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Composition of the Fresh Leaves and Stems of *Melissa officinalis* and Evaluation of Skin Irritation in a Reconstituted Human Epidermis Model

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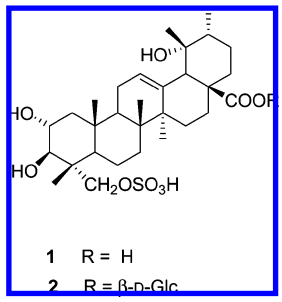
The composition of a centrifuged product obtained from the fresh leaves and stems of *Melissa officinalis* and skin irritation in the reconstituted human epidermis (Episkin model) have been investigated in comparison to the EtOH−H_2O (1:1) extract obtained by Soxhlet from the dried plant. Two new sulfated triterpenes (**1** and **2**) and two ionol derivatives have been isolated for the first time from *Melissa officinalis* together with caffeic and rosmarinic acids. The structures of compounds **1** and **2** were established by analysis of their spectroscopic data. Both the centrifuged material and its major constituents neither affected cell viability nor caused the release of pro-inflammatory mediators or the decrease of trans-epithelial electrical resistance (TEER) in the reconstituted human epidermis.

Lemon balm (*Melissa officinalis* L., Lamiaceae) is a very popular aromatic perennial herb growing in the Mediterriaean region and is used in the traditional medicine of Europe.[1] The volatile oil[1,2] has antibacterial and antifungal activities.[3,4] The hydroalcoholic extract possesses antioxidative properties related to the phenolic content, particularly of rosmarinic acid.[5−7] In previous work on *M. officinalis*, we reported the isolation of phenols and six new ursene and oleanene derivatives from the 1:1 EtOH−H_2O extract of the dried leaves and stems obtained by Soxhlet extraction, as well as the significant free-radical-scavenging and antimicrobial activities exhibited by the extract and its major constituent, rosmarinic acid.[8] Topical remedies prepared from extracts, juice, and plant homogenates of fresh lemon balm herb are used for its antiviral, antimicrobial, antioxidative, and antipruritic activities, in addition to the prevention of dandruff.[9−13] However, information on the constituents and bioactivity of the fresh *M. officinalis* plant is lacking. The present paper reports on the composition of the centrifuged material obtained using a tumble-dryer from the fresh leaves and stems of this species using EtOH−H_2O (1:1) and on the effect of its major constituents on tissue viability and release of pro-inflammatory mediators in a reconstituted human epidermis (RHE) model. In addition, two known ionol derivatives and two new sulfated triterpenes, **1** and **2**, have been isolated for the first time from *M. officinalis*. The structures of **1** and **2** were elucidated using spectroscopic methods including 1D- (^1H and ^{13}C) and 2D-NMR (DQF-COSY, HSQC, and HMBC) experiments as well as HRESIMS analysis. Differences in the composition as well as in the antioxidant and antimicrobial activities of the fresh centrifuged material with respect to the extract from dried plant were also evaluated.



Fresh stems and leaves of *M. officinalis* were centrifuged with EtOH−H_2O (1:1) using a tumble-dryer. The centrifuged product was dried in vacuo and then partitioned between water and *n*-BuOH. The *n*-BuOH-soluble fraction was subjected to purification over Sephadex LH-20 and by RP-HPLC to yield a new sulfated triterpene glucoside (**2**) and an α-ionol glucoside from fraction I, an α-ionol from fraction II, caffeic acid from fraction III, and rosmarinic acid and a new sulfated triterpene (**1**) from fraction IV. The known compounds were identified by comparison of their NMR data with those from the literature as 3,5,6-trihydroxydehydro-α-ionol,[14] 3,5,6-trihydroxydehydro-α-ionol 9-*O*-β-D-glucopyranoside,[15] caffeic acid,[16] and rosmarinic acid.[16] Rosmarinic acid has been previously isolated from the dried leaves and stems of the plant, but ionol derivatives are reported for the first time from *M. officinalis*.

The total phenolic content of the centrifuged material, as determined by the Folin−Ciocalteau method and expressed as a rosmarinic acid equivalent, and the concentrations of rosmarinic and caffeic acids, as determined by HPLC, were lower than in the extract from the dried plant (21.0 μg/mg, 0.08% and 0.01% in the fresh centrifuged product, compared with 250 μg/mg, 5.6% and 0.1%).[8] In the DPPH test, the well-recognized free-radical-scavenging activity of rosmarinic and caffeic acids was confirmed (EC_{50} 3.1 and 3.3 μg/mL, respectively), but no activity was shown by the ionol derivatives or by sulfated triterpenoids **1** and **2**, and the antioxidant activity of the centrifuged material was low compared to that of the extract from dried plant (EC_{50} 678.3 μg/mL vs 18.5 μg/mL, respectively) (see also Table S1, Supporting Information). No antimicrobial activity was observed, for either the centrifuged or isolated compounds, against several Gram-positive and Gram-negative bacteria, a yeast, and a mold, as tested by a broth microdilution method (see Table S2, Supporting Information). These findings demonstrate that the centrifuged material from *M. offinalis* possesses a rather low antioxidant and no antimicrobial activity, which may be correlated to its low total phenolic and rosmarinic acid content. Furthermore, the centrifuged material contained a series of characteristic compounds, such as ionol derivatives and sulfate triterpenes **1** and **2**, not found in the extract from the dried plant.

The structure elucidation of compounds **1** and **2** proceeded as follows. The HRESIMS of **1** showed a major ion peak at *m/z* 583.2915 $[M - H]^-$, consistent with the molecular formula $C_{30}H_{48}O_9S$ (calcd for $C_{30}H_{47}O_9S$, *m/z* 583.2941) and suggesting a triterpene derivative having a sulfate group in the molecule. MS-MS analysis of the ion peak showed a fragment ion at *m/z* 503.332 $[M - H - 80]^-$, indicating the loss of a sulfate group. Acid hydrolysis of **1**, followed by treatment with $BaCl_2$, gave a white precipitate, confirming the presence of a sulfate residue.[17] Inspection of the ^1H and ^{13}C NMR data (Table 1), assigned by $^1H-^1H$ DQF-

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

	1		2	
position	δ_C	δ_H (J in Hz)[b]	δ_C	δ_H (J in Hz)[b]
1	47.8	0.91, 1.97, m	47.8	0.93, 1.97, m
2	69.1	3.71, ddd, (3, 9.2, 13.0)	69.2	3.72, ddd, (3, 9.2, 13.0)
3	77.3	3.43, d, (9.2)	77.5	3.42, d, (9.2)
4	43.3		43.3	
5	48.1	0.94, m	48.2	0.95, m
6	18.7	1.42, 1.60, m	18.8	1.41, 1.58, m
7	33.5	1.31, 1.69, m	33.2	1.31, 1.68, m
8	40.7		40.8	
9	48.4	1.81, m	48.1	1.81, m
10	38.5		38.6	
11	24.9	2.04, 1.94, m	24.9	2.04, 0.98, m
12	128.7	5.30, t (3.5)	128.9	5.33, t (3.5)
13	140.9		140.0	
14	42.5		42.3	
15	29.5	0.98, 1.94, m	29.1	0.96, 1.86, m
16	26.7	2.46, 1.54, m	26.6	2.65, 1.65, m
17	49.4		49.1	
18	55.6	2.61, s	54.8	2.54, s
19	73.7		72.2	
20	42.9	1.39, m	42.9	1.35, m
21	27.3	1.18, 1.72, m	27.0	1.19, 1.62, m
22	39.4	1.63, 1.81, m	38.3	1.63, 1.87, m
23	70.2	3.76, d (9.6) 3.93, d (9.6)	70.3	3.76, d (9.6) 3.94, d (9.6)
24	13.6	0.78, s	13.6	0.76, s
25	17.3	1.06, s	17.5	1.05, s
26	18.1	0.88, s	17.4	0.79, s
27	24.5	1.34, s	24.5	1.35, s
28	177.7		179.5	
29	27.3	1.22, s	26.8	1.23, s
30	16.5	0.95, d (6.5)	16.7	0.95, d (6.5)
Glc-1′			95.3	5.35, d (7.6)
Glc-2′			73.6	3.34, dd (9.0, 7.6)
Glc-3′			78.2	3.36, t (9.0)
Glc-4′			71.0	3.40, t (9.0)
Glc-5′			77.8	3.27, m
Glc-6′			62.2	3.71, dd (12.0, 4.0) 3.83, dd (12.0, 2.5)

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

COSY, HSQC, and HMBC experiments, and comparison with chemical shift values of related glycosides isolated from the dried plant extract suggested a 23-hydroxytormentic acid derivative.[8] The hydrogen and carbon resonances were superimposable on those of the 3,23-disulfate ester of 23-hydroxytormentic acid,[8] except for the H-3ax and H-2ax (δ_H 3.43, d, $J = 9.2$ Hz and δ_H 3.71, ddd, $J = 3$, 9.2, 13.0 Hz, respectively) and C-3 (δ_C 77.3) signals. The upfield shifts observed for these signals with respect to the model compound[8] suggested the absence of the sulfate group at C-3. $^{1}H-^{1}H$ DQF-COSY experiments confirmed the proton sequence H-1a (δ_H 1.97), H-1b (δ_H 0.91), H-2 (δ_H 3.71), and H-3 (δ_H 3.43). The chemical shifts of the H-23a (δ_H 3.93) and H-23b (δ_H 3.76) signals and the HMBC correlations observed between the H-23a and H-23b resonance and C-4 (δ_C 43.3), C-3 (δ_C 77.3), C-5 (δ_C 48.1), and C-24 (δ_C 16.6) confirmed the placement of the sulfate group at C-23.[8] Thus, compound **1** was identified as the 23-sulfate ester of 2α,3β,19α,23-tetrahydroxyurs-12-en-28-oic acid (23-sulfate ester of 23-hydroxytormentic acid).

The HRESIMS of **2** (*m/z* 745.3502 $[M - H]^-$, calcd for $C_{36}H_{57}O_{14}S$, 745.3469) supported the molecular formula $C_{36}H_{58}O_{14}S$, suggesting a triterpene sulfate with an additional hexose unit in the molecule with respect to compound **1**. The MS-MS showed the most intense ion at *m/z* 583.289 $[M - H - 162]^-$, ascribable to the loss of a hexose unit. As in compound **1**, acid hydrolysis of **2**, followed by treatment with $BaCl_2$, again demonstrated the presence of a sulfate residue, and its position at C-23 was deduced by the chemical shifts of the pertinent hydrogen (H-23a and H-23b) and carbon (C-23) signals.[8] Full assignments of the proton and carbon resonances, obtained by $^{1}H-^{1}H$ DQF-COSY, HSQC, and HMBC spectra (Table 1), indicated that **2** is a β-glucopyranosyl (anomeric proton signal, δ_H 5.35, 1H, d, $J = 7.6$ Hz) derivative of compound **1**. The β-glucopyranosyl unit could be located at C-28 on the basis of the HMBC correlation observed between the anomeric proton signal and the carbon resonance at δ_C 179.5 (C-28). The sugar was determined to be D-glucose after hydrolysis of **2** with 1 N HCl and GC analysis. Thus, the structure of **2** was assigned as the 23-sulfate ester of 2α,3β,19α,23-tetrahydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (the 23-sulfate ester of niga-ichigoside F1).

To assess the safe topical use of the fresh centrifuged material of the *M. officinalis*, this and compound **1** were each tested on reconstituted human epidermis (RHE, Episkin-SM)[18] in vitro, in comparison to the extract from the dried plant and its major constituent, rosmarinic acid, at concentrations higher than those (0.01−1.00%) used in topical remedies.[12,13] The Episkin model has been reported to mimic morphologically and biochemically living skin and has been validated to classify skin irritants able to produce a decrease in cell viability, as evaluated by a MTT assay, below defined threshold levels (≤50%)[18,19] and an increased release of the pro-inflammatory mediator interleukin IL-1α, as evaluated by an ELISA assay,[18,19] after an acute exposure (15 min followed by 42 h of recovery).[18,19] To better discriminate nonirritant products, a third end point was evaluated, trans-epithelial electrical resistance (TEER), measuring the barrier function of the epithelium.[19,20] Solutions (2%) of the centrifuged plant material and of the extract from the dried plant, and 1% solutions of compound **1** (17.1 mM) and rosmarinic acid (27.7 mM) in PBS (phosphate buffer) were applied directly on the Episkin surface in comparison to PBS as control and to a surfactant (sodium dodecyl sulfate, SDS 5%), as an irritant reference compound. After topical exposure for 15 min followed by 42 h of recovery, tissue viability, release of IL-1α, and TEER values were determined. The epidermis viability, measured as optical density at 570 nm by the MTT assay[21] and calculated as percentage of cytotoxicity compared to the control (PBS-treated epidermis), was not significantly reduced after application of all product tested (viability >75%, see Figure S1, Supporting Information). No increased release of IL-1α, expressed as pg/mL in the medium underneath, was observed in tissue treated with all products at 42 h, compared to the control. Using the same test conditions, SDS (5%) caused an elevation of the pro-inflammatory mediator release to about 300 pg/mL (Figure 1). Finally, no significant decrease of TEER values was observed in epidermis treated with all products from *M. officinalis*, compared to the control. The irritant surfactant, SDS, impairing the barrier integrity of the epithelium, caused a reduction of −14 kΩ cm^2 in TEER (Figure 2). Although compound **1** possesses a surfactant-like structure, it showed neither cytotoxic nor pro-inflammatory effects or affected TEER reduction in the RHE. On the basis of the absence of skin-irritant effects at the concentrations tested, centrifuged lemon balm and compound **1**, as well as the dried balm extract and rosmarinic acid, appear to be safe for topical use.

Experimental Section

General Experimental Procedures. Melting points were determined using a Mettler-Toledo DSC822^e apparatus. 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 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. 1D- and 2D-NMR experiments were carried out using conventional pulse sequences. ESIMS was performed on a Finnigan LC-Q Deca instrument (Thermoquest, San Jose, CA) equipped with Xcalibur software. Exact masses (HRESIMS) were measured by a Q-TOF Premier (Waters) triple-

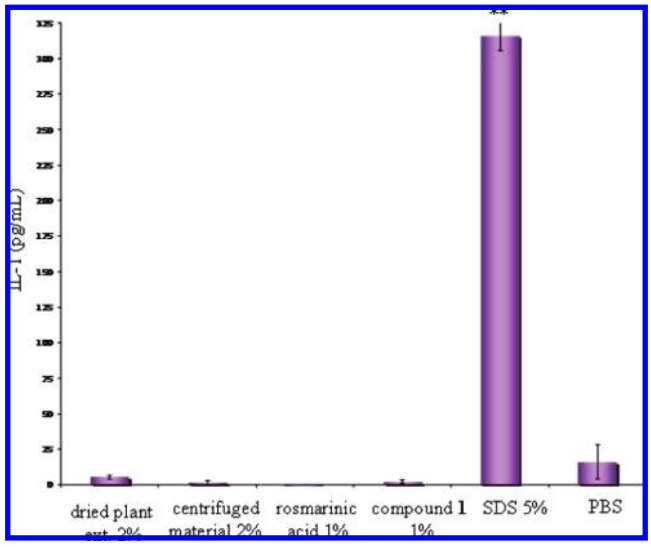

Figure 1. Effect of the centrifuged material (2%), the extract from dried plant (2%, dried plant ext.), rosmarinic acid (1%, 27.7 mM), and compound **1** (1%, 17.1 mM) on Il-1α release (pg/mL of medium), estimated by an ELISA assay, in reconstituted human epidermis (Episkin-SM) after 15 min of exposure followed by 42 h of recovery compared to a control (PBS) and a toxic reference compound, SDS (5%). Values are expressed as mean ± SD of three independent experiments. Comparison were performed using a one-way ANOVA test, $^{**}p < 0.01$ calculated vs control (PBS).

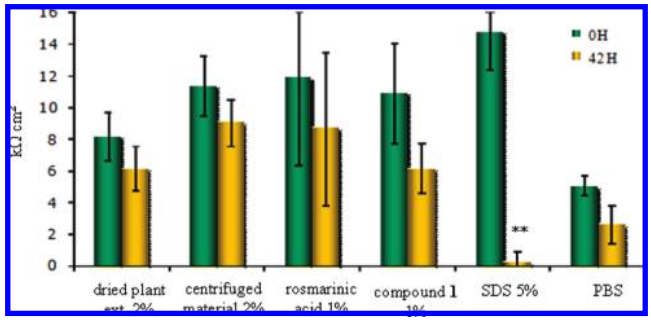

Figure 2. Effect of the centrifuged material (2%), the extract from dried plant (2%, dried plant ext.), rosmarinic acid (1%, 27.7 mM), and compound **1** (1%, 17.1 mM) on TEER (trans-epidermal electric resistance, kΩ cm^2), estimated using Millicel-ERS (resistance range 0−20 kΩ) volt/ohm-meter, in reconstituted human epidermis (Episkin-SM) at time 0 and after 15 min of exposure followed by 42 h of recovery compared to PBS and toxic reference compound SDS (5%). Values are expressed as mean ± SD of three independent experiments. Comparisons were performed using a one-way ANOVA test, $^{**}p < 0.01$ calculated vs control (PBS).

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 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 0049 MM) was deposited at the Herbarium of Respharma s.r.l., Trezzo sull'Adda, Italy.

Extraction and Isolation. Fresh aerial parts (stems and leaves) of *M. officinalis* (500 g) were centrifuged in a ALC-PK 120 (Thermo Electron Corporation) tumble-dryer apparatus with EtOH−H_2O 1:1 (1 L). The centrifuged material was dried in vacuo and then partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH-soluble portion (5.0 g). A portion (2.5 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 (silica gel, using *n*-BuOH−AcOH-H_2O (60:15:25) and $CHCl_3$−MeOH-H_2O (7:3:0.3) as solvents. Fractions with similar R_f values were combined, giving four major fractions (I−IV), which were further purified by RP-HPLC on a 30 cm × 7.8 mm i.d. μ-Bondapack column (flow rate 2.0 mL min^{-1}). Fraction I (413 mg) was chromatographed with MeOH−H_2O (35:65) as mobile phase to yield 3,5,6-trihydroxydehydro-α-ionol 9-*O*-*β*-D-glucopyranoside (4.0 mg) and compound **2** (4.6 mg). Fractions II (704 mg) and IV (223 mg) were purified using MeOH−H_2O (3:7) as mobile phase. Fraction II yielded 3,5,6-trihydroxydehydro-α-ionol (2.8 mg). Fraction IV gave rosmarinic acid (120 mg) and compound **1** (5.1 mg). Fraction III (316 mg) was purified with MeOH−H_2O (4:6) as the eluent to afford caffeic acid (4.0 mg).

Compound (1): white powder; mp 211 °C; $[\alpha]^{31}_D$ +19.6 (*c* 0.066, MeOH); ^{1}H (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150.9 MHz) data are reported in Table 1; HRESIMS *m/z* 583.2915 [M − H]$^-$ (calcd for $C_{30}H_{47}O_9S$, 583.2941).

Compound (2): white powder; mp 237 °C; $[\alpha]^{31}_D$ +5.9 (*c* 0.113, MeOH); ^{1}H (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150.9 MHz) data are reported in Table 1; HRESIMS *m/z* 745.3502 [M − H]$^-$ (calcd for $C_{36}H_{57}O_{14}S$, 745.3469).

3,5,6-Trihydroxydehydro-α-ionol: ^{1}H and ^{13}C NMR data were consistent with those previously reported;[14] ESIMS *m/z* 243 [M − H]$^-$.

3,5,6-Trihydroxydehydro-α-ionol 9-*O*-*β*-D-glucopyranoside: ^{1}H and ^{13}C NMR data were consistent with those previously reported;[15] ESIMS *m/z* 405 [M − H]$^-$.

Caffeic acid: ^{1}H and ^{13}C NMR data were consistent with those previously reported;[16] ESIMS *m/z* 179 [M − H]$^-$.

Rosmarinic acid: ^{1}H and ^{13}C NMR data were consistent with those previously reported;[16] ESIMS *m/z* 359 [M − H]$^-$.

Acid Hydrolysis. Compound **2** (0.8 mg) in 1 N HCl (0.25 mL) was subjected to hydrolysis, derivatized in 1-(trimethylsilyl)imidazole and pyridine (0.1 mL), and analyzed by GC using an L-Chirasil-Val column (0.32 mm × 25 m), according to a method previously reported.[8] The peak from the hydrolysate of **2** was detected at 14.74 min (D-glucose). The peak for standard D-glucose was detected at 14.71 min.

Sulfate Group Detection. Both compounds **1** and **2** (1 mg of each) were 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 ($BaSO_4$).[17]

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, elution gradient, and all experimental conditions were as reported in a previous paper.[8] Analysis was carried out in triplicate. Reference standard solutions of rosmarinic acid were prepared at three concentration levels in the range 0.25−2.00 mg/mL. The peak associated with rosmarinic acid was identified by retention time and spectroscopic UV and MS comparison with a standard, and confirmation was by co-injection. The centrifuged product and its *n*-BuOH-soluble portion were redissolved in MeOH and analyzed under the same chromatographic conditions.

Quantitative Determination of Total Phenols. The centrifuged material and its *n*-BuOH-soluble portion, dissolved in MeOH, were analyzed for their total phenolic content according to the Folin−Ciocalteau colorimetric method.[8] Total phenols were expressed as rosmarinic acid equivalents.

Bleaching of the Free Radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH Test). The antiradical activities of the centrifuged plant material, rosmarinic acid, and α-tocopherol were determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method, according to the procedure previously described by Mencherini et al.[8] Briefly, an aliquot (37.5 μL) of the MeOH solution containing different amounts of the centrifuged material and its *n*-BuOH-soluble portion, or rosmarinic acid, was added to 1.5 mL of DPPH solution (0.025 g/L in MeOH) daily prepared; the maximum concentration employed was 200 μg/mL. An equal volume (37.5 μL) of the vehicle alone was added to control tubes. Absorbances at 515 nm were measured on a Shimadzu UV-1601 UV−visible spectrophotometer 10 min after starting the reaction. 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[22] (see Table S1, Supporting Information).

Antimicrobial Activity. The centrifuged plant material, its *n*-BuOH-soluble portion, and rosmarinic acid were tested for antimicrobial activity using the broth microdilution method in 96-multiwell microtiter plates, in duplicate, as reported by Mencherini et al.[8] and as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2001),[23] using Gram-positive and Gram-negative bacteria, a yeast, and a mold, all from the American Type Culture Collection (ATCC). The lowest concentrations of the products at which microbial growth was inhibited after 24 h (MIC) and at which survival of any microbial cell was not possible after incubation for 48 (bacteria strains) and 5 days (yeasts and molds) (MBC) were determined and are reported in the Supporting Information (Table S2).

Toxicological Methods. Materials and Apparatus. 3-(4,5-Dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffer solution (PBS), and 2-propanol were from Sigma Chemical Co. (Milan, Italy). The reconstituted human epidermis (RHE) model (Episkin-SM, Episkin SNC, Lyon, France) of 0.63 cm^2 dimensions was used for skin-irritation testing according to the validated method (ESAC Statement 2007).[18,24]

For quantification of the IL-1α release in the medium underneath, an Elisa kit Quantikine DLA-50 (R&D Systems, San Diego, CA) and a M-200 Infinite microplate autoreader (Tecan) were used. For TEER (trans-epidermal electric resistance) determination), a Millicel-ERS (resistance range 0−20 kΩ) volt/ohm-meter (Millipore, Billerica, MA), able to measure membrane potential and resistance of epithelial cells grown on microporous membranes, was employed, equipped with a silver/silver chloride (Ag/AgCl) electrode to measure trans-epithelial voltage and the resistance of cells.

Skin Irritation on the Episkin Model. The extract of the dried plant prepared as reported by Mencherini et al.,[8] the centrifuged material from the fresh plant, rosmarinic acid, and compound **1** were dissolved in PBS to obtain 2% (extract) and 1% (pure compound) concentrations, respectively. PBS solutions (10 μL) of the test products were applied in duplicate, using a micropipet, to the surface of epidermis in a single well treated with 300 μL of its specific maintenance medium. Tissues were maintained at room temperature for 15 min, washed three times with PBS (1 mL), and then incubated at 37 °C in a 5% CO_2 atmosphere for 42 h. The culture medium was changed daily. The same volumes of PBS at pH 7.2 and sodium dodecyl sulfate (SDS 5%) were applied to control wells. After 42 h of incubation, three complementary end points were evaluated: (i) cellular parameter, the cytotoxicity by MTT assay; (ii) molecular parameter, the release of IL-1α; (iii) electrical resistance parameter, the TEER value. The tests were determined in triplicate.

Cell Viability. Cell viability was assessed according to Picerno et al.,[21] using the MTT assay, as previously reported.[21] Optical density was read at 570 nm against a blank of 2-propanol.

Release of Interleukin IL-1α. Underlying culture media were collected at 42 h after product application in triplicate. The release of IL-1α in the medium of each well was quantified by an Elisa Quantikine DLA-50 (R&D Systems) kit, as reported in a previous paper.[21]

Trans-Epidermal Electric Resistance (TEER). The resistance was measured (in kΩ) on the same tissues in triplicate at time 0 h (nontreated tissue R_{t0}) and after the treatment with the test products at 42 h (R_{t42}). Owing to the variability within tissues, the measurement performed at t 0 h has been taken as a basal value and the reference of each single tissue. R_{t0} and R_{t42} were calculated as follows: $R = R_{sample} - R_{blank}$ where $R_{blank} = R$ of the culture medium (PBS solution).

Since the resistance is inversely proportional to the area of the tissue, TEER in kΩ cm^2 was calculated, correcting for the area covered by the tissue, as the product of the resistance found in the experiments and the area of effective membrane diameter.

Statistical Analysis. Data on cell viability are expressed as percentage of viability versus negative control (PBS-treated epidermis). Data on IL-1α release are expressed in pg/mL in the medium underneath. TEER values are expressed in kΩ cm^2. All data were calculated from mean ± SD values of three independent determinations. Statistical analysis was performed by ANOVA test and multiple comparison by a Bonferroni test.[22] All experiments were made at least three times, each time with three or more independent observations.

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Supporting Information Available: 1D and 2D NMR spectra of the new compounds **1** (^{1}H, HSQC, and HMBC) and **2** (^{1}H, TOCSY, HSQC, and HMBC), as well as tables with the DPPH, the antimicrobial, and the MTT assay results. This information is available free of charge via the Internet at http://pubs.acs.org.

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