Triterpene, Antioxidant, and Antimicrobial Compounds from *Melissa officinalis*

Teresa Mencherini,[†] Patrizia Picerno,[†] Carla Scesa,[‡] and Rita Aquino*[,†]

Dipartimento di Scienze Farmaceutiche, University of Salerno, Via Ponte Don Melillo, 84084, Fisciano, Salerno, Italy, and Tekno Scienze, Viale Brianza 22, 20127, Milano, Italy

Received July 18, 2007

Six new triterpenes (**1**–**6**) and four known compounds have been isolated from dried stems and leaves of *Melissa officinalis*. The known compounds were identified as quadranoside III, salvianic acid A, rosmarinic acid, and luteolin. The structures of compounds **1**–**6** were established by analysis of spectroscopic data. Free radical scavenging and antimicrobial activities of the extracts and of rosmarinic acid, the major component, were evaluated.

Melissa officinalis L. (Labiatae) is a perennial edible herb native to the Mediterranean region. The essential oil is recommended for its antimicrobial activity,[1,2] and aqueous extracts exhibit antiviral[3,4] and antioxidative [5]properties. The leaves are used as a juice or as a herbal tea for their aromatic, digestive, and antispasmodic properties in nervous disturbance of sleep and for gastrointestinal disorders.[2,6] It was also reported that *M. officinalis* contains substances inhibiting protein biosynthesis in cancer cells.[7] These biological activities have been attributed to the essential oil,[6,8] flavonoids[6,9] and phenolic acids[6,7,10–14] such as rosmarinic and caffeic acids. Studies on the essential oil have been extensive, but information of the nonvolatile components is scarce.[13,15,16] Thus, we now describe the isolation and characterization of five new disulfated ursene or oleanene triterpenes and a new ursene glycoside from a polar extract of the stems and leaves. Their structures were elucidated by extensive spectroscopic methods including 1D (^{1}H and ^{13}C) and 2D NMR (DQF-COSY, HSQC, and HMBC) experiments as well as HRESIMS analysis. The polar extract (EtOH−H_2O 1:1) and the *n*-BuOH-soluble portion of the extract were both examined for their polyphenol content using the Folin−Ciocalteau colorimetric method, and for the rosmarinic acid content by HPLC. The *in vitro* free radical scavenging activity (DPPH° test) and antimicrobial (broth microdilution assay) effects of the extracts and of rosmarinic acid were also evaluated.

Results and Discussion

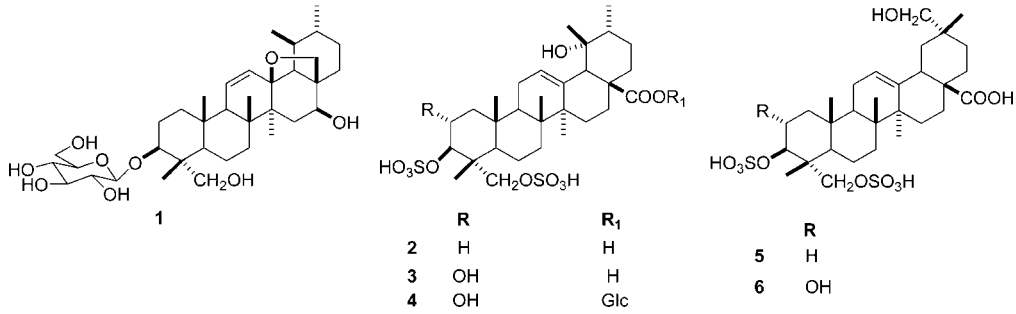
Dried stems and leaves of *M. officinalis* L. were extracted with EtOH−H_2O (1:1), and the dried extract was partitioned between water and *n*-BuOH. The *n*-BuOH-soluble fraction was chromatographed over Sephadex LH-20 to yield rosmarinic acid from fraction III. Triterpene glycosides (**1** and quadranoside III) were obtained from fraction I, salvianic acid A was obtained from fraction II, luteolin was obtained from fraction IV, and compounds **2**–**6** were obtained from fraction V by reversed-phase HPLC.

Compound **1** had the molecular formula $C_{36}H_{58}O_9$ on the basis of the HRESIMS molecular ion at 657.3920 $[M - Na]^+$ (calcd for $C_{36}H_{58}O_9Na$, 657.3979), suggesting a triterpene derivative with a hexose unit. In the ^{1}H NMR spectrum, signals were observed for two olefinic protons at δ_H 5.55 (1H, dd, J = 10.1, 2.8 Hz) and 5.86 (1H, d, J = 10.1 Hz), an AB system of methylene protons on a carbon bonded to an oxygen at δ_H 3.02 (1H, d, J = 7.7 Hz) and 3.89 (1H, d, J = 7.7 Hz), two carbinol protons (δ_H 3.67, 1H, dd, J = 11.9, 5.0 Hz and 4.23, 1H, dd, J = 10.1, 5.2 Hz), four tertiary methyl groups (δ_H 0.73, 1.08, 1.11, and 1.17), two secondary methyl groups (δ_H 0.99 and 1.03, 1H, d, J = 6.5 Hz), and a hydroxymethyl group (δ_H 3.70, 1H, m and 3.32, 1H, m). On the basis of the molecular formula and NMR (^{1}H, ^{13}C, HSQC, HMBC) data analysis, it was concluded that compound **1** was an ursane-type saikosapogenin, with a double bond ($\Delta^{11,12}$) and six rings, one of which is an epoxide bridge between C-28 (δ_C 72.7) and the quaternary carbon at C-13 (δ_C 86.0).[17,18] The HMBC correlations were used to place the glycosidated OH (δ_H 3.67) at C-3 (δ_C 83.1), the unglycosidated OH (δ_H 4.23) at C-16 (δ_C 66.2), and the hydroxymethyl group at C-23 (δ_C 64.6).[17,18] The OH groups at positions 3 and 16 must be equatorial, as the coupling constants of H-3 (J = 11.9, 5.0 Hz) and H-16 (J = 10.1, 5.2 Hz) were consistent with axial configurations for both protons. Thus, the aglycon of **1** was identified as a new ursane-type triterpene, 3β,16β,23-trihydroxy-13,28-epoxyurs-11-ene. The ^{1}H NMR for the sugar moiety of compound **1** showed an anomeric proton signal at δ_H 4.43 (1H, d, J = 7.6 Hz), three −CHOH signals between δ_H 3.20 and 3.36, and a $-CH_2OH$ signal at δ_H 3.70 and 3.88. On the basis of the ^{1}H and ^{13}C NMR data, the sugar moiety was identified as β-glucopyranosyl,[19,20] and it was located at C-3 on the basis of the HMBC correlation between the anomeric proton signal at δ_H 4.43 (H-1 glc) and the carbon resonance at δ_C 83.1 (C-3). The sugar unit was determined to be D-glucose after hydrolysis of **1** with 1 N HCl and GC analysis. Thus, the structure of **1** was 3β,16β,23-trihydroxy-13,28-epoxyurs-11-ene-3-*O*-β-D-glucopyranoside.

The HRESIMS of **2** showed a major ion peak at *m*/*z* 647.2623 $[M - H]^-$, consistent with the molecular formula $C_{30}H_{48}O_{11}S_2$ (calcd for $C_{30}H_{47}O_{11}S_2$, 647.2560) and suggesting a triterpene with two sulfate groups in the molecule. MS/MS analysis of the ion peak showed a fragment ion at *m*/*z* 567.277 $[M - H - 80]^-$, indicating the loss of a sulfate group. Acid hydrolysis of **2**, followed by treatment with $BaCl_2$, gave a white precipitate, confirming the presence of a sulfate residue.[21] The ^{1}H NMR spectrum of compound **2** showed signals corresponding to five tertiary methyls (δ_H 0.80, 0.87, 1.01, 1.20, and 1.32), a secondary methyl at δ_H 0.92 (1 H, d, J = 6.9 Hz), and an olefinic proton at δ_H 5.28 (1 H, t, J = 3.5 Hz). These data together with the ^{13}C NMR signals (Table 1) suggested that **2** was an ursolic acid derivative.[22] A signal typical of H-3ax at δ_H 4.39 (1 H, dd, J = 11.7, 4.8 Hz) shifted downfield with respect to ursolic acid models indicated the presence of a substituted 3 β-OH group, and two signals at δ_H 3.82 (1 H, d, J = 9.7 Hz) and 3.95 (1 H, d, J = 9.7 Hz) were assigned to protons of a primary OH function. Signals at δ_H 2.60 (1 H, s, H-18) and at δ_C 55.4 (C-18) and 74.3 (quaternary, C-19) indicated the presence of an OH at C-19.[23] Full assignments of the proton and carbon resonances were obtained by ^{1}H−^{1}H DQF-COSY and HSQC spectra, suggesting a 3β,19α-dihydroxyurs-12-en-28-oic acid derivative with one of the methyl groups substituted by a $-CH_2OR$ function (δ_H 3.82 and 3.95, and δ_C 69.6; C-23). HMBC correlations observed between H-23a (δ_H 3.95) and H-23b (δ_H 3.82) and between C-4 (δ_C 42.8) and C-3 (δ_C 80.3), C-5 (δ_C 48.2), and C-24 (δ_C 13.1) confirmed

* To whom correspondence should be addressed. Tel: ++39 089 969737. Fax: ++39 089 969602. E-mail: aquinorp@unisa.it.
[†] University of Salerno.
[‡] Tekno Scienze.

10.1021/np070351s CCC: $37.00
Published on Web 11/16/2007



	R	R_1
2	H	H
3	OH	H
4	OH	Glc

	R
5	H
6	OH

the $-CH_2OR$ at C-23, demonstrating that the aglycon of **2** was rotundic acid.[24,25] The sulfate groups were assigned to C-3 and C-23 on the basis of the downfield chemical shifts observed for H-3 (δ_H 4.39), and C-3 (δ_C 80.3) for H-23a (δ_H 3.95), and H-23b (δ_H 3.82), and C-23 (δ_C 69.6) with respect to rotundic acid, and were consistent with the presence of sulfate groups.[21] Thus, compound **2** was identified as the 3,23-disulfate ester of 3β,19α,23-trihydroxyurs-12-en-28-oic acid (3,23-disulfate ester of rotundic acid).

The HRESIMS of **3** (*m/z* 663.2456 $[M - H]^-$, calcd for $C_{30}H_{47}O_{12}S_2$, 663.2509) supported the molecular formula $C_{30}H_{48}O_{12}S_2$, suggesting a triterpene disulfate with an additional oxygen in the molecule with respect to compound **2**. The most intensive ion in the MS/MS spectrum was at *m/z* 583.092 $[M - H - 80]^-$, ascribable to the loss of a sulfate group. Analysis of the NMR data (^1H, ^{13}C, DQF-COSY, HSQC, HMBC) showed that **3** differed from **2** only in the presence of an additional secondary OH function. The 2α-OH substitution was suggested by the chemical shift and the *J* value of H-2 (δ_H 4.03, ddd, $J = 3$, 9.2, 13.0 Hz), by the multiplicity and *J* value of the H-3 signal (δ_H 4.32, d, $J = 9.2$ Hz), and by the chemical shift of C-3 (δ_C 86.1). $^1H-^1H$ DQF-COSY experiments showed the proton sequence H-1a (δ_H 2.02), H-1b (δ_H 0.98), H-2 (δ_H 4.03), and H-3 (δ_H 4.32), and HMBC correlations confirmed placement of the $-CH_2OR$ group (δ_H 3.82 and 3.97, δ_C 69.7) at C-23, suggesting a 23-hydroxytormentic acid derivative.[26] The downfield shifts observed for H-3 (δ_H 4.32)/C-3 (δ_C 86.1) signals and H-23a (δ_H 3.97), H-23b (δ_H 3.82)/C-23 (δ_C 69.7) signals with respect to 23-hydroxytormentic acid indicated the presence of sulfate groups at C-3 and C-23. Thus, the structure of **3** was the 3,23-disulfate ester of 2α,3β,19α,23-tetrahydroxyurs-12-en-28-oic acid (3,23-disulfate ester of 23-hydroxytormentic acid).

The HRESIMS of **4** (*m/z* 825.3028 $[M - H]^-$, calcd for $C_{36}H_{57}O_{17}S_2$, 825.3037) supported the molecular formula $C_{36}H_{58}O_{17}S_2$, suggesting a triterpene disulfate with an additional hexose unit in the molecule with respect to compound **3**. The MS/MS spectrum showed the most intense ion at *m/z* 745.341 $[M - H - 80]^-$, ascribable to the loss of a sulfate group. As in compound **3** the positions of the sulfate groups (at C-3 and C-23) were deduced by the downfield shifts of the pertinent hydrogen (H-3, H-23a, and H-23b) and carbon (C-3 and C-23) signals with respect to niga-ichigoside F1.[27] The ^1H and ^{13}C NMR data indicated that the sugar unit was a β-glucopyranosyl unit (anomeric proton signal, δ_H 5.34, 1H, d, $J = 7.6$ Hz) at C-28 on the basis of the HMBC correlation observed between the anomeric proton signal at δ_H 5.34 (H-1 glc) and the carbon resonance at δ_C 179.5 (C-28). The sugar was determined to be D-glucose after hydrolysis of **4** with 1 N HCl and GC analysis. Thus, the structure of **4** was the 3,23-disulfate ester of 2α,3β,19α,23-tetrahydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (3,23-disulfate ester of niga-ichigoside F1).

The HRESIMS of **5** showed a major ion peak at *m/z* 647.2500 $[M - H]^-$, ascribable to the molecular formula $C_{30}H_{48}O_{11}S_2$, suggesting that it was a triterpene having two sulfate groups as in compound **2**. Also the MS/MS analysis of the ion exhibited the most intense ion at *m/z* 567.289 $[M - H - 80]^-$ as in compound **2**, ascribable to the loss of a sulfate group. The ^1H NMR spectrum of **5** showed signals corresponding to five tertiary methyl groups (δ_H 0.81, 0.89, 0.97, 1.03, and 1.21), an olefinic proton (δ_H 5.26), and signals for H-18 at δ_H 2.94 (1H, dd) and for H-3ax at δ_H 4.39 (1H, dd, $J = 4.4$, 11.7 Hz) typical of a 3β-hydroxyolean-12-en-28-oic acid derivative.[28] Two signals at δ_H 3.84 (1H, d, $J = 9.5$ Hz) and 3.98 (1H, d, $J = 9.5$ Hz) and a further signal at δ_H 3.18 (2H, s) were ascribable to protons of two primary OH functions. $^1H-^1H$ DQF-COSY, HSQC, and HMBC experiments led to unambiguous assignment of all proton and carbon signals and indicated that it was a hederagenin derivative[28] having an additional $-CH_2OH$ function (δ_H 3.18 and δ_C 74.6). The HMBC correlations observed between H-29 (δ_H 3.8) and C-20 (δ_C 37.1), C-21 (δ_C 28.8), and C-30 (δ_C 19.3) indicated the placement of the $-CH_2OH$ group at C-29 (δ_C 74.6), demonstrating that **5** was a 29-hydroxyhederagenin.[29] The sulfate groups were assigned to C-3 and C-23 on the basis of the downfield chemical shifts observed for the H-3 (δ_H 4.39)/C-3 (δ_C 80.5) signals and H-23a (δ_H 3.98), H-23b (δ_H 3.84)/C-23 (δ_C 69.7) with respect to hederagenin. Thus, compound **5** is the 3,23-disulfate ester of 3β,23,29-trihydroxyolean-12-en-28-oic acid (3,23-disulfate ester of 29-hydroxyhederagenin).

The HRESIMS of **6** supported the molecular formula $C_{30}H_{48}O_{12}S_2$, suggesting a triterpene disulfate with an additional oxygen in the molecule with respect to compound **5**. The MS/MS spectrum showed a major ion peak at *m/z* 583.298 $[M - H - 80]^-$, due to the loss of a sulfate group. The NMR data of **6**, in comparison to those of **5**, revealed that **6** differed from **5** only in the presence of an additional secondary OH (δ_H 4.01, δ_C 68.9). The 2α-OH substitution was suggested by the chemical shifts and *J* values of H-2 (δ_H 4.01, ddd, $J = 3$, 9.3, 13.0 Hz) and H-3 (δ_H 4.31, d, $J = 9.3$ Hz) and by the chemical shift of C-3 (δ_C 86.2). $^1H-^1H$ DQF-COSY experiments confirmed this hypothesis showing the proton sequence H-1a (δ_H 2.01), H-1b (δ_H 0.97), H-2 (δ_H 4.01), and H-3 (δ_H 4.31), suggesting a stachlic acid A derivative.[30] The sulfate groups were again deduced to be at C-3 and C-23 from the downfield shifts of the pertinent carbon (C-3 and C-23) and proton (H-3 and H-23) signals. Thus, compound **6** was identified as the 3,23-disulfate ester of 2α,3β,23,29-tetrahydroxyolean-12-en-28-oic acid (3,23-disulfate ester of stachlic acid A).

The known compounds were identified by comparison of their NMR data with those from the literature to be quadranoside III,[31] salvianic acid A,[32] luteolin,[33] and rosmarinic acid.[34] To confirm that the antioxidative properties reported for *M. officinalis* L.[5,14,35,36] relate primarily to the rosmarinic acid content,[14,37] both the extracts and their major component, rosmarinic acid, were tested for free radical scavenging activity using the DPPH° test. Significant concentration-dependent free radical scavenging activity was shown by the EtOH$-H_2O$ (1:1) extract, its *n*-BuOH-soluble portion, and rosmarinic acid (EC_{50} values were 18.5, 15.2, and 3.1 μg/mL, respectively). The latter value was in good agreement with data reported for rosmarinic acid by Moreno et al.[38]

Antimicrobial effects of the essential oil of *M. officinalis* L. have been reported;[1,2] however, antimicrobial activity of nonvolatile extracts and compounds have not. Thus, rosmarinic acid and both extracts (EtOH$-H_2O$ (1:1) and *n*-BuOH) were tested for antibacterial and antifungal activities by a broth microdilution method against several Gram-positive and Gram-negative bacteria, a yeast, and a

Table 1. ^{13}C and ^{1}H NMR Data of Compounds **2**, **3**, **4** (aglycon moiety), **5**, and **6** in CD_3OD[a]

	2		**3**		**4**		**5**		**6**	
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]	δ_C	δ_H (J in Hz)[b]	δ_C	δ_H (J in Hz)[b]
1	39.1	1.01, 1.65, m	47.4	0.98, 2.02, m	47.3	0.97, 2.02, m	38.7	1.02, 1.66, m	47.1	0.97, 2.01, m
2	24.4	1.79, 2.16, m	69.0	4.03, ddd, (3, 9.2, 13.0)	68.8	4.01, ddd, (3, 9.2, 13.0)	24.5	1.81, 2.19 m	68.9	4.01, ddd,, (3.0, 9.3, 13.0)
3	80.3	4.39, dd, (4.8, 11.7)	86.1	4.32, d, (9.2)	86.2	4.31, d, (9.2)	80.5	4.39, dd, (4.4, 11.7)	86.2	4.31, (d, 9.3)
4	42.8		43.8		43.9		42.6		43.9	
5	48.2	1.41, m	48.1	1.51, m	48.1	1.51, m	48.7	1.48, m	48.1	1.48, m
6	18.6	1.40, 1.63, m	18.7	1.43, 1.66, m	18.8	1.43, 1.66, m	18.5	1.41, 1.68, m	18.6	1.43, 1.55, m
7	33.3	1.28, 1.70, m	33.4	1.32, 1.73, m	33.2	1.29, 1.74, m	33.0	1.29, 1.58, m	33.5	1.31, 1.69, m
8	40.4		40.6		40.8		40.3		40.7	
9	48.6	1.75, m	48.4	1.81, m	48.4	1.82, m	48.9	1.68, m	49.1	1.66, m
10	37.3		37.8		37.6		37.7		38.5	
11	24.3	1.98, 1.73, m	24.7	2.04, 1.94, m	24.6	2.04, 1.81, m	24.1	1.62, 1.95, m	24.7	1.71, 1.98, m
12	128.6	5.28, t (3.5)	128.3	5.31, t (3.5)	129.7	5.33, t (3.5)	122.7	5.26, t (3.5)	123.1	5.28, t (3.5)
13	141.2		140.9		140.0		146.9		147.3	
14	42.3		42.4		42.8		42.9		43.3	
15	29.5	0.93, 1.94, m	29.7	0.96, 1.98, m	29.4	0.96, 1.98, m	29.5	1.12, 1.46, m	28.9	0.95, 1.98, m
16	26.7	1.52, 2.40, m	26.7	2.44, 1.54, m	26.3	2.44, 1.54, m	23.7	1.76, 2.10, m	23.7	1.76, 2.10, m
17	49.2		49.0		49.1		48.9		49.0	
18	55.4	2.60, s	55.4	2.63, s	54.9	2.54, s	42.0	2.94, dd, (3.6, 13.7)	42.4	2.96, dd (3.6, 13.7)
19	74.3		74.0		73.6		41.9	1.06, 1.77, m	42.2	1.04, 1.74, m
20	42.8	1.37, m	43.0	1.40, m	43.0	1.37, m	37.1		36.9	
21	27.4	1.16, 1.69, m	27.5	1.18, 1.72, m	27.0	1.18, 1.72, m	28.8	1.05, 1.88, m	29.7	1.04, 1.86, m
22	39.0	2.15, 1.70, m	39.4	1.63, 1.81, m	38.2	1.63, 1.81, m	33.2	1.69, 1.76, m	32.0	1.70, 1.78, m
23	69.6	3.95, d (9.7) 3.82, d (9.7)	69.7	3.97, d (9.6) 3.82, d (9.6)	69.4	3.98, d (9.6) 3.82, d (9.6)	69.7	3.98, d (9.5) 3.84, d (9.5)	70.1	3.97, d (9.7) 3.81, d (9.7)
24	13.1	0.80, s	14.3	0.84, s	14.2	0.84, s	13.0	0.81, s	14.3	0.84, s
25	16.3	1.01, s	17.1	1.08, s	16.9	1.08, s	16.0	1.03, s	17.5	1.08, s
26	17.3	0.87, s	17.6	0.89, s	17.3	0.89, s	17.8	0.89, s	18.2	0.92, s
27	24.7	1.32, s	24.8	1.35, s	24.6	1.37, s	26.4	1.21, s	26.4	1.20, s
28	176.7		177.7		179.5		177.8		177.8	
29	27.0	1.20, s	27.1	1.23, s	26.6	1.23, s	74.6	3.18, s	75.4	3.19, s
30	16.4	0.92, d (6.9)	16.7	0.95, d (6.5)	16.9	0.95, d (6.5)	19.3	0.97, s	19.5	0.98, s

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

mold. Antimicrobial activities were expressed as MIC (minimum inhibitory concentration) and as MBC (minimal bactericide concentration). Major bacteriostatic effects were exerted against Gram-positive bacteria, *S. aureus* and *S. epidermidis*, by rosmarinic acid (MIC 0.12 mg/mL) and against *B. spizizenii* by both of the extracts (MIC 0.5 mg/mL). In the case of Gram-negative bacteria, yeasts, and molds the MICs were greater than 2.0 mg/mL. In previous work[38] no antimicrobial effect was observed for rosmarinic acid because it was tested at lower concentrations (5–250 μg/mL). Growth inhibition in this study (MBC ranging from 0.12 to 16 mg/mL) was observed, however, indicating that rosmarinic acid does have some bactericidal and fungicidal effects, and rosmarinic acid appears to be the major bioactive compound present in *Melissa* extracts.

Experimental Section

General Experimental Procedures. Melting points were determined using a DSC822^e (Mettler-Toledo) apparatus. Optical rotations were measured in MeOH solution on a JASCO DIP-1000 digital polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. 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 in δ (parts per million) referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD; coupling constants, *J*, are in hertz; ^{1}H−^{1}H DQF-COSY, ^{1}H−^{13}C HSQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature.[19,39] Exact masses (HRESIMS) were measured by a Q-TOF Premier (Waters) triple-quadrupole orthogonal time-of-flight (TOF) instrument equipped with an electrospray ionization source used in TOF mode at 10.000 resolving power. Samples were dissolved in MeOH, mixed with the internal calibrant, and introduced directly into the ion source by direct infusion. Calibration was performed on the peaks of cesium iodide and synthetic peptide (TOF positive ion calibration solution Bachem) at *m/z* 132.9054 and 829.5398, respectively. 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, Rheodyne model G-1322A loop (20 μL), and DAD G-1315 A detector. Peak areas were calculated with an Agilent integrator.

Plant Material. The aerial parts (stems and leaves) of *M. officinalis* L. were collected at Trezzo sull'Adda, Italy, in May 2005 and identified by Dr. A. Facchini, Respharma. A voucher sample (RES 0441-2 CT) was deposited at the Herbarium of Respharma s.r.l., Trezzo sull'Adda, Italy.

Extraction and Isolation Procedure. Dried and powdered aerial parts (stems and leaves) of *M. officinalis* L. (500 g) were extracted in a Soxhlet with EtOH−H_2O (1:1) to give 15.2 g of residue. This was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH-soluble portion (3.0 g). A portion (2.7 g) of the *n*-BuOH extract was chromatographed over a Sephadex LH 20 column (1 m × 3 cm i.d.) using MeOH as eluent (flow rate 0.5 mL min^{-1}). Fractions (8 mL each) were collected and checked by TLC (Si gel, *n*-BuOH−AcOH−H_2O (60:15:25), $CHCl_3$−MeOH−H_2O (7:3:0.3)). Fractions with similar R_f values were combined, giving five major fractions. Fraction III (238.6 mg) contained pure rosmarinic acid. Fraction I (271.0 mg) was chromatographed by RP-HPLC using MeOH−H_2O (6:4) as mobile phase (flow rate 2.0 mL min^{-1}) to yield quadranoside III (2.1 mg) and compound **1** (5.7 mg). Fractions II, IV, and V were chromatographed by RP-HPLC using MeOH−H_2O (2:8) as mobile phase (flow rate 2.0 mL min^{-1}). Fraction II (70.7 mg) yielded salvianic acid (3.2 mg). Fraction IV (82.5 mg) gave luteolin (3.5 mg). Fraction V (80.7 mg) afforded sulfated triterpenes **5** (5.8 mg), **6** (5.5 mg), **3** (5.4 mg), **4** (5.7 mg), and **2** (6.0 mg).

Compound (1): white powder; mp 298–300 °C; $[\alpha]^{31}_D$ +31.2 (*c* 1.5, MeOH); ^{1}H NMR data (CD_3OD, 600 MHz) for the aglycon moiety δ 5.86 (1H, d, *J* = 10.1 Hz, H-12), 5.55 (1H, dd, *J* = 2.8, 10.1 Hz, H-11), 4.23 (1H, dd, *J* = 5.2, 10.1 Hz, H-16), 3.89 (1H, d, *J* = 7.7 Hz, H-28a), 3.70 (1H, m, H-23a), 3.67 (1H, dd, *J* = 5.0, 11.9 Hz, H-3), 3.32 (1H, m, H-23b), 3.02 (1H, d, *J* = 7.7 Hz, H-28b), 2.19 (1H, m, H-22a), 2.04 (1H, m, H-2a), 1.99 (1H, brs, H-9), 1.86 (1H, m, H-1a), 1.82 (1H, m, H-2b), 1.77 (1H, m, H-19), 1.64 (1H, dd, *J* = 10.1, 12.5 Hz, H-15a), 1.60 (1H, m, H-21a), 1.57 (1H, m, H-6), 1.53 (1H, m, H-7), 1.47 (1H, dd, *J* = 5.2, 12.5 Hz, H-15b), 1.40 (1H, d, *J* = 11.7 Hz, H-18), 1.37 (1H, m, H-22b), 1.25 (2H, m, H-21b, H-5), 1.17 (3H, s, H-27), 1.11 (3H, s, H-26), 1.08 (3H, s, H-25), 1.03 (3H, d, *J* = 6.5 Hz, H-29), 0.99 (3H, d, *J* = 6.5 Hz, H-30), 0.98 (1H, m, H-20), 0.97 (1H, m, H-1b), 0.73 (3H, s, H-24); ^{1}H NMR data (CD_3OD, 600 MHz) for the sugar moiety δ 4.43 (1H, d, *J* = 7.6 Hz, H-1 Glc), 3.88 (1H, dd, *J* = 2.5, 12.0 Hz, H-6a Glc), 3.70 (1H, dd, *J* = 4.0, 12.0 Hz, H-6b Glc), 3.36 (1H, t, *J* = 9.0 Hz, H-3 Glc), 3.31 (1H, t, *J* = 9.0 Hz, H-4 Glc), 3.29 (1H, m, H-5 Glc), 3.20 (1H, dd, *J* = 7.6, 9.0 Hz, H-2 Glc); ^{13}C NMR data (CD_3OD, 150 MHz) for the aglycon moiety: δ 132.6 (CH, C-11), 131.6 (CH, C-12), 86.0 (C, C-13), 83.1 (CH, C-3), 72.7 (CH_2, C-28), 66.2 (CH, C-16), 64.6 (CH_2, C-23), 63.3 (CH, C-18), 53.8 (CH, C-9), 48.0 (CH, C-5), 47.0 (C, C-14, C-17), 44.2 (C, C-4), 43.1 (C, C-8), 41.9 (CH, C-20), 39.3 (CH_2, C-1), 39.2 (CH, C-19), 38.4 (CH_2, C-22), 37.2 (C, C-10), 36.1 (CH_2, C-15), 32.2 (CH_2, C-7), 32.1 (CH_2, C-21), 25.9 (CH_2, C-2), 20.1 (CH_3, C-26), 19.2 (CH_3, C-30), 18.7 (CH_3, C-29), 18.4 (CH_3, C-27), 17.6 (CH_2, C-6), 17.5 (CH_3, C-25), 12.8 (CH_3, C-24); ^{13}C NMR data (CD_3OD, 150 MHz) for the sugar moiety δ 105.9 (CH, C-1 Glc), 78.3 (CH, C-3 Glc), 77.5 (CH, C-5 Glc), 75.3 (CH, C-2 Glc), 71.3 (CH, C-4 Glc), 62.4 (CH_2, C-6 Glc); HRESIMS *m/z* 657.3920 [M − Na]$^+$ (calcd for $C_{36}H_{58}O_9Na$, 657.3979).

Compound (2): white powder; mp 258 °C; $[\alpha]^{31}_D$ −24.9 (*c* 0.066, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are reported in Table 1; HRESIMS *m/z* 647.2623 [M − H]$^-$ (calcd for $C_{30}H_{47}O_{11}S_2$, 647.2560).

Compound (3): white powder; mp 266 °C; $[\alpha]^{31}_D$ +20.1 (*c* 0.16, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are reported in Table 1; HRESIMS *m/z* 663.2456 [M − H]$^-$ (calcd for $C_{30}H_{47}O_{12}S_2$, 663.2509).

Compound (4): white powder; mp 278 °C; $[\alpha]^{31}_D$ +8.1 (*c* 0.027, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) for the aglycon moiety are reported in Table 1; for the sugar portion ^{1}H NMR (CD_3OD, 600 MHz) δ 5.34 (1H, d, *J* = 7.6 Hz, H-1 Glc), 3.82 (1H, dd, *J* = 2.5, 12.0 Hz, H-6a Glc), 3.70 (1H, dd, *J* = 4.0, 12.0 Hz, H-6b Glc), 3.67 (1H, t, *J* = 9.0, H-4 Glc), 3.40 (1H, t, *J* = 9.0 Hz, H-3 Glc), 3.34 (1H, m, H-5 Glc), 3.33 (1H, dd, *J* = 7.6, 9.0 Hz, H-2 Glc); ^{13}C NMR (CD_3OD, 150 MHz) δ 95.4 (CH, C-1 Glc), 78.3 (CH, C-5 Glc), 77.9 (CH, C-3 Glc), 73.8 (CH, C-2 Glc), 70.6 (CH, C-4 Glc), 62.2 (CH_2, C-6 Glc); HRESIMS *m/z* 825.3028 [M − H]$^-$ (calcd for $C_{36}H_{57}O_{17}S_2$, 825.3037).

Compound (5): white powder; mp 242 °C; $[\alpha]^{31}_D$ −13.5 (*c* 0.047, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) are reported in Table 1; HRESIMS *m/z* 647.2500 [M − H]$^-$ (calcd for $C_{30}H_{47}O_{11}S_2$, 647.2560).

Compound (6): white powder; mp 263 °C; $[\alpha]^{31}_D$ +31.7 (*c* 0.08, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) are reported in Table 1; HRESIMS *m/z* 663.2498 [M − H]$^-$ (calcd for $C_{30}H_{47}O_{12}S_2$, 663.2509).

2α,3β,23,29-Tetrahydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (quadranoside III): white powder; mp 269 °C; $[\alpha]^{31}_D$ −10.5 (*c* 0.133, MeOH); ^{1}H and ^{13}C NMR data were consistent with those previously reported;[31] HRESIMS *m/z* 689.3870 [M + Na]$^+$ (calcd for $C_{36}H_{59}O_{11}Na$, 689.3901).

Salvianic acid A: ^{1}H and ^{13}C NMR data were consistent with those previously reported;[32] ESIMS *m/z* 197 [M − H]$^-$.

Luteolin: ^{1}H and ^{13}C NMR data were consistent with those previously reported;[33] ESIMS *m/z* 285 [M − H]$^-$.

Rosmarinic acid: ^{1}H and ^{13}C NMR data were consistent with those previously reported;[34] ESIMS *m/z* 359 [M − H]$^-$.

Acid Hydrolysis. A solution (0.8 mg each) of **1** and **4** in 1 N HCl (0.25 mL) was stirred at 80 °C for 4 h. After cooling, the solutions were concentrated by blowing with N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.1 mL), and the solution was stirred at 60 °C for 5 min After drying the solution with a stream of N_2, the residue was partitioned between H_2O and CH_2Cl_2 (1 mL, 1:1 v/v). The CH_2Cl_2 layer was analyzed by GC using an L-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. The peaks from the hydrolysates of both **1** and **4** were detected at 14.72 min. The peak for standard D-glucose was detected at 14.71 min.

Table 2. Total Phenol Content and Free Radical Scavenging Activity of the EtOH−H_2O (1:1) and *n*-BuOH Extracts and Rosmarinic Acid from *M. officinalis* L.

extract and compounds	phenol content[a] (μg/mg extract)[b]	DPPH test [EC_{50} (μg of extract or compound/mL)]
EtOH−H_2O (1:1)	250.0 ± 2.8	18.5 (16.5–20.5)[c]
n-BuOH soluble portion	356.0 ± 4.0	15.2 (13.0–17.4)[c]
rosmarinic acid		3.1 (2.7–3.5)[c]
α-tocopherol[d]		10.1 (8.8–11.4)[c]

[a] Mean ± SD of three determinations. [b] Rosmarinic acid equivalents. [c] 95% confidence limits. [d] Positive control.

Detection of the Sulfate Group. A 1–2 mg aliquot of each sample of compounds **2**–**6** was refluxed with 10% HCl (4 mL) for 4 h and then extracted with Et_2O. An aliquot of the aqueous layer of each was treated with 70% $BaCl_2$ to give a white precipitate ($BaSO_4$).[21]

Quantitative HPLC Analysis of the Extracts. Quantitative HPLC was conducted using a 150 × 3.9 mm i.d. C_{18} μ-Bondapack column. The solvents were TFA 0.1% in H_2O (solvent A) and MeOH (solvent B). The elution gradient used was as follows: 0 → 30 min, 10 → 55% B; 30 → 32 min, 55 → 100% B. Analyses were carried out in triplicate, at a flow rate of 0.8 mL min^{-1} with a DAD detector set at λ 320 nm. Reference standard solutions of rosmarinic acid were prepared at three concentration levels in the range 0.25–2.00 mg/mL. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area (regression equation $y = 1992.5x + 6279.7$, $r = 0.999$, where y is the peak area and x the concentration). The peak associated with rosmarinic acid was identified by retention time. UV and mass spectra were compared with the standard and confirmed by co-injection. The extracts, EtOH−H_2O (1:1) and *n*-BuOH, were redissolved in MeOH and analyzed under the same chromatographic conditions. The results showed that rosmarinic acid represented 5.6% and 10.0% w/w, respectively.

Quantitative Determination of Total Phenols. The 1:1 EtOH−H_2O extract and its *n*-BuOH-soluble portion, dissolved in MeOH, were analyzed for their total phenolic content according to the Folin−Ciocalteu colorimetric method.[40] Total phenols were expressed as rosmarinic acid equivalents (250 ± 2.8 and 356 ± 4.0 μg/mg extract, respectively).

Bleaching of the Free Radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH° Test). The antiradical activities of the *M. officinalis* L. extracts, rosmarinic acid, and α-tocopherol were determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH°) and the procedures described by Aquino et al.[40] In its radical form, DPPH° has an absorption band at 515 nm, which disappears upon reduction by an antiradical compound. An aliquot (37.5 μL) of the MeOH solution containing different amounts of the EtOH−H_2O (1:1) extract and its *n*-BuOH-soluble portion, or rosmarinic acid, from *M. officinalis* L. was added to 1.5 mL of daily prepared DPPH° solution (0.025 g/L in MeOH); the maximum concentration employed was 200 μg/mL. An equal volume (37.5 μL) of the vehicle alone was added to control tubes. Absorbance at 515 nm was measured on a Shimadzu UV-1601 UV–visible spectrophotometer 10 min after starting the reaction. The DPPH° concentration in the reaction medium was calculated from a calibration curve analyzed by linear regression. The percentage of remaining DPPH° (% $DPPH°_{REM}$) was calculated as follows:

$$\% \ DPPH°_{REM} = [DPPH°]_T/[DPPH°]_0 \times 100$$

where T is the experimental duration time (10 min). α-Tocopherol 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 by using the Litchfield and Wilcoxon test.[41] Results are reported in Table 2.

Antimicrobial Activity. The EtOH−H_2O (1:1) extract, *n*-BuOH-soluble portion, and rosmarinic acid were tested for antimicrobial activity using the broth microdilution method in 96-well microtiter plates, in duplicate, as reported by Koneman[42] and Camporese[43] and recommended by the National Committee for Clinical Laboratory Standard (NCCLS, 2001).[44] The following microorganisms from American Type Culture Collection (ATCC) were utilized: Gram-positive bacteria (*S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *B. spizizenii* ATCC 6633), Gram-negative bacteria (*P. aeruginosa* ATCC 9027, *E. coli* ATCC 8739), a yeast (*C. albicans* ATCC 10231), and a mold (*A. niger* ATCC 16404). For susceptibility testing, the first step was to place 50 μL of Triptone soya broth in wells 2–12. Dry extracts and rosmarinic acid were initially dissolved in 100 μL of dimethyl sulfoxide (DMSO) and then in Triptone to a final concentration of 32 mg/mL for the extracts and 4 mg/mL for rosmarinic acid. Then, 100 μL of these suspensions was added to the first test well of each microtiter line, and then 50 μL of scalar dilutions was transferred from the second to the 11th well. The 12th tube was considered as growth control, since no test solutions were added. Then, 50 μL of a suspension of test organism (10^5 colony forming unit (CFU)/mL) was added to each well. The final concentration varied from 16 (first well) to 0.031 mg/mL (11th well) for the extracts and from 2 to 0.0078 mg/mL for rosmarinic acid. Plates were incubated for 24 h at 37 °C and then examined from below using a reflective viewer. MIC was the lowest concentration of extract or compound at which microbial growth was inhibited after 24 h. The minimum bactericide concentration (MBC) or the minimum fungicide concentration (MFC) was the lowest concentration of the extract or compound at which survival of any microbial cell was not possible after incubation for 48 h (for bacteria strains) and 5 days (for yeasts and molds) and was determined by inoculating a portion of the broth culture on agar plates, where MIC values were previously defined.[38] A blank control was taken using DMSO alone (100 μg/mL) added to a series of tubes, and the MIC was evaluated as described above. No growth inhibition was observed at DMSO concentrations lower or equal to 25 μg/mL. The determination of the MICs of known antimicrobial compounds, gentamicin and nystatin, for all the reference strains was simultaneously carried out (gentamicin, MIC 1 μg/mL for Gram-positive bacteria, 4 μg/mL for Gram-negative bacteria; nystatin, MIC 1 μg/mL for *C. albicans*).

References and Notes

(1) Larrondo, J. V.; Agut, M.; Calvo-Torras, M. A. *Microbios* **1995**, *82*, 171–172.
(2) Mimica-Dukic, N.; Bozin, B.; Sokovic, M.; Simin, N. *J. Agric. Food Chem.* **2004**, *52*, 2485–2489.
(3) Dimitrova, Z.; Dimov, B.; Manolova, N.; Pancheva, S.; Ilieva, D.; Shishkov, S. *Acta Microbiol. Bulg.* **1993**, *29*, 65–72.
(4) Yamasaki, K.; Nakano, M.; Kawahata, T.; Mori, H.; Otake, T.; Ueba, N.; Oishi, I.; Inami, R.; Yamane, M.; Nakamura, M.; Murata, H.; Nakanishi, T. *Biol. Pharm. Bull.* **1998**, *21*, 829–833.
(5) Hohmann, J.; Zupko, I.; Redei, D.; Csanyi, M.; Falkay, G.; Mathe, I.; Janicsak, G. *Planta Med.* **1999**, *65*, 576–578.
(6) Carnat, A. P.; Carnat, A.; Fraisse, D.; Lamaison, J. L. *Pharm. Acta Helv.* **1998**, *72*, 301–305.
(7) Galasinski, W.; Chlabicz, J.; Paszkiewicz-Gadek, A.; Marcinkiewicz, C.; Gindzienski, A. *Acta Pol. Pharm.* **1996**, *53*, 311–318.
(8) Adzet, T.; Ponz, R.; Wolf, E.; Schulte, E. *Planta Med.* **1992**, *58*, 562–564.
(9) Patora, J.; Klimek, B. *Acta Pol. Pharm.* **2002**, *59*, 139–143.
(10) Caniova, A.; Brandsteterova, E. *J. Liq. Chromatogr. Relat. Technol.* **2001**, *24*, 2647–2659.
(11) Isao, A.; Hijiri, K.; Tsutomu, H.; Sansei, N.; Takuo, O. *Chem. Pharm. Bull.* **1993**, *41*, 1608–16011.
(12) Tüth, J.; Mrlianová, M.; Tekeová, D.; Koreňová, M. *Acta Fac. Pharm. Univ. Comenianae* **2003**, 139–145.
(13) Herode, S. S.; Hadolin, M.; Skerget, M.; KnezŽ. *Food Chem.* **2003**, *80*, 275–282.
(14) Lamaison, J. L.; Petitjean-Freytet, C.; Carnat, A. *Pharm. Acta Helv.* **1991**, *66*, 185–188.
(15) Colceru-Mihul, S.; Nita, S.; Panteli, M.; Armatu, A.; Ocnaru, D.; Manaila, N.; Bazdoaca, C.; Rasit, I.; Ichim, M. *Roum. Biotechnol. Lett.* **2007**, *12*, 3045–3057.
(16) Brieskorn, C. H.; Krause, W. *Arch. Pharm.* **1974**, *307*, 603–612.
(17) Chen, I. H.; Chang, F. R.; Wu, C. C.; Chen, S. L.; Hsieh, P. W.; Yen, H. F.; Du, Y. C.; Wu, Y. C. *J. Nat. Prod.* **2006**, *69*, 1543–1546.
(18) Fujioka, T.; Yoshida, K.; Fujii, H.; Nagao, T.; Okabe, H.; Mihashi, K. *Chem. Pharm. Bull.* **2003**, *51*, 365–372.
(19) Safir, O.; Fkih-Tetouani, S.; De Tommasi, N.; Aquino, R. *J. Nat. Prod.* **1998**, *61*, 130–134.
(20) Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy,* 3rd rev. ed.; VCH: Weinheim, 1989.
(21) Perrone, A.; Masullo, M.; Bassarello, C.; Hamed, A. I.; Belisario, M. A.; Pizza, C.; Piacente, S. *J. Nat. Prod* **2007**, *70*, 584–588.
(22) De Tommasi, N.; De Simone, F.; Pizza, C.; Mahmood, N.; Moore, P. S.; Conti, C.; Orsi, N.; Stein, M. L. *J. Nat. Prod.* **1992**, *55*, 1067–1073.
(23) Aquino, R.; De Simone, F.; Vincieri, F. F.; Pizza, C. *J. Nat. Prod.* **1990**, *53*, 559–564.
(24) Nakatani, M.; Miyazaki, Y.; Iwashita, T.; Naoki, H.; Hase, T. *Phytochemistry* **1989**, *28*, 1479–1482.

(25) Sahpaz, S.; Gupta, M. P.; Hostettmann, K. *Phytochemistry* **2000**, *54*, 77–84.
(26) Reher, G.; Reznicek, G.; Baumann, A. *Planta Med.* **1991**, *57*, 506.
(27) Um, B. H.; Pouplin, T.; Lobstein, A.; Weniger, B.; Litaudon, M.; Anton, R. *Fitoterapia* **2001**, *72*, 591–593.
(28) Magid, A. A.; Voutquenne, L.; Moretti, C.; Long, C.; Lavaud, C. *J. Nat. Prod.* **2006**, *69*, 196–205.
(29) Lavaud, C.; Crublet, M. L.; Pouny, I.; Litaudon, M.; Sévenet, T. *Phytochemistry* **2001**, *57*, 469–78.
(30) Yang, J. H.; Wang, Y. S.; Huang, R.; Luo, S. D.; Zhang, H. B.; Li, L. *Helv. Chim. Acta* **2006**, *89*, 2830–2835.
(31) Adnyana, I. K.; Tesuka, Y.; Banskota, A. H.; Xiong, Q.; Tran, K. Q.; Kadota, S. *J. Nat. Prod.* **2000**, *63*, 496–500.
(32) Huang, H.; Sun, H.-D.; Wang, M.-S.; Zhao, S.-X. *J. Nat. Prod.* **1996**, *59*, 1079–1080.
(33) Heitz, A.; Carnat, A.; Fraisse, D.; Carnat, A.-P.; Lamaison, J.-L. *Fitoterapia* **2000**, *71*, 201–202.
(34) Dapkevicius, A.; van Beek, T. A.; Lelyveld, G. P.; van Veldhuizen, A.; de Groot, A.; Linssen, J. P. H.; Venskutonis, R. *J. Nat. Prod.* **2002**, *65*, 892–896.
(35) Lugasi, A.; Dworschak, E.; Hovari, J. *Special Publication-R. Soc. Chem.* **1996**, *179*, 372–375.
(36) Triantaphyllou, K.; Blekas, G.; Boskou, D. *Int. J. Food Sci. Nutr.* **2001**, *52*, 313–317.
(37) Chen, J. H.; Ho, C. T. *J. Agric. Food Chem.* **1997**, *45*, 2374–2378.
(38) Moreno, S.; Scheyer, T.; Romano, C. S.; Vojnov, A. A. *Free Radical Res.* **2006**, *40*, 223–231.
(39) Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 2821–2823.
(40) Aquino, R.; Morelli, S.; Lauro, M. R.; Abdo, S.; Tomaino, A. *J. Nat. Prod.* **2001**, *64*, 1019–1023.
(41) Tallarida, R. J.; Murray, R. B. *Manual of Pharmacological Calculations*; Springer-Verlag: New York, 1984.
(42) Koneman, E. W. *Testo Atlante di Microbiologia Diagnostica*, 2nd ed.; A. Delfino: Roma, 1995; pp 550–605.
(43) Camporese, A. *Riv. Ital. EPPOS* **1997**, *21*, 4.
(44) Performance standards for anti-microbial susceptibility testing: eleventh informational supplement. National Committee for Clinical laboratory Standard, NCCLS, document M100-S11, 2001.

NP070351S