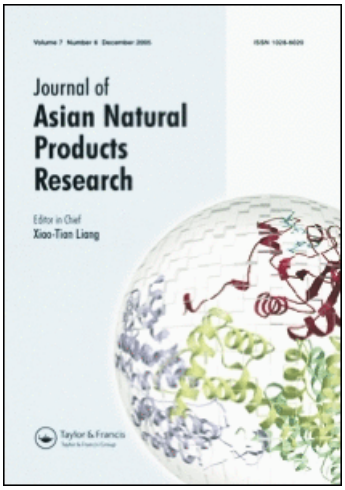

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L. Zheng[a]; J. Zheng[a]; L. -J. Wu[a]; Y. -Y. Zhao[b]
[a] Department of Natural Products Chemistry, Shenyang Pharmaceutical University, Shenyang, China [b] State Key Laboratory of Natural and Biomimetic, School of Pharmaceutical Sciences, Health Science Center, Peking University, Beijing, China

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Julibroside J_8-induced HeLa cell apoptosis through caspase pathway

L. ZHENG†‡, J. ZHENG‡, L.-J. WU‡ and Y.-Y. ZHAO†*

†State Key Laboratory of Natural and Biomimetic, School of Pharmaceutical Sciences, Health Science Center, Peking University, Xueyuan Road No. 38, Beijing 100083, China
‡Department of Natural Products Chemistry, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenhe District, Shenyang 110016, China

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The *julibroside* J_8 was isolated from the *Albizia julibrissin* and evaluated for antiproliferatived on six cancer cell lines (BGC-823, Bel-7402, HeLa, PC-3MIE8, MDA-MB-435 and LH-60) *in vitro*. *Julibroside* J_8 at 100 $\mu g\,mL^{-1}$ (46.08 $\mu mol{\cdot}L^{-1}$) significantly inhibited growth in the first three cell lines. In addition, in HeLa cells typical apoptotic changes in morphology were observed, and further, nuclear damage was observed by Giemsa staining and DNA fragmentation was exhibited. Effects of *julibroside* J_8 on induction of DNA fragmentation, caspase-3 activation and downregulation of ICAD expression were effectively inhibited by a caspase-3 inhibitor, z-DEVD-fmk. In addition, apoptosis induced with julibroside J_8 was associated with an increase in expression of the apoptosis inducer Bax, and a significant reduction in expression of the apoptosis suppressor Bcl-2 in mitochondria. These results suggest that julibroside J_8 induces HeLa death through caspase pathway.

Keywords: *Julibroside J_8*; HeLa cell; Apoptosis; Caspase; Bax

1. Introduction

The stem bark of *Albizia julibrissin* (Leguminosae) has been utilized as a sedative and anti-inflammatory drug to treat swelling and pain in the lung and for skin ulcers and wounds [1]. The isolation and identification of several triterpenoid saponins from this plant have been reported by our groups [2]. It was reported that the triterpenoid saponins isolated from the *Albizia julibrissin* showed significant inhibitory activity on cancer cell lines [3] in vitro. Julibroside J_8 is a new triterpenoid saponin isolated from *Albizia julibrissin* [4]. We further investigated its mechanism of inducing cancer cell death for the first time in this study.

Apoptosis is an essential and highly organized cell death process that requires the active participation of endogenous cellular enzymes and oncogene expression. Morphologically, this process is characterized by a dramatic execution phase that induces loss of cell volume, plasma membrane blebbling, chromatin condensation, DNA degradation and formation of

*Corresponding author. Tel.: +86-10-82801592. E-mail: nmechem@bjmu.edu.cn

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apoptotic bodies [5]. Several anticancer drugs and a variety of cell differentiation inducers have been shown to induce apoptosis in susceptible cancer cell lines. Among the numerous proteins and genes involved, members of caspase family and the Bcl-2 family play important roles in inhibiting or promoting apoptosis. The Bcl-2 family functions to either promote or inhibit apoptosis. Some of them suppress apoptosis, including Bcl-2 and Bcl-X_L, and others promote apoptosis, such as Bax and Bid, and the subtle balance of the Bcl-2/Bax complex can modulate the anti- or pro-apoptotic effect. Once apoptosis was initiated, Bcl-2 was cleaved by caspase-3 to attenuate its anti-apoptotic effects [6]. In this study, we evaluated the inhibitory activity of julibroside J_8 against six cancer cell lines. The effect of the compound on apoptosis was also investigated in HeLa cell.

2. Results and discussion

2.1 Inhibitory activity of julibroside J_8 against cancer cell lines

The chemical structure of julibroside J_8 is shown in figure 1. The inhibitory activity of julibroside J_8 against six cancer cell lines (Bel-7402, HeLa, BGC-823, PC-3MIE8, MDA-MB-435 and LH-60) in vitro were detected by MTT and SRB methods. Julibroside J_8 at 100 $\mu g\,mL^{-1}$ (46.08 $\mu mol\,L^{-1}$) significantly inhibited growth in the first three cell lines, and showed good dose-dependence (table 1). A dose-dependent response was observed at concentration of 0–25 $\mu mol\,L^{-1}$ for 0–48 h. The treatment of HeLa cells with 15 $\mu mol\,L^{-1}$ of julibroside J_8 for 12 h and 24 h induced approximately 70% and 79% of HeLa cell death. The treatment of HeLa cells with 20 $\mu mol\,L^{-1}$ of julibroside J_8 for 12 h and 24 h induced approximately 72% and 87% of HeLa cell death (figure 2). Whether the inhibitory effect of julibroside J_8 was mediated by an apoptotic mechanism was further investigated.

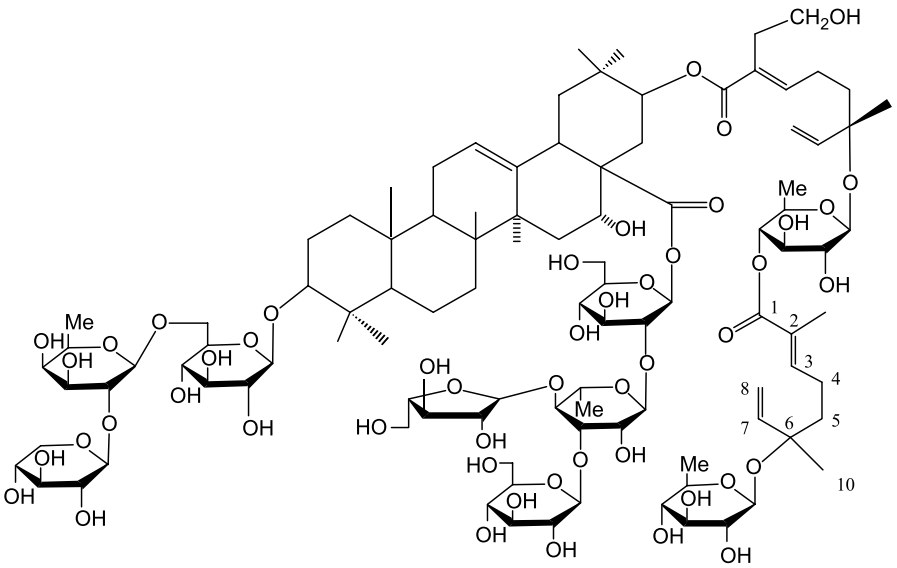

Figure 1. The structure of *julibroside* J_8

Table 1. The Inhibition and IC $_{50}$ against cancer cells of *julibroside* J_8.

	The Inhibition (%) against cancer cells		
Concentrations of julibroside J_8 *(μmol L^{-1})*	*BGC-823*	*Bel-7402*	*HeLa*
(0.46)	13.61	39.75	35.10
(4.60)	29.46	58.32	60.17
(46.08)	87.44	86.66	88.75
IC $_{50}$	15.80	3.06	3.57

2.2 *Julibroside J_8 induces apoptotic cell death in HeLa cells*

When cancer cells were cultured with of julibroside J_8 (15 μmol L^{-1}) for 6 to 24 h compared with untreated cells, typical apoptotic changes in morphology were observed. After 6 h culture time, julibroside J_8-treated HeLa cells underwent retraction of cellular processes and became round in shape (figure 3B), and by 24 h the majority of HeLa cells had become round with shrunken nuclei (figure 3C, D). Untreated cells did not show these apoptotic characteristics (figure 3A). Morphological changes were further confirmed by Giemsa staining of cell nuclei. In control group, nuclei of HeLa cells were round and stained homogeneously (figure 4A). However, julibroside J_8 (15 μmol L^{-1})-treated cells showed marked blebbling of nuclei and granular apoptotic bodies (Figure 4B).

To further identify julibroside J_8-induced apoptotic cell death in HeLa cells, treated cells were subjected to DNA fragmentation analysis, another well-known marker of apoptosis. Results showed that DNA fragmentation was clearly observed when HeLa cells were treated with julibroside J_8 at concentration of 10 μmol L^{-1} or higher for 24 h (Figure 5). The appearance of an increasing level of cleavage of internucleosomal DNA fragments showed dose-dependent julibroside J_8-induced apoptosis of HeLa cells from 5 to 15 μmol L^{-1}. Based

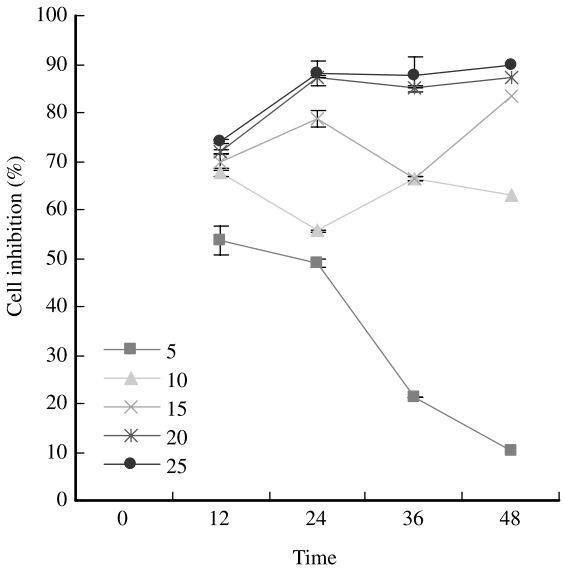

Figure 2. Time courses of the induction of cell death by *julibroside* J_8 HeLa cells were treated with *julibroside* J_8 at various concentrations (5–25 μmol L^{-1}) for 12, 24 36 and 48 h, $P < 0.01$ at concentrations (10–25 μmol L^{-1}).

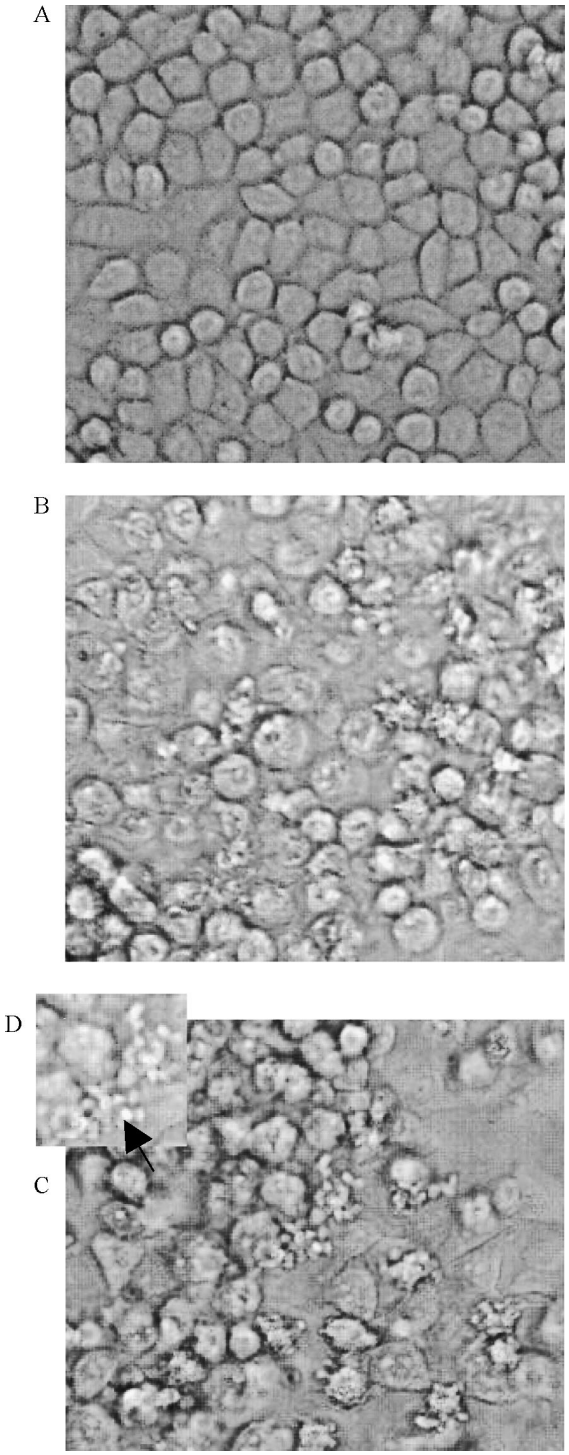

Figure 3. *Julibroside* J_8-induced morphological changes of HeLa cells. The cells were incubated in a 6-well culture plate. Changes of cellular morphology were examined at 6 h (B), 12 h (C) (× 100 magnification), and (D) (× 200 magnification)in the absence (A) (× 100 magnification) or the presence of 15 μmol L^{-1} of *julibroside* J_8 (× 100 magnification).

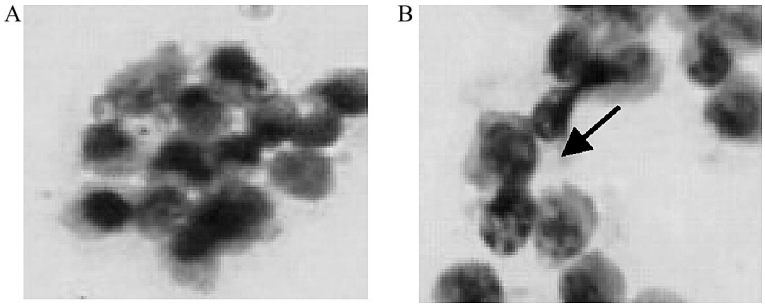

Figure 4. Morphological changes of cell nuclei. HeLa cells were incubated in the medium alone (A) or the medium containing 15 μmol L^{-1} of *julibroside* J_8 for 24 h (B), then the cells were stained with Giemsa with × 200 magnification.

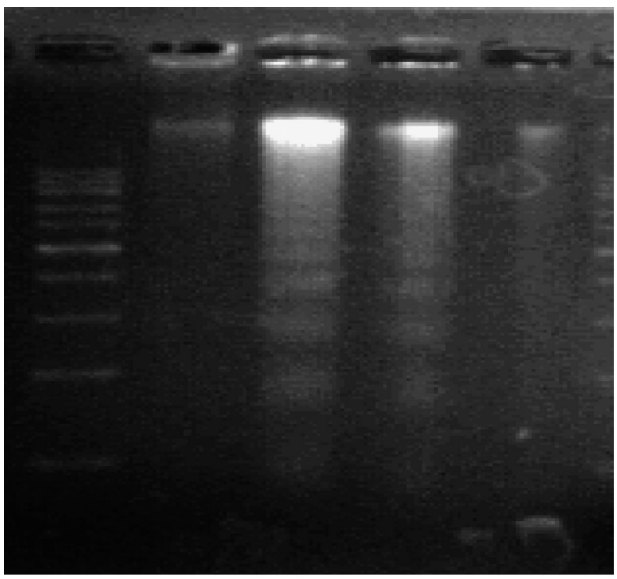

Figure 5. Julibroside J_8 -induced DNA fragmentation of HeLa cells. The cells (1×10^6 cells) were cultured in the absence or presence of *julibroside* J_8 (5, 10, 15 μmol L^{-1}) for 24 h. The molecular weight of Marker: 97, 66, 45, 30, 20, 14 KDa.

on the changes in cellular morphology and DNA fragmentation, it was concluded that julibroside J_8 caused apoptosis in HeLa cells.

2.3 Different expression of Bcl-2, Bcl-x_L and Bax proteins, and the activation of caspase-3/ICAD pathway involved in julibroside J_8*-induced HeLa cells apoptosis*

It has been shown that proteins of the Bcl-2 family play an essential role in HeLa apoptosis [7] and that several inflammatory mediators regulate apoptosis by altering the expression of anti-apoptotic Bcl-x_L and pro-apoptotic Bax proteins [8]. To confirm whether such a mechanism is involved in julibroside J_8-induced apoptosis, western blot analysis was performed to observe changes in the expression of Bcl-2, Bax and Bcl-x_L

proteins during julibroside J_8-induced apoptosis. A significant downregulation of Bcl-2 and Bcl-x_L expression in HeLa cells after treatment with julibroside J_8 for 24 h was detected, while that of Bax protein began to increase (Figure 6). Bcl-2 is the prototypic family member and binds to the adapter CED-4 to prevent it from activating caspase CED-3. It is well known that in the caspase family, caspase-3 plays the central role. Once activated, caspase-3 performed a number of executioner functions, including the activation of a latent cytosolic endonuclease, poly (ADP-ribose) polymerase (PARP) and inhibitor of the caspase-activated DNase (ICAD). The ICAD cleavage is consistent with DNA degradation, which is marker for apoptotic cell death [9]. Results showed that ICAD expression decreased as the functional isoform of M_γ 45 kDa with time in julibroside J_8-treated cells. This decline was effectively prevented by caspase-3 inhibitor (z-DEVD-fmk 20 μmol L^{-1}) at 12 h (Figure 7). Together, these observations indicated that julibroside J_8-promoted apoptosis in HeLa cells involved caspase-3 activation and cleavage of its substrate ICAD, and that the balance between Bcl-2, Bcl-x_L and Bax expression is essential for julibroside J_8-induced apoptosis.

3. Experimental

3.1 Materials and methods

3.1.1 General experimental procedures. ^1H and ^{13}C NMR spectra were taken on a Bruker AM-500 spectrometers with Me_4Si as internal standard. Mass spectra (MS) were measured on Mos mate spectrometer.

3.1.2 Plant material. The dried stem bark of *Ablizia julibrissin* (Leguminosae) were collected from Shanxi Province of China in July 2000, and was identified by Professor Junhua Zheng, Departmemt of Natural Medicines, School of Pharmaceutical Sciences, Peking University. A voucher specimen is deposited in the Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University. All the chemicals used were analytical grade reagents.

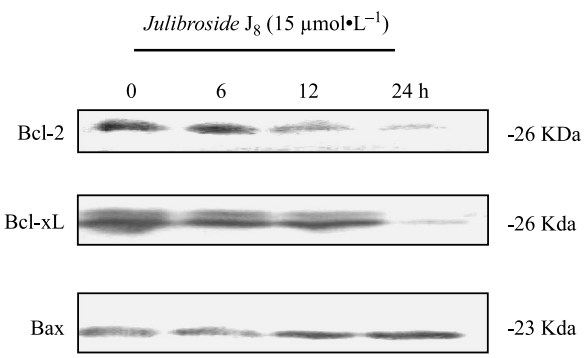

Figure 6. The expression of Bcl-2, Bax and Bcl-x_L in Julibroside J_8-treated HeLa cells. The cells were treated with 15 μmol L^{-1} *julibroside* J_8 for 0, 6, 12 and 24 h. Cell lysates were separated by 12% SDS-PAGE electrophoresis, and the protein bands were detected by Western blot analysis.

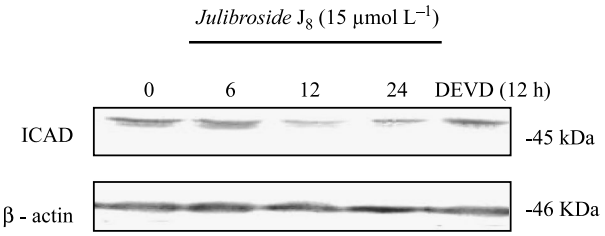

Figure 7. The expression of ICAD in *julibroside* J_8-treated HeLa cells. The cells were treated with 15 μmol L^{-1} *julibroside* J_8 for 0, 6, 12 and 24 h. Cell lysates were separated by 12% SDS-PAGE electrophoresis, and ICAD protein bands was detected by Western blot analysis.

3.1.3 Isolation and purification of active compound. The stem barks of A. julibrissin were extracted three times with boiling water. After the removal of solvents under reduced pressure the residual extract was partitioned with *n*-BuOH and water. The *n*-BuOH extract (200 g) was subjected to silica gel column chromatography and eluted with $CHCl_3$–MeOH–H_2O (65:35:10, v/v/v) to yield fractions A and B. Fraction A was separated over HP-20 macroporous resin column using 0–100% of MeOH as an eluting solvent to give fractions 1 to 6. Fraction 4 was further separated by chromatography over silica gel column and RP C18 (55–75% of MeOH) to afford fractions a to c. Fraction b was further separated by preparative HPLC on a C-18 column (22 × 10 mm, flow rate 2.8 mL min^{-1}, UV deterset at 216 nm) with MeOH–H_2O (60:40) as eluent to yield julibroside J_8 (80 mg). The structure of julibroside J_8 was determined by comparing the chemical and spectral data (^{1}H NMR, ^{13}C NMR) with those reported in the literature [4]. Its purity was measured by HPLC equipped with an UV detector and a Phenomenex 100A ODS-18C column (5 μm, 10 mm × 250 mm). The mobile phase was composed of MeOH–H_2O (74:26, v/v), flow rate 6.0 mL min^{-1}, UV deterset at 216 nm, at room temperature, t_R of julibroside J_8 52.3 min. The purity of the compound was about 99.3%.

3.2 Biological materials

3.2.1 Cell cultures. The HeLa cell lines for use of the apoptotic mechanism was purchased from American Type Culture Collection (ATCC, no. CRL1872; Rockville, MD, USA). The cells were cultured in RPMI-1640 medium (Gibco, NY, USA), supplemented with 10% fetal bovine serum (FBS) and 0.03% L-glutamine (Gibco), and maintained at 37°C with 5% CO_2 in a humidified air. Caspase-3 inhibitor, Z-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk) was from Calbiochem (CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma (MO, USA). We evaluated the inhibitory activity of julibroside J_8 against six cancer cell lines. The cell lines for evaluating inhibitory activity were from the cell lines stock of Modern Research Center of Traditional Chinese Medicine, Peking University, Beijing.

3.3 Drug solutions

Julibroside J_8 was dissolved in DMSO to make a stock solution, then diluted in cell culture medium at different concentrations and was kept below 0.01% in all the culture media.

3.4 Inhibitory activity against HeLa cells and dose-dependence

The inhibitory activity of julibroside J_8 on cancer cells was determined by a colorimetric MTT assay as described previously [10]. The cells were dispensed in 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) at a density of 1×10^4 cells per well. After 12 h incubation, they were treated with various concentrations of the test samples, followed by 12, 24, 36 and 48 h cell culture. After these treatments, 20 μL MTT solution (5.0 mg ml^{-1}) were added to each well and the cells were incubated for 4 h. The resulting crystals were dissolved in DMSO. The growth inhibition was determined by an ELISA reader (Tecan Spectra, Wetzlar, Germany). The inhibitory activity was expressed as a relative percentage of inhibition calculated as follows: relative% inhibition $= [(A_{570(control)} - A_{570(experimental)}]/(A_{570(control)} - A_{570(blank)}] \times 100\%$.

3.5 Observation of morphological changes

HeLa cells in RPMI-1640 containing 10% FBS were seeded into six-well culture plates and cultured for 12 h. *Julibroside J_8* (15 μmol L^{-1}) was added to the cell culture and the cellular morphology was observed using phase contrast microscopy at 24 h (Leica, Wetzlar, Germany).

3.6 Nuclear damage observed by Giemsa staining

Apoptotic nuclear morphology was assessed using Giemsa staining. Cells were fixed with ethanol–chloroform–acetic acid (6:3:1, v/v) for 10 min at room temperature, then washed and stained with diluted Giemsa (1:20) for 10 min at room temperature. Cellular morphology was observed using phase-contrast microscopy at 24 h (Leica).

3.7 DNA extraction and detection of DNA fragments

HeLa cells (1×10^6 cells) were seeded and treated with of *julibroside J_8* (15 μmol L^{-1}). Both floating and adherent cells were scraped off and collected in medium, washed three times with PBS, and resuspended in 100 μL lysis buffer (Tris–HCl 10 mmol L^{-1}, pH 7.4, EDTA 10 mmol L^{-1}, pH 8, and 0.5% Triton X-100) and kept at 50°C for 2 h. The lysate was centrifuged at 7200*g* for 20 min. The supernatant fraction was incubated with 2 μL RNase A (2.0×10^4 mg L^{-1}) at 37°C for 60 min, then incubated with 2 μL proteinase K 40 μg L^{-1} (Merk, USA) at 37°C for 60 h, then extracted with 0.5% (5 mol L^{-1}) NaCl and 50% 2-propanol, incubated overnight at −20°C, and centrifuged at 7200*g* for 15 min. After drying, DNA was dissolved in TE buffer, pH 7.8 (Tris–HCl 10 mmol L^{-1}, pH 7.4, and edetic acid 1 mmol L^{-1}, pH 8.0), separated by agarose (2%) gel electrophoresis at 100 V for 40 min and stained with ethidium bromide (0.1 mg L^{-1}) [11].

3.8 Western blot analysis

HeLa cells were treated with julibroside J_8 (15 μmol L^{-1}) for 0, 6, 12, 24 and 48 h. Both adherent and floating cells were collected, and then washed in PBS. The cell pellets were resuspended in lysis buffer, including HEPES 50 μmol L^{-1}, pH 7.4, Triton X-100 1%,

sodium orthovanadate 2 μmol L^{-1}, sodium fluoride 100 mmol L^{-1}, edetic acid 1 mmol L^{-1}, PMSF 1 mmol ;L^{-1}, aprotinin (Sigma) 10 mg L^{-1}, leupeptin (Sigma) 10 mg L^{-1}, and lysis at 4°C for 1 h. After 13,000*g* centrifugation for 15 min, the protein content of the supernatant fraction was determined by Bio-Rad (USA) protein assay reagent. The protein lysates were separated by electrophoresis in 12% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane. Proteins were detected with antibodies against Bcl-2, Bax, Bcl-x_L (rabbit IgG, Oncogene) and ICAD (rabbit IgG;, Santa Cruz, CA, USA), followed by addition of horseradish peroxidase-conjugated secondary antibody [12].

3.9 Statistical analysis

All results were confirmed in at least three separate experiments. Differences between experimental groups comprised normally distributed data, which were analysed for statistical significance using the Student's *t*-test. *P*-values of less than 0.05 were considered significant.

Acknowledgements

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