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Isolation from *Eucalyptus occidentalis* and Identification of a New Kaempferol Derivative that Induces Apoptosis in Human Myeloid Leukemia Cells

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In this paper we report the isolation and structural elucidation of a new flavonoid (**1**) and three known compounds, 6,8-di-*C*-methylkaempferol 3-methyl ether (**2**), oleanolic acid, and 2α,3*β*-dihydroxyurs-12-en-28-oic acid, from aerial parts of *Eucalyptus occidentalis* collected in Algeria. Flavonoids **1** and **2** were used to study their biological activities on the human promyelocytic leukemia cell line, HL-60. Our data show that these compounds induce morphological changes and internucleosomal DNA fragmentation characteristic of apoptotic cell death, which is mediated by caspase-8/caspase-3 activation and cytochrome *c* release.

The genus *Eucalyptus* comprises more than 523 species and 138 varieties, and new species and varieties are still being described.[1] *Eucalyptus* is mainly native to Australia, but a few species are also native to the Philippines, Papua New Guinea, and Timor. *Eucalyptus* species have been utilized for medicinal purposes; the bark and leaves have been used to treat colds, toothache, fever, diarrhea, and other complaints.[2] Polyphenolic compounds (flavonoids, anthocyanidins, ellagic acids, and tannins) occur in *Eucalyptus* species,[3] as well as unusual *C*-methyl flavones.[4]

Flavonoids are a class of more than 8000 phenylbenzopyrones[5] that occur in many edible plants such as fruit and vegetables. These polyphenolic compounds display a remarkable spectrum of biochemical activities including some that may possibly influence processes that are dysregulated during cancer development.[6]

Apoptosis, or programmed cell death, is a form of cell suicide central to the development and homeostasis of multicellular organisms. Many death signals converge into mitochondria.[7] Previous studies have demonstrated that mitochondria transduce proapoptotic signals by release of cytochrome *c* into the cytoplasm.[8] Cytochrome *c* associates with cytoplasmic Apaf-1 and thereby activates procaspase-9. In turn, caspase-9 cleaves to and activates caspase-3. A central role for caspase-3 in cell death is supported by involvement of this executioner protease in the apoptotic response to diverse stimuli. Although cytochrome *c* release and activation of caspase-9 represent one pathway for caspase-3 cleavage, other studies have shown that caspase-8 can activate caspase-3 directly.[7]

This paper reports the isolation and structural elucidation of a new flavonoid (**1**) and three known compounds, 6,8-di-*C*-methylkaempferol 3-methyl ether (**2**),[9] oleanolic acid,[10] and 2α,3*β*-dihydroxyurs-12-en-28-oic acid,[11] from the

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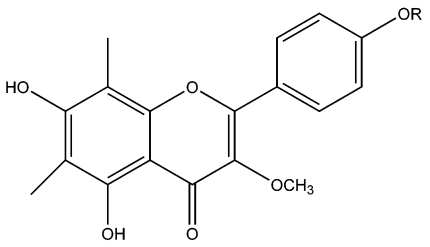
Table 1. ^{1}H and ^{13}C NMR Data of **1** (DMSO-d_6)

position	δ_C[a]	δ_H[b]
2	155.4	
3	138.6	
4	179.0	
5	156.8	
6	105.6	
7	157.9	
8	100.7	
9	152.2	
10	105.5	
1′	123.3	
2′, 6′	130.0	8.11, d (8.5)
3′, 5′	114.1	7.03, d (8.5)
4′	161.6	
CH_3-6	7.2	2.18 s
CH_3-8	7.7	2.32, s
OCH_3-3	60.1	3.85, s
OCH_3-4′	55.4	3.89, s

[a] The number of protons directly attached to each carbon was verified by DEPT and HSQC experiments. [b] Multiplicities are in parentheses.

aerial parts of *Eucalyptus occidentalis* from Algeria. The cytotoxicity of flavonoids **1** and **2** on human promyelocytic leukemia HL-60 cells was also studied, and these compounds proved to suppress survival and proliferation by triggering typical apoptosis mediated by caspase-8/caspase-3 activation and cytochrome *c* release.



1 R = CH_3
2 R = H

Results and Discussion

The leaves of *E. occidentalis* were extracted with aqueous ethanol, followed by extraction with chloroform, and this extract was chromatographed on silica gel followed by gel filtration on Sephadex LH-20 and preparative TLC, yielding a new flavonoid, **1**, along with three known compounds.

The EIMS of the newly isolated flavonoid **1** showed a molecular ion peak at *m/z* 342, which, taken in conjunction with the ^{13}C NMR spectroscopic analysis, indicated the molecular formula $C_{19}H_{18}O_6$. The ^{1}H and ^{13}C NMR data (Table 1) for **1** are very similar to those of 6,8-di-*C*-methylkaempferol 3-methyl ether (**2**),[9] the only difference being a methoxy group (δ_H 3.85, 3H) instead of a hydroxy group at C-4′. The nature and identity of this 6,8-di-*C*-methylflavonoid was deduced from the NMR data, which together with published data[4,9] identified the parent moiety of **1** as di-*C*-methylkaempferol. The ^{13}C NMR data of **1** revealed the presence of *C*-methyls at δ_C 7.2 and 7.7, and the placement of the former at C-6 and the latter at C-8 was based on HMBC. The rest of the structure was assigned and confirmed using HMBC and ROESY data (Figure 1). Compound **1** was thus determined to be a 6,8-di-*C*-methylkaempferol 3,4′-dimethyl ether. The postulated structure was further validated by single-crystal X-ray measurements (Figure 2).

Flavonoids **1** and **2** were found to inhibit the proliferation of human HL-60 myeloid leukemia cells in a dose-dependent manner as determined by the MTT assay (Figure 3). Flavonoid **1** (IC_{50} 1.7 ± 0.1 μM) was more potent than **2** (IC_{50} 7.4 ± 2.3 μM).

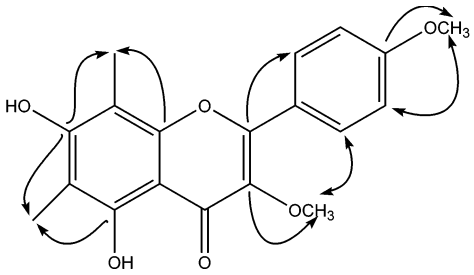


Figure 1. Correlations of **1**. Double-ended arrows indicate NOESY, and single arrows indicate HMBC correlations.

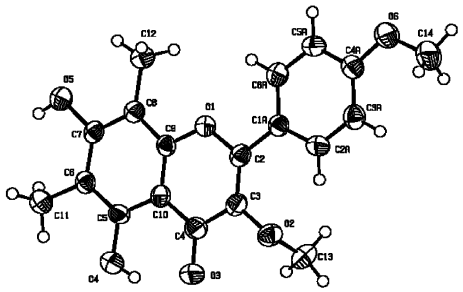


Figure 2. ORTEP diagram of **1**.

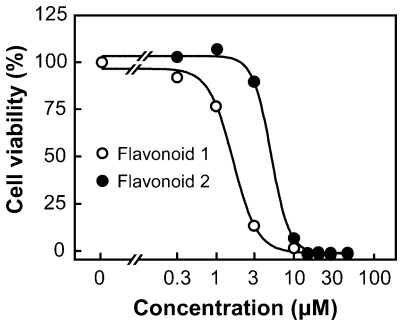


Figure 3. Effect of flavonoids **1** and **2** on human HL-60 cell viability. Cells were cultured in the presence of the indicated doses of flavonoids for 72 h, and thereafter cell viability was determined by the MTT assay as described in the Experimental Section. The results of a representative experiment are shown. Each point represents the average of triplicate determinations.

To determine whether this cytotoxic effect was due to apoptosis, human HL-60 myeloid leukemia cells were treated with these compounds and the typical appearance of this kind of cell death (i.e., shrinkage and apoptotic bodies as well as fragmented apoptotic nuclei) was examined by fluorescence microscopy after DNA staining with Hoechst 33258. As shown in Figure 4A, flavonoids **1** and **2** (30 μM, 12 h) induced morphological changes typical of apoptotic cells. These compounds (10−30 μM, 12 h) increased the percentage of apoptotic cells in a dose-dependent manner (Figure 4B), again determined by fluorescence microscopy. At the higher concentrations (30 μM), the percentages of apoptotic cells were 47.8 ± 4.2% and 26.6 ± 1.1% for **1** and **2**, respectively, and were greater than controls (5.5 ± 0.8%). The percentage of apoptotic cells in etoposide-treated cells, used as a positive control, was 65 ± 1% (Figure 4B).

We also wanted to see if these flavonoids would induce DNA fragmentation, considered the end point of the apoptotic pathway, and fragments formed by intranucleosomal hydrolysis of chromatin were observed after 12 h of treatment with 10 μM of **1** or **2** (Figure 4C).

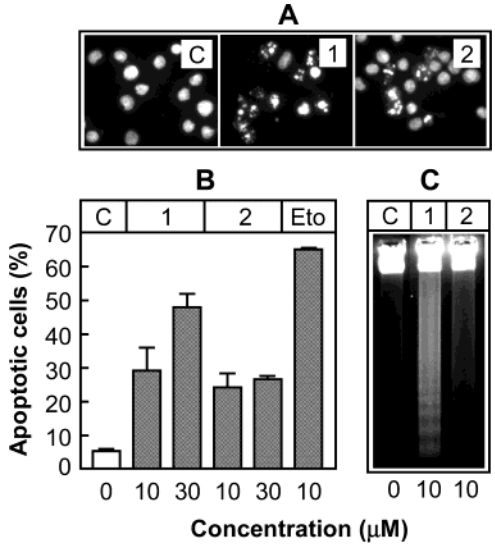


Figure 4. Induction of apoptosis in HL-60 cells by flavonoids **1** and **2**. (A) Cells were cultured in the absence (C, control) or presence of 30 μM of the indicated flavonoid for 12 h, then they were stained with Hoechst 33258 and nuclei were visualized using fluorescence microscopy. (B) Cells were incubated with the indicated doses of flavonoids **1** and **2** (or 10 μM etoposide as a positive control) for 12 h, and the percentages of apoptotic cells were determined by quantitative fluorescence microscopy. The results of a representative experiment are shown, and each point represents the average ± SE of triplicate determinations. (C) Cells were treated with 10 μM flavonoids **1** and **2** for 24 h, and DNA fragmentation was monitored by electrophoresis in 2% agarose gels.

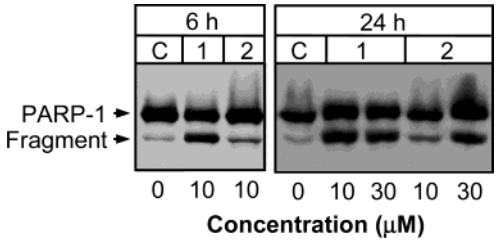


Figure 5. Western blot analysis of poly(ADP-ribose)polymerase-1 (PARP-1) cleavage in HL-60 cells. Cells were incubated in the absence (C, control) or presence of the indicated concentrations of flavonoids **1** or **2** for 6 and 24 h. Total cell lysates were analyzed by immunoblotting with anti-PARP-1 antibody.

These flavonoids also induced poly(ADP-ribose) polymerase-1 (PARP-1) cleavage, a hallmark of apoptosis indicating caspase activation. PARP-1 catalyzes the transfer of the ADP ribose moiety from its substrate, NAD^+, to a limited number of protein acceptors involved in chromatin architecture or in DNA metabolism. The cleavage of PARP-1 inactivates the enzyme, thereby making DNA repair impossible. Two bands corresponding to the remaining intact PARP-1 protein (116 kDa) and the typical apoptotic 85 kDa fragment were to be expected (Figure 5), and this effect was observed for flavonoid **1** (10 μM) after 6 h of treatment. A longer incubation time was needed for PARP-1 cleavage by flavonoid **2** (Figure 5).

To define which caspases are involved during apoptosis induced by flavonoid **1**, the enzymatic activity of HL-60 cell lysates on tetrapeptide substrates DEVD-*p*NA (for caspase-3) and IETD-*p*NA (for caspase-8) was analyzed after 12 h exposure to 1−30 μM of this compound. The relative hydrolytic activity toward the respective substrates increased significantly depending on the flavonoid concentration (Figure 6). Maximal caspase-3 activity was obtained with 30 μM of the tested compound (1.9-fold compared to untreated cells; Figure 6A), while maximal level of caspase-8

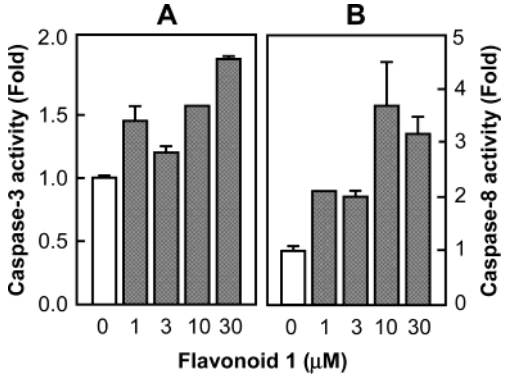


Figure 6. Activation of caspase-3 and caspase-8 in response to flavonoid **1**. HL-60 cells were treated with the indicated concentrations of flavonoid and harvested at 12 h. Total cell lysates were assayed for caspase-3 (A) or caspase-8 (B) activity. The results from a representative experiment performed in duplicate are shown and expressed as *x*-fold increases of caspase activity compared with control.

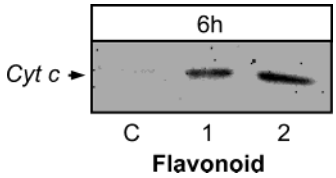


Figure 7. Flavonoids **1** and **2** induce release of cytochrome *c*. HL-60 cells were treated with 10 μM flavonoids and harvested at 6 h. Cytosolic lysates were analyzed by immunoblotting with an anti-cytochrome *c* (Cyt *c*, cytochrome *c*).

activity was observed using only 10 μM of product (3.7-fold compared to control; Figure 6B).

For a better understanding of the mechanisms underlying flavonoid-induced apoptosis in human HL-60 myeloid leukemia cells, the effect of both the flavonoids (**1** and **2**) on cytochrome *c* translocation from the mitochondria into the cytosol was studied. The representative Western blot analysis showed that the monoclonal antibody for cytochrome *c* detected a single band of the expected size, 15 kDa (Figure 7). Cytochrome *c* release was observed in the presence of doses as low as 10 μM of either compound.

In conclusion, these kaempferol derivatives were shown to induce apoptosis via caspase activation. Although more research must be carried out of the detailed pathway of programmed cell death used by these compounds, the kaempferol derivative **1** is a potentially interesting compound for development as an antitumor agent.

Experimental Section

General Experimental Procedures. Melting points were determined on a Büchi B-540 apparatus and are uncorrected. UV spectra were recorded using a JASCO model V-560 spectrophotometer. IR spectra were recorded using a Bruker model IFS-55 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker model AMX-400 spectrometer with standard pulse sequences operating at 400 MHz in ^{1}H and 100 MHz in ^{13}C NMR. $CDCl_3$ and DMSO-d_6 were used as solvents. EIMS and HREIMS were taken on a Micromass model Autospec (70 eV) spectrometer. Column chromatography (CC) was carried out on silica gel 60 (Merck 230−400 mesh), and preparative TLC on silica gel 60 $PF_{254+366}$ plates (20 × 20 cm, 1 mm thickness) and Sephadex LH-20 (Aldrich).

Plant Material. Leaves of *Eucalyptus occidentalis* were collected from the arboretum of Bekira, Constantine (Eastern Algeria), in November 1998. The plant was identified by a eucalypt botanist. A voucher specimen has been deposited in the Department of Forestry, University of Constantine.

Extraction and Isolation. Dried leaves (1.8 kg) were extracted with (EtOH−H_2O, 8:2) (24 h) at room temperature

and filtered. The extract was concentrated under reduced pressure. The residue was dissolved in water (300 mL) and the solution kept in a refrigerator overnight. After filtering to remove the precipitate, the solution was extracted with $CHCl_3$ to give 4 g of extract. This extract was chromatographed on a silica gel (900 g) column packed into CH_2Cl_2 eluted with a gradient of CH_2Cl_2−EtOAc (95:5 → 90:10 → 75:25 → 50:50, 300 mL each) to give four main fractions. Fraction 1 (30 mg) was subjected to gel filtration on Sephadex LH-20 using *n*-hexane−CH_2Cl_2−MeOH (1:1:1) followed by silica gel CC using *n*-hexane−EtOAc (3:1) to afford compound **1** (7 mg). Fraction 2 (100 mg) was further column chromatographed on Sephadex LH-20 using *n*-hexane−CH_2Cl_2−MeOH (1:1:1) followed by silica gel CC using *n*-hexane−EtOAc (3:2) to yield **2** (8.7 mg). Fraction 3 (100 mg) was eluted with *n*-hexane−EtOAc (7:3) followed by silica gel TLC CH_2Cl_2−MeOH (98:2) to afford oleanolic acid (3.6 mg). Fraction 4 (89 mg) was column chromatographed on silica gel CH_2Cl_2−MeOH (94:6) (16 mg), and then silica gel TLC with CH_2Cl_2−CH_3COCH_3 (7:3) yielded 2α,3*β*-dihydroxyurs-12-en-28-oic acid (2.1 mg).

6,8-Di-*C*-methylkaempferol 3,4′-dimethyl ether (1): yellow needles (MeOH) mp 193−194 °C; UV (EtOH) λ_{max} log (ϵ) 324 nm (4.64), 277 (4.82), 271 (4.83), 261 (4.94), 255 (5.0), 250 (4.98) nm; IR (film) ν_{max} 3344 (OH), 2927 (unsaturated carbon), 1649 (C═C−C═O), 1560, 1482, 1360, 1261, 1172, 1033, 802 cm^{-1}; ^1H and ^{13}C NMR spectra, see Table 1; EIMS (70 eV) *m/z* 342 $[M]^+$ (100), 323 (24), 313 (10), 299 (42), 157 (12), 135 (10); HREIMS *m/z* 342.1093 (calcd for $C_{19}H_{18}O_6$, 342.1103).

X-ray Structure Determination of 1. X-ray data were collected at 293 K with use of a Nonius Kappa CCD single-crystal diffractometer, using graphite-monochromated Mo Kα radiation (λ = 0.71070 Å) to θ max of 27.8. The structure was solved by direct methods using SIR97.[11] Refinement was performed with SHELXL-97[13] using full-matrix least squares with anisotropic thermal parameters for all non-H atoms. The hydrogen atoms were placed and refined at idealized positions. The final refinement converged at R_1 = 5.48% and with a goodness of fit of 1.062 for 1593 reflections with $F_o > 4\sigma F_o$ and wR_2 = 12.98 for the 2135 unique measurements. The largest peak on the final difference map was 0.21 e/Å^3.

Crystal Data for 1. $C_{19}H_{18}O_6$, mol wt = 342.3, orthorhombic, space group $P2_12_12_1$ (No. 19), a = 4.054(1) Å, b = 16.202(6) Å, c = 23.979(9) Å, V = 1575.1(1) Å^3, Z = 4, D_c = 1.44 g cm^{-3}, $F(000)$ = 720, μ(Mo Kα) = 0.11 mm^{-1}. A single crystal approximately 0.35 × 0.25 × 0.20 mm was used for all X-ray measurements. Data reduction and cell refinement were carried out with the programs COLLECT[14] and DENZO.[15] The crystallographic data of **1**, including atomic coordinates, are on file at the Cambridge Crystallographic Data Centre (deposit number CCDC 230055). Copies of the data can be obtained free of charge upon application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44-(0)1223-306033 or e-mail: deposit@ccdc.cam.ac.uk].

Cell Culture. HL-60 cells were cultured in suspension in RPMI-1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were maintained at a density of $<1 \times 10^6$ cells/mL. Stock solutions of 10 mM flavonoids were made in DMSO, and aliquots were frozen at −20 °C. Further dilutions were made in culture medium immediately prior to use. In all experiments, the final concentration of DMSO did not exceed 0.5% (v/v), a concentration that was nontoxic to the cells. Cell viability was determined using the trypan blue exclusion test. To ensure an exponential growth, cells were resuspended in fresh medium 24 h before each treatment.

Assay of Cytotoxicity. Cytotoxic assays were performed using an MTT assay.[16] Cells (1×10^4/well) were continuously exposed to different concentrations of the compounds in 96-well plates for 72 h at 37 °C. Flavonoids were applied in DMSO. Controls were always treated with the same amount of DMSO as used in the corresponding experiments. Surviving cells were detected on the basis of their ability to metabolize 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into formazan crystals. Optical density at 570 nm was used as a measure of cell viability. Cell survival was calculated as the fraction of cells alive relative to control for each point as cell survival (%) = mean absorbance in treated wells/mean absorbance in control wells × 100. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically for each experiment using the curve-fitting routine of the computer software Prism 2.0 (GraphPad) and the equation established by DeLean.[17]

Quantitative Fluorescence Microscopy. To detect apoptotic cells, cells were fixed in 3% paraformaldehyde for 10 min at room temperature and then stained with 10 μg/mL bisbenzimide trihydrochloride (Hoechst 33258) for 30 min at 37 °C before fluorescence microscopic analysis. Apoptotic cells were identified by their fragmented chromatin. Stained nuclei with condensed chromatin (supercondensed chromatin at the nuclear periphery) or nuclei that were fragmented into multiple smaller dense bodies were considered to be apoptotic. Nuclei with uncondensed and dispersed chromatin were considered to be nonapoptotic. A minimum of 500 cells was counted for each sample, and each experiment was carried out in triplicate.

Analysis of DNA Fragmentation. DNA fragmentation followed earlier protocols with slight modifications.[18] Briefly, cells (1×10^6) were washed with PBS and incubated in 20 μL of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, and 1 μg/μL RNase A (Sigma) at 37 °C for 1 h. Then, 10 μg/μL proteinase K (Sigma) (2 μL) was added, and the mixture was incubated at 50 °C for 2 h more. DNA was extracted with 100 μL of phenol−chloroform−isoamyl alcohol (24:24:1) and mixed with 5 μL of loading solution (10 mM EDTA, pH 8.0, containing 1% (w/v) low-melting-point agarose, 0.25% bromophenol blue, and 40% sucrose). Samples were separated by electrophoresis in 2% agarose gels and visualized by UV illumination after ethidium bromide staining.

Western Blot Analysis of PARP-1 Hydrolysis. Induction of apoptosis was also tested by proteolytic cleavage of PARP-1.[19] Exponentially growing HL-60 cells ($\sim 7 \times 10^5$) were treated with flavonoids at the indicated concentrations for different periods of time at 37 °C. Cell lysates were prepared in a buffer containing 20 mM Hepes (pH 7.4), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, aprotinin, and pepstatin A. Insoluble materials were removed by centrifugation at 10 000*g* for 10 min. The proteins extracted (50 μg/well) were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels and were electrophoretically transferred onto Immobilon-P membranes. The loading and transfer of equal amounts of protein were confirmed by staining the membrane with Ponceau S. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h, followed by incubation with anti-PARP-1 monoclonal antibody (BD PharMingen; 1:1000 dilution in TBST supplemented with 3% nonfat milk) overnight. After washing and incubation with anti-mouse antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech), the antigen−antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) using the manufacturer's protocol. The appearance of an 85 kDa cleavage product was used as a measure of apoptosis.

Isolation of the Cytosolic Fraction and Immunoblot Analyses of Cytochrome *c* Release. Cells were washed twice with PBS and then resuspended in ice-cold buffer (20 mM Hepes, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL leupeptin, aprotinin, and pepstatin A containing 250 mM sucrose). After 30 min incubation on ice, cells were lysed by being pushed several times through a 22-gauge needle and the lysate spun down at 1000*g* for 5 min at 4 °C. The supernatant fraction was centrifuged at 105 000*g* for 45 min at 4 °C, and the resulting supernatant was used as the soluble cytosolic fraction. Cytosolic proteins (50 μg) were resolved on an SDS/15% polyacrylamide gel and electrotransferred onto a PVDF membrane. The membrane

was probed with monoclonal anti-cytochrome *c* (BD Transduction Laboratories) (1:250 dilution) antibody and then with secondary antibody conjugated to horseradish peroxidase. Protein bands were detected by chemiluminescence (ECL, Amersham Pharmacia Biotech) as described above.

Assays of Caspase Activity. After treatment, cells were harvested by centrifugation at 1000*g* for 5 min at 4 °C and washed with PBS, and the cell pellets were kept on ice. The cells were resuspended in cell lysis buffer (50 mM Hepes, pH 7.4, 1 mM DTT, 0.1 mM EDTA, 0.1% Chaps) and held on ice for 5 min. After centrifugation for 10 min at 17 000*g* at 4 °C the supernatants were analyzed for protein concentration by the Bradford dye-binding assay and stored at −20 °C until used to study caspase colorimetric enzymatic activity. Equal amounts of protein (∼20 μg) from different treatments were used, and the assays were set up on ice. The net increase of absorbance at 405 nm after incubation at 37 °C was indicative of enzyme activity. Specific labeled substrates for caspase-3 and -8 activities were DEVD-*p*NA and IETD-*p*NA, respectively.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) Brooker, M. I. H.; Kleining, D. A.; Kleinig, D. A. *Field Guide to Eucalypts: Northern Australia*; Butterworth-Heinemann: Camberra, 2000. (b) Penford, A. R.; Willis, J. L. The *Eucalyptus*−*Botany, Cultivation, Chemistry, and Utilization*; Interscience Publishers: New York, 1961; p 551.
(2) (a) Lassak, E. V.; McCarthy, T. *Australian Medicinal Plants*; Methuen: Australia, 1983. (b) Barr, A. *Traditional Aboriginal Medicines in the Northern Territory of Australia*; Conservation Commission of the Northern Territory of Australia: Darwin, Australia, 1993.
(3) Hill, W. E. *Phytochemistry* **1967**, *6*, 845−856.
(4) Harborne, J. B.; Williams, C. A.; Greenham, E. J. *Phytochemistry* **1994**, *35*, 1475−1480.
(5) Cotelle, N. *Curr. Top. Med. Chem.* **2001**, *1*, 569−590.
(6) Middleton, E.; Kandaswami, C.; Theoharides, T. C. *Pharmacol Rev.* **2000**, *52*, 673−751.
(7) Van-Gurp, M.; Festjens, N.; van-Loo, G.; Saelens, X.; Vandenabeele, P. *Biochem. Biophys. Res. Commun.* **2003**, *304*, 487−497.
(8) Cain, K.; Bratton, S. B.; Cohen, G. M. *Biochimie* **2002**, *84*, 203−214.
(9) Ibewuike, J. C.; Ogundaini, A. O.; Ogungbamila, F. O.; Martin, M. T.; Gallard, J. F.; Bohlin, L. *Phytochemistry* **1996**, *43*, 687−690.
(10) Siddiqui, B. S.; Sultana, I.; Begum, S. *Phytochemistry* **2000**, *54*, 861−865.
(11) Khare, M.; Srivastava, S. K.; Singh, A. K. *Indian J. Chem. B* **2002**, *41*, 440−445.
(12) Altomare, A.; Burla, M. C.; Camalli, M.; Cascarano, G.; Giacovazzo, C.; Guagliardi. A. R. *J. Appl. Crystallogr.* **1999**, *32*, 115−119.
(13) Sheldrick, G. M. *SHELXL-97, Program for the Refinement of Crystal Structures*; University of Göttingen: Göttingen, Germany, 1997.
(14) *Nonius Kappa CCD Server Software*; Nonius, B.V.: Delft, The Netherlands, 1998.
(15) Otwinowsky, Z.; Minor, W. In *Methods in Enzymology, Macromolecular Crystallography Part A*; Carter, C. E., Sweet, R. M., Eds.; Academic Press: University of Texas, Southwestern Medical Centre at Dallas, 1997; Vol. 276, pp 307−326.
(16) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55−63.
(17) DeLean, A.; Munson, P. J.; Rodbard, D. *Am. J. Phys.* **1978**, *235*, E97−E102.
(18) Ito, Y.; Pandey, P.; Place, A.; Sporn, M. B.; Gribble, G. W.; Honda, T. *Cell Growth Differ.* **2000**, *11*, 261−267.
(19) Tewari, M.; Quan, L. T.; O'Rourke, K.; Desnoyers, S.; Zeng, Z.; Beidler, D. R. *Cell* **1995**, *81*, 801−809.

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