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Triterpenoid Constituents from the Roots of *Paeonia rockii* ssp. *rockii*

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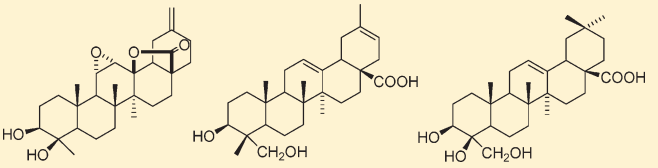
Supporting Information

ABSTRACT: An investigation of a chloroform-soluble extract from the roots of *Paeonia rockii* ssp. *rockii* yielded three new noroleanane triterpenoids (**1**−**3**) together with 19 known compounds. Their structures were established by analysis of the spectroscopic data. The effects of this chloroform-soluble extract and its major constituents on cell proliferation and apoptosis of a panel of human cancer cell lines (melanoma M-14, colon cancer HT-29, breast cancer MCF-7) were evaluated by the MTT bioassay and propidium iodide staining, respectively, in comparison with normal human embryonic kidney cells (HEK-293). Two of the triterpenoids, betulinic acid (**4**) and oleanolic acid (**5**), and the crude extract were cytotoxic and induced apoptosis selectively in the M-14 melanoma cell line. This effect was reversed by the caspase-inhibitor z-VAD-fmk, suggesting that such action is mediated by caspase-3 activation.



Paeonia rockii (Haw et Lauener) Hong et Li subsp. *rockii* is one of the eight species recognized in the section *Moutan* of the genus *Paeonia*, and it is distributed mainly in the northwest of mainland China. Tree peonies, commonly known as the "King of Flowers", are endemic to the People's Republic of China and are cultivated in the temperate regions of the world for their medicinal, ornamental, and economic value.[1] The roots of various *Paeonia* species (e.g., *P. suffruticosa*, *P. lactiflora*, *P. albiflora*, *P. ovata*) are important crude drugs in China. Traditional Chinese medicine utilizes these species as analgesic, sedative, anti-inflammatory, and antimicrobial agents and to treat cardiovascular and female genital diseases.[2] Recent reviews on the genus have summarized the main constituents (mono- and triterpenoids, polyphenols such as tannins) and biological activities (antioxidant, anti-inflammatory, cytotoxic, cardioprotective) during the past six decades.[3,4] Several biological effects may be attributed to the characteristic chemotaxonomic markers, paeoniflorin and its derivatives, which are monoterpenes with a pinane skeleton.[2] Whereas investigations on the *Paeonia* genus are extensive,[5−7] up to now phytochemical and pharmacological information on *P. rockii* ssp. *rockii* is limited to reports on anthocyanins and flavonoids in the flowers[8] as well as on nutritional components (sugars, lipids, proteins, mineral elements, and amino acids) in the seeds.[9] Furthermore, extracts and oils from the fruits and root bark of *P. rockii* ssp. *rockii* are components of moisturizing cosmetic preparations and health-care capsules.[10,11]

This paper reports on the composition of a crude chloroform-soluble extract from the roots of *P. rockii* ssp. *rockii* and its antiproliferative activity. Three new nortritepenoids (**1**−**3**) have been isolated, and their structures elucidated using spectroscopic methods including 1D- and 2D-NMR experiments as well as HRESIMS analysis. The effects of this plant crude extract and its major constituents on cell proliferation and apoptosis of a panel of human cancer cell lines (melanoma M-14, colon cancer HT-29, breast cancer MCF-7) were evaluated by the MTT bioassay and propidium iodide staining, respectively, in comparison with normal human embryonic kidney cells (HEK-293). Finally, involvement of caspase-3 in the apoptotic process was established by Western blotting analysis and the use of the caspase inhibitor z-VAD-fmk in the M-14 melanoma cell line.

RESULTS AND DISCUSSION

Air-dried roots of *P. rockii* ssp. *rockii* were defatted with *n*-hexane and extracted with chloroform, which was evaporated to give a chloroform-soluble extract. A portion of this extract was subjected to purification by silica gel column chromatography followed by RP-HPLC to yield three new compounds (**1**−**3**) together with 19 known compounds, including phenolic and triterpenoid derivatives as well as monoterpenes, which are characteristic of the genus.

The structure elucidation of compounds **1**−**3** proceeded as follows. The HRESIMS of **1** showed a major ion peak at m/z 479.2800 $[M + Na]^+$ consistent with the molecular formula $C_{28}H_{40}O_5$ (calcd for $C_{28}H_{40}O_5Na$, 479.2773). This suggested **1** is a dinortriterpenoid derivative, as confirmed by the presence of 28 carbon signals in the ^{13}C NMR spectrum (Table 1). The ^1H NMR spectrum of compound **1** showed signals corresponding to four tertiary methyl groups at δ_H 1.05, 1.07 (×2), and 1.19, as well as a typical H-3ax signal (δ_H 3.38, 1H, dd, J = 4.5, 11.1 Hz) of a pentacyclic triterpene bearing a β-OH group at the C-3 position.[12] The $^1H-^1H$ DQF-COSY and HSQC spectra (see Table 1 and Supporting Infirmation) suggested that compound **1** is a 24,30-dinor-triterpenoid, analogous to paeonenoide A.[13] In particular, the spectra showed signals for an epoxide ring (δ_H 3.05, 1H, d, J = 3.7 Hz, and 3.12, 1H, dd, J = 2.0, 3.7 Hz) in an α position at C-11 and C-12.[12] The multiplicity of H-12 (d, J = 3.7 Hz) suggested

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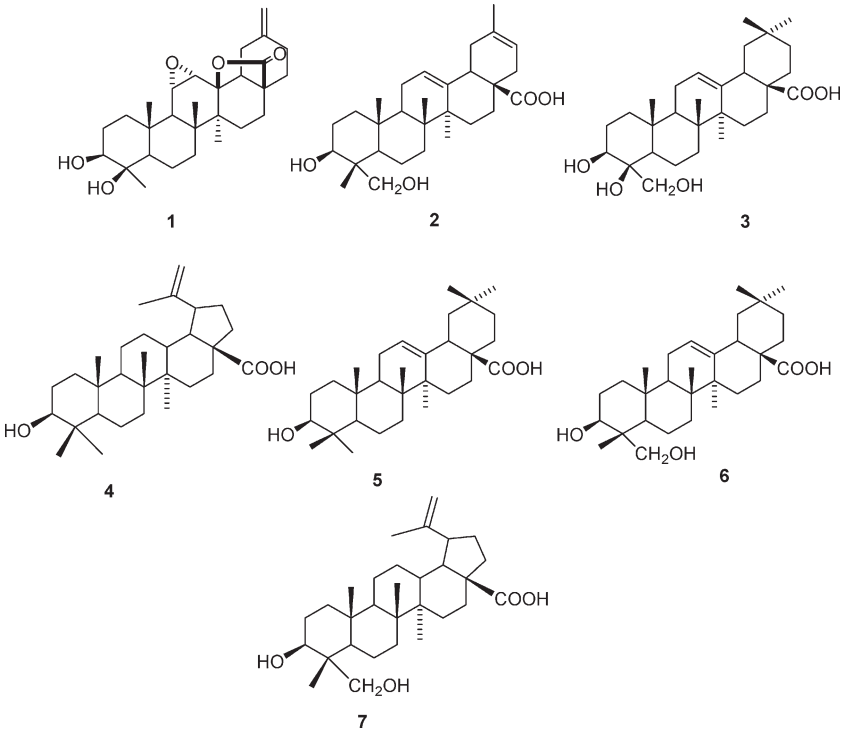
Chart 1



that it is adjacent to an oxygenated quaternary carbon (C-13, δ_C 88.4). Hence, a lactone ring was presumed to occur between C-13 and C-17, and this conclusion was supported by the chemical shifts of quaternary carbons at δ_C 88.4 (C-13), 45.3 (C-17), and 180.9 (C-28). In addition, an exomethylene group (δ_H 4.76, 2H, br s; δ_C 147.0, quaternary C, and 109.4, olefinic CH_2) could be located at the C-20(29) position on the basis of the long-range C−H correlations observed in the HMBC spectrum between the proton signal at δ_H 4.76 and C-19 (δ_C 35.0) and C-21 (δ_C 30.6). With respect to paeonenoide A,[13] compound **1** seems to possess the typical $-CH_2OH$ function at C-23 substituted by a methyl group (δ_H 1.07, δ_C 15.9) linked to a C-4 carbinol unit (δ_C 76.1). The HMBC spectrum confirmed the proposed structure, showing long-range correlations between this methyl signal and the adjacent C-4, C-5 (δ_C 55.9), and C-3 (δ_C 79.7) signals. The relative configuration as Me-23α was determined by a NOESY experiment (see Supporting Information) showing main NOE interactions between H-3α/Me-23α and H-5α. Consequently, compound **1** was determined structurally as 11α,12α-epoxy-3β,4β,13β-trihydroxy-24,30-dinor-olean-20(29)-en-28,13β-olide, or 23-deoxypaeonenoide A.

Compound **2** gave the molecular formula $C_{29}H_{44}O_4$, as indicated by the HRESIMS molecular ion at m/z 455.3186 $[M - H]^-$ (calcd for $C_{29}H_{43}O_4$, 455.3161), suggesting it is also a nortriterpenoid derivative. This deduction was confirmed by the ^{1}H NMR spectrum, showing signals for five tertiary methyl groups (δ_H 0.74, 0.91, 1.00, 1.26, and 1.61), and by the ^{13}C NMR spectrum, exhibiting 29 carbon signals (Table 1). Analysis of the ^{1}H−^{1}H DQF-COSY and HSQC spectra (see the Supporting Information) suggested **2** to be a norhederagenin derivative, in which the A/B/C/D ring signals were in good agreement with those reported for hederagenin.[14] In particular, this conclusion was supported from the HMBC spectrum, which showed long-range correlations between the typical hydroxymethyl group (δ_H 3.31, 1H, m, and 3.55, 1H, m) at C-23 of hederagenin and C-4 (δ_C 42.9), C-3 (δ_C 74.1), C-5 (δ_C 48.1), and C-24 (δ_C 12.5). Moreover, the NOESY spectrum showed that the $-CH_2OH$ at C-23 is in an α position on the basis of the NOE interactions observed between H-3α/$-CH_2OH$ and H-5α. In relation to the ring E, the ^{1}H NMR spectrum showed a methyl singlet signal at comparatively downfield position (δ_H 1.61), suggesting that it could be attached to an olefinic carbon,[15] and a signal for an additional olefinic proton (δ_H 5.27), which was positioned at C-20(21) on the basis of the long-range correlations observed in the HMBC spectrum. In fact, the downfield shifted methyl signal (δ_H 1.61) correlated with the methylene signal at δ_C 38.1 (C-19), as well as with two olefinic carbon signals at δ_C 133.0 (C-20) and 118.3 (C-21). Thus, compound **2** was identified as 3β,23-dihydroxy-30-nor-olean-12,20-dien-28-oic acid.

The HRESIMS of **3** (m/z 497.3300 $[M + Na]^+$, calcd for $C_{29}H_{46}O_5Na$, 497.3243) supported the molecular formula $C_{29}H_{46}O_5$, suggesting this compound to be a further nortriterpenoid derivative. The ^{1}H NMR spectrum of **3** showed signals corresponding to five tertiary methyl groups at δ_H 0.90 (×2), 0.95, 1.11, and 1.16 and for a H-3ax (δ_H 3.59, dd, J = 4.5, 11.2 Hz) and a $-CH_2OH$ function (δ_H 3.42, 1H, d, J = 9.6 Hz and 3.61, 1H, d, J = 9.6 Hz). In turn, the ^{13}C NMR spectrum displayed an oxygenated quaternary carbon signal assigned to C-4 (δ_C 75.4). Overall scrutiny of the ^{1}H and ^{13}C NMR resonances (Table 1), ascertained by ^{1}H−^{1}H DQF-COSY, HSQC, and HMBC experiments, indicated that **3** is a norhederagenin, in which a methyl group in ring A is substituted by a quaternary hydroxy function. The HMBC correlations observed between $-CH_2OH$ at C-23 and C-3 (δ_C 71.5), C-5 (δ_C 48.0), and an oxygenated quaternary C-4 (δ_C 75.4) as well as a NOESY experiment indicated the placement of a β-OH group at C-4 instead of the typical Me-24 of

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Table 1. ^{13}C and ^{1}H NMR Spectroscopic Data of Compounds 1, 2, and 3 in CD_3OD[a]

	1		2		3	
position	δ_C	δ_H (J in Hz)[b]	δ_C	δ_H (J in Hz)[b]	δ_C	δ_H (J in Hz)[b]
1	38.5	1.21, 1.93, m	39.3	1.02, 1.65, m	38.9	0.97, 1.65, m
2	27.5	1.19, 1.61, m	26.7	1.70, 2.07, m	26.4	1.60, 1.79, m
3	79.7	3.38, dd (4.7, 12.0)	74.1	3.62, dd (4.5, 11.2)	71.5	3.59, dd (4.5, 11.2)
4	76.1		42.9		75.7	
5	55.9	1.05, m	48.1	1.18, m	48.0	1.16, m
6	17.2	1.54, 1.84, m	18.8	1.47, 1.61, m	18.3	1.35, 1.58, m
7	31.3	1.18, 1.40, m	27.5	1.28, 1.61, m	33.0	1.32, 1.58, m
8	42.1		42.0		40.4	
9	51.6	1.68, m	49.1	1.66, m	48.0	1.62, m
10	36.7		37.0		37.3	
11	53.1	3.12, dd (2.0, 3.7)	24.4	1.68, 1.93, m	23.9	1.62, 1.90, m
12	57.5	3.05, d (3.7)	122.5	5.30, t (3.5)	123.0	5.15, t (3.5)
13	88.4		145.6		145.8	
14	41.7		40.2		42.3	
15	27.8	1.60, 1.67, m	26.3	1.02, 1.18, m	28.5	1.04, 1.85, m
16	22.2	1.41, 2.40, m	28.7	1.02, 1.93, m	23.7	1.76, 2.10, m
17	45.3		44.8		49.2	
18	55.6	2.26, dd (3.0, 14.0)	43.4	2.89, dd (3.6, 13.7)	42.7	2.89,dd (3.6, 13.7)
19	35.0	2.52, 2.82, m	38.1	1.86, 2.26, m	47.4	1.11, 1.67, m
20	147.0		133.0		31.9	
21	30.6	1.32, 2.26, m	118.3	5.27, dd (1.5, 6.0)	34.9	1.18, 1.39, m
22	32.5	1.51, 1.86, m	37.6	1.82, dd (1.5, 12.0)	33.6	1.53, 1.74, m
				2.36, dd (6.0, 12.0)		
23	15.9	1.07, s	66.9	3.31, d (9.6)	63.3	3.42, d (9.6)
				3.55, d (9.6)		3.61, d (9.6)
24			12.5	0.74, s		
25	16.6	1.05, s	15.9	1.00, s	15.2	1.11, s
26	18.8	1.19, s	17.4	0.91, s	17.7	0.90, s
27	20.3	1.07, s	27.5	1.26, s	26.1	1.16, s
28	180.9		180.0		177.8	
29	109.4	4.76, br s	23.1	1.61, s	23.9	0.95, s
30					33.3	0.90, s

[a] Assignments confirmed by 2D COSY, HSQC, and HMBC experiments. [b] $^{1}H-^{1}H$ coupling constants (Hz) were measured from the COSY spectra.

hederagenin.[14] Thus, compound **3** was identified as 3β,4β,23-trihydroxy-24-nor-olean-12-en-28-oic acid.

Nineteen known compounds isolated from *P. rockii* ssp. *rockii* were identified by comparison of their NMR data with those from the literature, including six phenolic compounds, acetovanillone,[16] paeonol,[17] resacetophenone,[18] benzoic acid, and vanillic acid,[19] and a neolignan, lawsonicin.[20] Two monoterpenes, predominant bioactive constituents within the genus *Paeonia*, were confirmed as paeoniflorigenone[21] and benzoylpaeoniflorin,[17] and a terpenoid was confirmed as palbinone.[22] Moreover, six nor-triterpenoids with oleanane skeletons, 3β,4β,23-trihydroxy-24,30-dinor-olean-12,20(29)-dien-28-oic acid,[23] paeonenoides A and C,[13] akebonic acid,[13] 30-nor-hederagenin,[24] and 11α,12α-epoxy-3β,23-dihydroxy-30-nor-olean-20(29)-en-28,13β-olide,[25] were characterized along with two triterpenes of the lupane series, betulinic acid[16] (**4**) and 23-hydroxybetulinic acid (**7**),[26] and two triterpenes of the oleanane series, oleanolic acid[16] (**5**) and hederagenin[14] (**6**).

The concentrations in the chloroform-soluble extract of *P. rockii* ssp. *rockii* of the chemotaxonomic markers, benzoic acid and the monoterpenes paeoniflorigenone and benzoylpaeoniflorin, reported from a number of *Paeonia* species, were determined by a HPLC direct calibration method (see Experimental Section), as 10.4%, 15.1%, and 6.5% w/w of the extract, respectively. The total phenolic content of this crude extract, as determined by the Folin−Ciocalteau method and expressed as a benzoic acid equivalent, was 110.0 μg/mg. A significant concentration-dependent free-radical-scavenging activity, evaluated by a DPPH test, was shown by this extract (EC_{50} 57.3 μg/mL) and may be correlated to its polyphenol content.[3,4]

In recent years, the antiproliferative activities of some triterpenes of the oleanane or lupane series have been reviewed extensively.[27,28] Therefore, the antiproliferative activity of the crude chloroform extract of *P. rockii* ssp. *rockii* (1−100 μg/mL) was evaluated against a panel of human cancer cells (melanoma, M-14; colon cancer, HT-29; and breast cancer, MCF-7) using the MTT assay in comparison with a cell line derived from normal human tissue (human embryonic kidney, HEK-293). Results showed that the crude extract exhibited a significant ($p < 0.01$) concentration-dependent inhibitory effect selectively on M-14 cell growth (IC_{50} 96 μg/mL), whereas it showed a less

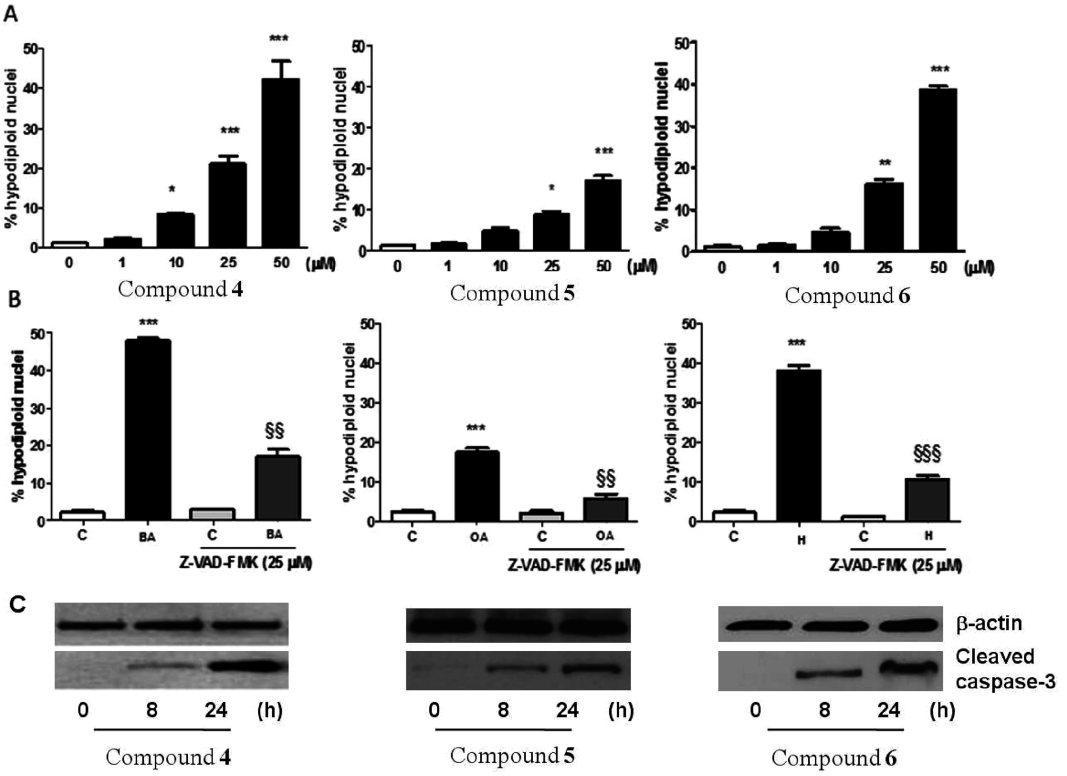

Figure 1. (A) Effects of compounds **4**−**6** on apoptosis of the M-14 melanoma cells. Analysis of percentage of cells in apoptosis was performed with propidium iodide staining. M-14 cells were incubated with different concentrations of compounds **4**, **5**, and **6** (1−50 μM) isolated from *P. rockii* ssp. *rockii*, for 24 h. Cells were then collected, and the percentage of hypodiploid nuclei was analyzed by flow cytometry ($^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$ vs control cells). (B) Effects of the caspase inhibitor z-VAD-fmk on compound **4**-, **5**-, or **6**-induced apoptosis in M-14 melanoma cells. z-VAD-fmk (25 μM) was administered 30 min before incubation with compounds (each 50 μM) for 24 h, and the percentage of apoptotic cell was evaluated by flow cytometry (means ± SEM of three experiments performed in triplicate; $^{***}p < 0.001$ vs control cells (C), $^{\S\S\S}p < 0.001$, $^{\S\S}p < 0.01$ vs **4**-, **5**-, or **6**-treated cells. Results are expressed as the means ± SEM of three experiments performed in triplicate. (C) Western blotting analysis of caspase-3 expression after incubation with compounds **4**−**6** at different times. Blots are representative of three different experiments.

potent effect (from 10% to 20%) on HT-29 and MCF-7 cell proliferation only at the highest concentration (100 μg/mL). No cytotoxicity was observed on the HEK-293 normal cell line. To localize the activity within the extract, a set of 13 major compounds isolated from this extract were tested by the MTT method on the most responsive cancer cells. The M-14 cell line was somewhat sensitive to some triterpenes such as **4**, **5**, **6**, and **7** (the IC_{50} values were 50, 82, 58, and 50 μM, respectively). On the contrary, other terpenoids (palbinone, paeonenoides A and C, and akebonic acid), polyphenols (benzoic acid and paeonol), and monoterpenes (paeoniflorigenone and benzoylpaeoniflorin) were not cytotoxic to the M-14 cells. The neolignan lawsonicin (IC_{50} 100 μM) was found to be more than 2 times less active than compound **4**.

The effects of triterpenes **4**−**7** have not been previously investigated on M-14, a very aggressive human melanoma cell line, also called UCLA-SO-M14, derived from a lesion metastatic to the buttock of a patient.[29] In fact, only a recent paper assessed the effect of oleanolic acid (**5**) on inducing apoptosis in a murine melanoma cell line (B16F-10).[30] As for betulinic acid (**4**), Pisha et al. published a key paper reporting its cytotoxic effect on different human melanoma cell lines (MEL 1−4);[31] moreover, Zuco et al. reported that Me665/2/60 and Me665/2/21 melanoma cells are sensitive to betulinic acid.[32] However, their mechanism of action is still subjected to extensive research.[27,28,30] Induction of apoptosis in cancer cells, determined by various methods such as cell shrinkage, DNA fragmentation, nuclear condensation, and caspase activation, has been recognized as a possible property of triterpenes as chemotherapeutic agents.[27,28,30] To verify this hypothesis, the percentage of apoptotic cancer cells, incubated with the crude chloroform-soluble extract (1−100 μg/mL) and cytotoxic triterpenes **4**−**7** (each 1−50 μM), for 24 h, was determined using propidium iodide staining by flow cytometry. The crude extract induced a dose-dependent increase of the percentage of hypodiploid M-14 cells, and this effect appeared significant ($p < 0.05$) up to 50 μg/mL. Interestingly, the results shown in Figure 1A indicate that compound **4** (betulinic acid) increased the percentage of the M-14 cells in apoptosis in a dose-dependent manner from 10 μM, and it was more potent than **5** (oleanolic acid) and **6** (hederagenin), which showed a significant ($p < 0.01$) effect up to 25 μM. As a matter of fact, no pro-apoptotic effect was evidenced for compound **7** (23-hydroxybetulinic acid; data not shown), although it was as cytotoxic as betulinic acid (IC_{50} was 50 μM for both lupane derivatives as determined by the MTT bioassay).

To determine whether the activation of caspase-3, a cysteine aspartate-specific protease, was involved in the induction of apoptosis, the M-14 cells were cultured in the presence and absence of a polycaspase inhibitor, z-VAD-fmk, 30 min before the addition of the crude plant extract (75 μg/mL) or **4**−**6** (50 μM). Pretreatment with z-VAD-fmk resulted in inhibition of both the crude extract- and compound-induced apoptosis (Figure 1B),

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indicating that their ability to induce cell death was dependent upon caspases. Meanwhile, the selective activation of caspase-3 was evaluated by Western blotting analysis. Results show that compounds **4**−**6** (Figure 1C) induced activation of caspase-3 after 8−24 h of treatment, confirming that the role of this protein was critical in their pro-apoptotic activity.

Although *P. rockii* ssp. *rockii* is used as a traditional medicine remedy, no study on its bioactivity and little published phytochemical data have been reported before. Polyphenolic compounds together with known and new triterpenoids are the main constituents of this species. A very promising finding of the present study was the ability of the crude chloroform extract and certain of its triterpenoid constituents to selectively inhibit the growth of human M-14 melanoma cells, inducing apoptosis mediated by caspase-3.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell in MeOH solution. For NMR experiments, a Bruker DRX-600 NMR spectrometer was used, operating at 599.2 MHz for ^{1}H and at 150.9 MHz for ^{13}C, and using the UXNMR software package. Chemical shifts are expressed as δ (parts per million) values, referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD; coupling constants, *J*, are in Hz. 1D- and 2D-NMR experiments were carried out using conventional pulse sequences.[33] ESIMS was performed on a Finnigan LC-Q Deca instrument (Thermoquest, San Jose, CA, USA), equipped with Xcalibur software. Exact masses (HRESIMS) were measured by a Q-TOF Premier (Waters) triple-quadrupole orthogonal time-of-flight (TOF) instrument having an electrospray ionization source. HPLC separations were performed with a Waters 590 series pumping system equipped with a Waters R401 refractive index detector, a μ-Bondapak C_{18} column (300 × 7.8 mm i.d.), and a U6K injector. Quantitative HPLC analysis was carried out on an Agilent 1100 series system equipped with a model G-1312 pump, a Rheodyne model G-1322A loop (20 μL), and a DAD G-1315 A detector. Peak areas were calculated with an Agilent integrator.

Plant Material. The roots of *P. rockii* ssp. *rockii* were purchased from Whitessence Srl, Viterbo (Italy), in October 2005. A voucher sample (PR101) was deposited at the Herbarium of the Department of Pharmaceutical and Biomedical Sciences, University of Salerno. The authentication of herbal material was supported as a HPLC trace of the crude plant extract (Supporting Information, Figure S1).

Extraction and Isolation. Powdered, dried roots of *P. rockii* ssp. *rockii* (2.73 kg) were defatted with *n*-hexane (17.17 g) and then extracted with chloroform and dried under vacuum, giving 12.8 g of dried extract. A portion (2.6 g) was chromatographed on a silica gel column (130 g) using a step gradient of $CHCl_3$ and MeOH (from 7:3 to 1:1). Fractions of 20 mL were collected, analyzed by TLC (silica 60 F_{254} gel-coated glass sheets with $CHCl_3$ or mixtures of $CHCl_3$−MeOH (95:5, 93:7, 9;1, 4:1), and combined to 10 major fractions (I−X) based on TLC patterns. Fractions II and III were purified using MeOH−H_2O (8:2) as mobile phase (flow rate 2.0 mL min^{-1}). Fraction II (309.3 mg) yielded compounds **4** (11.9 mg, t_R = 41.0 min) and **5** (49.3 mg, t_R = 46.0 min), while fraction III (134.6 mg) afforded compound **5** (24.9 mg, t_R = 46.0 min). Fraction V (106.9 mg), separated using MeOH−H_2O (6.5:3.5), consisted of compound **1** (1.6 mg, t_R = 21.0 min). Fraction VI (121.7 mg) was chromatographed using MeOH−H_2O (7:3), giving compounds **2** (1.7 mg, t_R = 26.0 min), **7** (2.4 mg, t_R = 32.0 min), and **6** (55.2 mg, t_R = 37.0 min). A portion of fraction IX (128.0 mg) was separated using MeOH−H_2O (7.5:2.5) to obtain compound **3** (1.7 mg, t_R = 19.0 min).

Compound ***1****:* white powder; $[\alpha]^{21}_D$ +50.8 (*c* 0.13, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150.9 MHz), see Table 1; HRESIMS *m/z* 479.2800 $[M + Na]^+$ (calcd for $C_{28}H_{40}O_5Na$, 479.2773).

Compound ***2****:* white powder; $[\alpha]^{21}_D$ +11.4 (*c* 0.14, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150.9 MHz), see Table 1; HRESIMS *m/z* 455.3186 $[M - H]^-$ (calcd for $C_{29}H_{43}O_4$, 455.3161).

Compound ***3****:* white powder; $[\alpha]^{21}_D$ +16.6 (*c* 0.14, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150.9 MHz), see Table 1; HRESIMS *m/z* 497.3300 $[M + Na]^+$ (calcd for $C_{29}H_{46}O_5Na$, 497.3243).

Compounds ***4***−***7***. ^{1}H and ^{13}C NMR data were consistent with those previously reported; ESIMS $[M - H]^+$ *m/z* 457, betulinic acid (**4**);[16] *m/z* 457, oleanolic acid (**5**);[16] *m/z* 473, hederagenin (**6**);[14] *m/z* 473, 23-hydroxybetulinic acid (**7**).[26]

Quantitative HPLC Analysis of the Chloroform-Soluble Extract of *P. rockii* ssp. *rockii*. Quantitative HPLC was conducted using a 150 × 3.9 mm i.d. C_{18} μ-Bondapack column. The solvents were HCOOH 0.1% in H_2O (solvent A) and MeOH (solvent B), and gradient elution used was as follows: 0 → 7 min, 5% B; 7 → 12 min, 5 → 15% B, 12 → 20 min, 15% B; 20 → 22 min, 15 → 25% B; 22 → 52 min, 25 → 35% B; 52 → 82 min, 35 → 65% B; 82 → 85 min, 65 → 100% B. Analysis was carried out in triplicate at a flow rate of 1.0 mL min^{-1} with a DAD detector set at 230 nm. Benzoic acid (from Sigma-Aldrich, Italy), benzoylpaeoniflorin (from 3B Scientific Corporation, USA), and paeoniflorigenone (isolated from the *P. rockii* spp. *rockii* extract and characterized by UV, NMR, and MS data) were used to prepare standard solutions at three concentration levels in the range 6.25−1.50 mg/mL for benzoic acid, 6.00−1.50 mg/mL for paeoniflorigenone, and 2.50−0.25 mg/mL for benzoylpaeoniflorin. The standard curves were analyzed using the linear least-squares regression equation derived from the peak area (regression equation $y = 1006.3x + 53473$, $r = 0.9999$ for benzoic acid; $y = 700.64x + 12355$, $r = 0.9990$ for paeoniflorigenone, and $y = 1907.8x - 252.89$, $r = 1.000$ for benzoylpaeoniflorin, where *y* is the peak area and *x* the concentration). The peaks associated with the three compounds were identified by retention time. UV and mass spectra were compared with the standards and confirmed by co-injections. The crude chloroform-soluble extract of *P. rockii* ssp. *rockii* was dissolved in MeOH and analyzed under the same chromatographic conditions.

Quantitative Determination of Total Phenols. The crude chloroform-soluble extract of *P. rockii* ssp. *rockii*, dissolved in MeOH, was analyzed for its total phenolic content according to the Folin−Ciocalteau colorimetric method.[34] Total phenols were expressed as benzoic acid equivalent (110.0 ± 1.1 μg/mg extract).

Bleaching of the Free Radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH Test). The antiradical activity of the crude plant chloroform-soluble extract and the polyphenols under investigation was determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), according to the procedure previously described by Mencherini et al.[34] α-Tocopherol (EC_{50} 10.1 ± 1.3 μg/mL) was used as a positive control in the test. All experiments were carried out in triplicate, and the mean effective scavenging concentrations (EC_{50}) were calculated using the Litchfield and Wilcoxon protocol.[35]

Cell Cultures. Human melanoma (M-14), breast (MCF-7), and colon (HT-29) cancer cells and human embryonic kidney 293 cells (HEK-293) were cultured in DMEM medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin (all from Cambrex Bioscience, Verviers, Belgium). Cells were plated at a density of 3× 10^3 cells/well and, at the end of the incubation period (24 h, 37 °C in humidified 5% CO_2), were processed. The cells were used up to a maximum of 10 passages.

MTT Bioassays. Aliquots of serial dilutions of the crude plant chloroform-soluble extract (1−100 mg/mL) or isolated compounds

(1−50 μM) were added to human normal and cancer cells, and the cells were reincubated for 48 h. Cell viability was assessed through the MTT assay according to Picerno et al.[36] Optical density was read at 620 nm.

Analysis of Apoptosis. Cancer cells treated and untreated with serial dilutions of the crude plant chloroform-soluble extract or isolated compounds, with or without pretreatment for 30 min of 25 mM general caspases inhibitor z-VAD-fmk (BioVision Inc., Mountain View, CA), were analyzed using the method of propidium iodide staining by flow cytometry, as previously described.[37] The percentage of the cells in apoptosis (hypodiploid nuclei) was calculated with Becton Dickinson FACScan flow cytometer using the Cells Quest program.

Western Blotting Analysis. Total intracellular proteins were extracted from the lysed cancer cells (M-14), and Western blot analysis was performed according to Petrella et al.[38] Protein samples (30 μg) were resolved by SDS-PAGE and then transferred electrophoretically. Blots were blocked with 5% non-fat dry-milk poweder, before incubation with primary antibody rabbit anticleaved-caspase-3 (Cell Signaling, Euroclone, Milan). After washing in TBS/0.1% Tween, the secondary antibody anti-rabbit (diluted 1:5000) (both from Sigma-Aldrich, Italy) was added for 1 h at room temperature. Immunoreactive protein bands were detected by chemiluminescence using enhanced chemiluminescence reagents and exposed to Hyperfilm (both from Amersham Biosciences, Italy). The blots were then scanned and analyzed (Gel-Doc 2000, BIO-RAD).

Statistical Analysis. All results are shown as means ± SEM of three experiments performed in triplicate. The optical density of the protein bands detected by Western blotting was normalized on β-actin levels. Statistical comparison between groups was made using ANOVA followed by the Bonferroni parametric test.[35] Differences were considered significant if $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information. HPLC fingerprint of the plant crude chloroform-soluble extract, 1D and 2D NMR spectra (^{1}H, ^{13}C, NOESY, HSQC, HMBC, and DQF-COSY) of the new compounds **1**−**3**, and the isolation procedure for the known compounds. This information is available free of charge via the Internet at http://pubs.acs.org.

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