m</i> , 1H) [#]	126.0
11		136.0
12	2.02 (<i>m</i> , 2H)	40.9
13	2.18 (<i>m</i> , 2H)	27.2
14	5.40 (<i>app t</i> , 1H, $J=6.6, 7.2$)	131.3
15		132.5
16	1.79 (<i>br s</i> , 3H)	22.0
17	4.31, (<i>d</i> , 1H, $J=11.4$ Hz) 4.22 (<i>d</i> , 1H, $J=11.4$ Hz)	68.2
18	1.61 (<i>br s</i> , 3H)	16.1
19	1.61 (<i>br s</i> , 3H)	16.2
20	1.25 (<i>br s</i> , 3H)	27.6
Glc I		
1	4.35 (<i>d</i> , 1H, $J=7.8$)	101.1
2	3.67 (<i>m</i> , 1H)	81.9
3	3.55 (<i>t</i> , 1H, $J=7.8, 9.0$)	77.8
4	3.28 (<i>m</i> , 1H)	71.5
5	3.35 (<i>m</i> , 1H)	76.7
6a, 6b	3.97 (<i>d</i> , 1H, $J=10.8$ Hz) 3.84 (<i>app t</i> , 1H, $J=1.2$ Hz)	67.6
Glc II		
1	4.63 (<i>d</i> , 1H, $J=7.8$ Hz)	104.8
2	3.24 (<i>m</i> , 1H)	75.9
3	3.35 (<i>m</i> , 1H) [#]	78.3
4	3.35 (<i>m</i> , 1H)	71.3
5	3.50 (<i>t</i> , 1H, $J=7.8, 9.0$ Hz) [#]	78.2
6a, 6b	3.84, 3.67 (<i>m</i> , 1H, 1H)	62.8
Rha		
1	4.74 (<i>s</i> , 1H)	102.1
2	3.67 (<i>m</i> , 1H)	72.2
3	3.67 (<i>m</i> , 1H)	72.4
4	3.38 (<i>m</i> , 1H)	74.0
5	3.67 (<i>m</i> , 1H)	69.8
6 (-CH ₃)	1.27 (<i>d</i> , 3H, $J=6.0$ Hz)	18.1

[#] Assignments may be interchangeable.

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Figure S1. ^1H NMR (600 MHz, CD_3OD) spectrum of the new compound **1**.

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Figure S2. ^{13}C NMR (75 MHz, CD_3OD) spectrum of the new compound **1**.

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Figure S3. Key HMBC and COSY correlations observed in **1**.

Apoptosis and cell cycle analysis of compound **1**

Compound **1** showed strong cytotoxicity (IC_{50} : 8.3 μ M) against MCF-7, therefore, to identify the mechanism behind compound **1**'s cytotoxicity, its apoptosis-inducing potential was evaluated. Following treatment of cells for 24 h with compound **1** at 8.3 μ M the number of cells (45.5%) that underwent apoptosis (AV^+/PI) was significantly ($p < 0.05$) increased. In comparison paclitaxel, a known apoptotic compound resulted in 39.9% of cells undergoing apoptosis at 0.023 μ M (Figure S4). Interestingly treatment of MCF-7 cells for 48 h with **1** did not further increase the extent of apoptosis (% AV^+/PI) for compound **1**. Further analysis of compound **1**'s cytotoxic and apoptosis-inducing activity using propidium iodide (PI) staining revealed only a slight accumulation of cells in the G1 phase (7.9% increase in comparison to untreated cells), with a concurrent decrease of cells in the S and G2/M phase (Figure S5). This suggests that the strong cytotoxic activity of **1** can likely be attributed to its apoptosis-inducing capacity (and possibly signal transduction pathways) rather than strong effects on the cell cycle.

Figure S4. Apoptosis induction of compound **1** (8.3 μ M) in MCF-7 cells after 24 hr incubation in comparison to paclitaxel (0.023 μ M). Values are means $\pm$ standard deviations of two independent experiments, each experiment performed in triplicate.

Figure S5. Cell cycle arresting potential of the new compound **1** (8.3 μ M) on MCF-7 cells in comparison to paclitaxel (0.023 μ M) after 24 h incubation. Figure represents percentage of cell distribution in G1, S and G2/M phases after 24 h treatment. Values are means $\pm$ standard deviations of two independent experiments, each experiment performed in triplicate.