Sulfated Triterpenes from Lemon Balm

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Dedicated to Prof. *Otto Sticher*, ETH Zurich, Switzerland, for his pioneering work in phytochemistry and pharmacognosy

The hydroalcoholic ($EtOH/H_2O$) extract of matured leaf margins of lemon balm (*Melissa officinalis* L.) afforded a new 3,23-disulfate of 2α,3β-23,29-tetrahydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (**1**) and a new 23-monosulfate of 2α,23-dihydroxyurs-12-en-28-oic acid 3-*O*-β-D-glucopyranoside (**2**), along with six known compounds, *i.e.*, 23-monosulfate of 2α,3β,19α,23-tetrahydroxyurs-12-ene-28-oic acid 28-*O*-β-D-glucopyranoside (**3**), 3,5,6-trihydroxydehydro-α-ionol 9-*O*-β-D-glucopyranoside (**4**), quadranoside III (**5**), rosmarinic acid (**6**), caffeic acid (**7**), and luteolin (**8**). All the isolated compounds were evaluated for their antioxidant, antimicrobial, antimalarial, and cytotoxic activities. Only rosmarinic acid exhibited substantial antioxidant and antimicrobial activities, whereas sulfated terpenes showed considerably lower or no antimicrobial activity.

Introduction. – Lemon balm (*Melissa officinalis* L.) is an important aromatic plant, cultivated for its lemon-scented leaves used for seasoning and in medicine. There are many reports in the literature on the essential oils of lemon balm [1]. Mostly the volatile oil, its chemical profile, and its different pharmacological properties such as antifungal, antibacterial, and spasmolytic activities [2–4] are well-documented. The alcoholic extracts of lemon balm exhibited antioxidant properties, because of high phenolic content such a rosmarinic acid [5]. Biosynthesis of proteins in cancer cells by lemon balm-containing substances has been reported. Studies on the volatile oils of the balm are extensive, but properties of secondary metabolites have not been investigated in detail. Herein, we describe the chemical profiling of leaf margins of lemon balm. The hydroalcoholic extraction of the leaf margins led to the isolation of two sulfated ursane-type triterpenes, one sulfated oleanane-type triterpene, one oleanane triterpene, an α-ionol derivative, rosmarinic acid, caffeic acid, and luteolin. Further, the essential oil from leaf margins was also analyzed. This is a part of the work, in accordance with 'Quality by Design' approach of medicinal and aromatic plants (MAPs) of Western

Himalayas, undertaken by our Centre. All the compounds were evaluated for their antioxidant, antimicrobial, antimalarial, and cytotoxic activities. The characterization of the compounds was accomplished by ^{1}H- and ^{13}C-NMR, ^{1}H,^{1}H-DFQ-COSY, HMBC, HSQC, HMQC, TOCSY, and HR-ESI-MS experiments.

Results and Discussion. – The fresh matured leaf margins of lemon balm were separated from main leaf with scissors, and then dried in the shade. The dried plant material was coarsely powdered, extracted with EtOH/H_2O 80:20, and the residue obtained was partitioned between H_2O and ^{i}PrOH. The ^{i}PrOH soluble portion was chromatographed over silica gel (40 μm), followed by *Sephadex LH-20* and *RP-C8*/*C18*, which led to the isolation of compounds **1**–**8**.

The mass spectra of compound **1** (*Fig. 1*) displayed a molecular ion peak at *m*/*z* 827.1723 ($[M+H]^+$) corresponding to the molecular formula $C_{36}H_{58}O_{17}S_2$, supported by ^{13}C-NMR spectra, which showed 36 C-atom resonances suggesting a triterpene derivative with a hexose unit. The DEPT experiment revealed the presence of five Me, twelve CH_2, and eleven CH groups. Acid hydrolysis of **1**, followed by treatment with $BaCl_2$, gave a white precipitate, confirming the presence of a sulfate [6]. This was supported by an intense IR absorption band at 1265 cm^{-1} for S–O bond stretching [7]. The ^{1}H-NMR (*Table 1*) exhibited signals corresponding to five tertiary Me groups (δ(H) 1.01, 1.07, 0.98, 1.12, and 1.03), an olefinic H-atom (δ(H) 5.31 (*t*, *J* = 8.0)), and signals for H–C(18) (δ(H) 2.92) and for H_{ax}–C(3) (δ(H) 4.17 (*d*, *J* = 9.3)), typical of a 3β-oxyolean-12-en-28-oic acid skeleton [8]. The signals at δ(H) 4.01 (*dd*, *J* = 3.0, 9.3) and 3.18 (*s*) indicated the presence of secondary and primary OH functions [8]. The presence and attachment of OH groups was confirmed by the following correlations observed in HMBC and HSQC spectra: δ(H) 4.01 (*dd*, *J* = 3.0, 9.3)/δ(C) 89.4 (C(3)), 42.1 (C(4)), and 37.7 (C(10)), and δ(H) 3.18 (*s*)/δ(C) 37.1 (C(20)), 42.2 (C(19)), and 28.2 (C(21)). The attachment of HO_3SO moieties with the aglycone skeleton was confirmed by the downfield chemical shifts observed for C(3) and H–C(3), and C(23) and CH_2(23) (δ(C) 89.4 (C(3)/δ(H) 4.17 (*d*, *J* = 9.3, H–C(3)), and δ(C) 70.8 (C(23))/δ(H) 3.92, 3.79 (CH_2(23)). These values correlated with those in the literature and were consistent with the presence of sulfate groups [9]. The signals in the ^{13}C-NMR spectra at δ(C) 102.4 (C(1′)), 76.6 (C(2′)), 78.8 (C(3′)), 71.7 (C(4′)), 75.4 (C(5′) and 61.9 (C(6′)

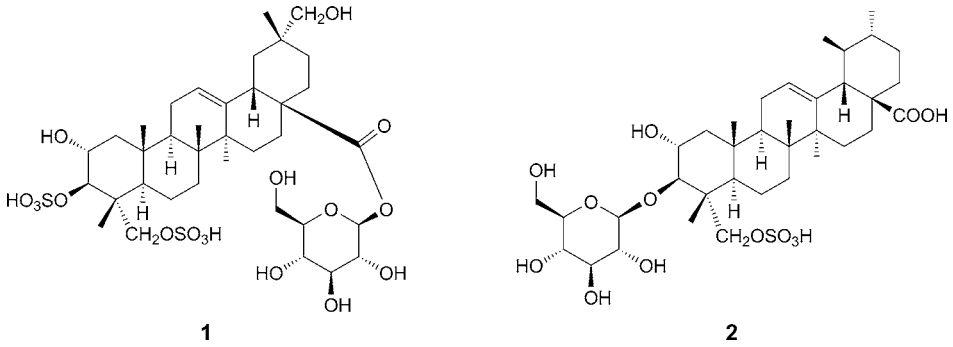


Fig. 1. *Structures of compounds* **1** *and* **2**

Table 1. *^{1}H- and ^{13}C-NMR Data* (500 and 125 MHz, resp., in CD_3OD) *of* **1** *and* **2**. δ in ppm, J in Hz.

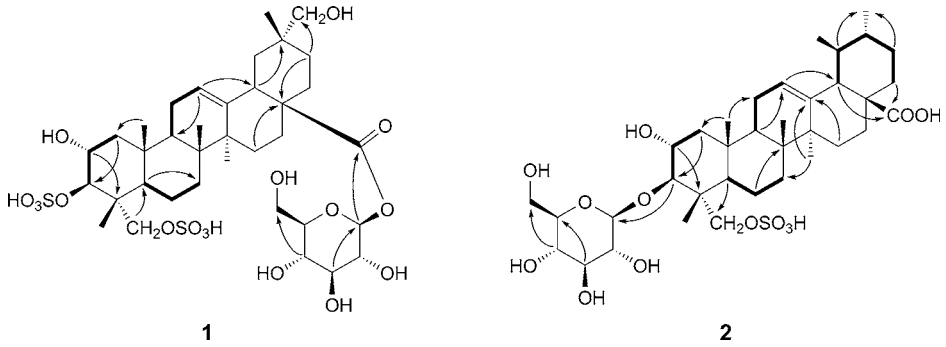
Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
1	1.01–1.95 (*m*)	46.9 (*t*)	0.98–1.95 (*m*)	46.5 (*t*)
2	4.01 (*dd*, *J* = 3.0, 9.3)	69.1 (*d*)	3.99 (*dd*, *J* = 9.0, 12.0)	68.9(*d*)
3	4.17 (*d*, *J* = 9.3)	89.4 (*d*)	4.30 (*d*, *J* = 9.0)	84.2 (*d*)
4		42.1 (*s*)		43.1 (*s*)
5	1.39–1.43 (*m*)	48.2 (*d*)	1.49 (*m*)	47.5 (*d*)
6	1.46–1.55 (*m*)	19.2 (*t*)	1.41–1.62 (*m*)	19.1 (*t*)
7	1.29–1.58 (*m*)	32.7 (*t*)	1.28–1.71 (*m*)	33.3 (*t*)
8		41.3 (*s*)		37.9 (*s*)
9	1.55–1.63 (*m*)	48.9 (*d*)	1.75–1.83 (*m*)	45.7 (*d*)
10		37.7 (*s*)		36.6 (*s*)
11	1.62–1.89 (*m*)	23.5 (*t*)	1.81–1.99 (*m*)	25.1 (*t*)
12	5.31 (*t*, *J* = 8.0)	124.0 (*d*)	5.29 (*t*, *J* = 4.5)	126.5 (*d*)
13		143.3 (*s*)		142.3 (*s*)
14		42.9 (*s*)		41.2 (*s*)
15	1.01–1.92 (*m*)	27.8 (*t*)	0.95–1.95 (*m*)	28.8 (*t*)
16	1.65–2.02 (*m*)	24.3 (*t*)	1.51–2.30 (*m*)	25.0 (*t*)
17		48.8 (*s*)		48.8 (*s*)
18	2.92 (*dd*, *J* = 3.5, 12.0)	41.5 (*d*)	2.48–2.52 (*m*)	42.4 (*d*)
19	1.02–1.85 (*m*)	42.2 (*t*)	1.75–1.79 (*m*)	39.1 (*d*)
20		37.1 (*s*)	1.02–1.12 (*m*)	42.2 (*d*)
21	1.69–1.71 (*m*)	28.2 (*t*)	1.17–1.69 (*m*)	32.3 (*t*)
22	1.68–1.82 (*m*)	30.2 (*t*)	1.60–1.79 (*m*)	37.5 (*t*)
23	3.92 (*d*, *J* = 9.5), 3.79 (*d*, *J* = 9.5)	70.8 (*t*)	3.92 (*d*, *J* = 9.5), 3.71 (*d*, *J* = 9.5)	70.2 (*t*)
24	1.01 (*s*)	16.2 (*q*)	1.01 (*s*)	15.1 (*q*)
25	1.07 (*s*)	18.9 (*q*)	1.07 (*s*)	17.1 (*q*)
26	0.98 (*s*)	18.1 (*q*)	1.02 (*s*)	18.2 (*q*)
27	1.12 (*s*)	25.4 (*q*)	1.30 (*s*)	25.0 (*q*)
28		179.2 (*s*)		178.1 (*s*)
29	3.18 (*s*)	74.4 (*t*)	1.13 (*d*, *J* = 4.5)	29.2 (*q*)
30	1.03 (*s*)	19.3 (*q*)	1.13 (*d*, *J* = 4.7)	17.3 (*q*)
1′	4.97 (*d*, *J* = 7.7)	102.4 (*d*)	4.95 (*d*, *J* = 7.7)	101.3 (*d*)
2′	4.51 (*t*, *J* = 8.4)	76.6 (*d*)	4.48 (*t*, *J* = 8.2)	75.8 (*d*)
3′	4.19–4.23 (*m*)	78.8 (*d*)	4.18–4.22 (*m*)	77.7 (*d*)
4′	4.23–4.29 (*m*)	71.7 (*d*)	4.22–4.27 (*m*)	72.7 (*d*)
5′	3.91–3.95 (*m*)	75.4 (*d*)	3.88–3.91 (*m*)	75.2 (*d*)
6′	3.43–4.35 (*m*)	61.9 (*t*)	3.42–4.33 (*m*)	62.0 (*t*)

evidenced the presence of a glucose moiety [10]. The attachment of sugar to aglycone through a ester linkage was confirmed by a HMBC δ(H) 4.97 (*d*, *J* = 7.7, H–C(1′))/δ(C) 179.2 (C(28)). The ESI-MS/MS peaks at *m*/*z* 747.3678, 667.5633, and 505.4880 were attributed to fragment ions $[M - SO_3 + H]^+$, $[M - 2\,SO_3 + H]^+$, and $[M - 2\,SO_3 - \text{sugar} + H]^+$, respectively, indicating the loss of mass units 79.8045 and 164.0833. This fragmentation pattern in ESI-MS/MS further supported the presence of two sulfate groups and one glucose moiety in the molecule. Based on the above evidences, compound **1** was identified as 3,23-disulfate of 2α,3β-23,29-tetrahydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (*Fig. 1*).

The HR-ESI-MS of compound **2** (*m/z* 731.2157 ($[M+H]^+$)) revealed the molecular formula $C_{36}H_{58}O_{13}S$, indicating a molecule with the same skeletal pattern as that of **1**. The 96 lesser mass units of **2** than that of **1** suggested the possibility that **2** contained four O-atoms and one S-atom less in the molecule than **1**. The ESI-MS/MS fragment ion peak at *m/z* 79.8041 indicated 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 moiety, which also was supported by the intense absorption band at 1259 cm^{-1} for S–O bond stretching in IR spectrum. The ^{1}H-NMR signals of six tertiary Me groups (δ(H) 1.01 (*s*), 1.02 (*s*), 1.30 (*s*), 1.07 (*s*), 1.13 (*d*, *J* = 4.5), and 1.13 (*d*, *J* = 4.7) indicated an ursane-type of triterpene [11] which was further supported by the DEPT resonances of six Me, ten CH_2, and 13 CH groups, and seven quaternary C-atoms. The downfield resonances at δ(H) 5.29 (*t*, *J* = 4.5), 3.99 (*dd*, *J* = 9.0, 12.0), and 3.92 (*d*, *J* = 9.5), 3.71 (*d*, *J* = 9.5) corresponding to the olefinic H-atom (H–C(12)), a secondary carbinol H-atom (H–C(2)), and primary CH_2(23)O H-atoms. The downfield signal of H–C(3) at δ(H) 4.30 (*d*, *J* = 9.0) indicated that the C(3) was glycosylated, which was further supported by the HMBC δ(H) 4.95 (*d*, *J* = 7.7)/δ(C) 84.2 (C(3)). The signal at δ(C) 178.1 (*s*) was assigned to carboxy C=O group, C(28), by HMBC δ(H) 2.48–2.52 (*m*, H–C(18)/δ(C) 178.1 (C(28)) (*Table 1* and *Fig. 2*). The series of resonances at δ(C) 101.3 (C(1′)), 75.8 (C(2′)), 77.7 (C(3′)), 72.7 (C(4′)), 75.2 (C(5′)), and 62.0 (C(6′)) identified the glucose moiety as hexopyranose [12]. Based on the above evidence the compound **2** was elucidated as 23-monosulfate ester of 2α,23-dihydroxyurs-12-ene-28-oic acid 3-*O*-β-D-glucopyranoside (*Fig. 1*).

The other six compounds, **3**–**8** (*Fig. 3*), were characterized as 23-monosulfate ester of 2α,3β,19α,23-tetrahydroxyurs-12-en-28-oic acid 28-*O*-β-D-glucopyranoside (**3**) [6], 3,5,6-trihydroxydehydro-α-ionol 9-*O*-β-D-glucopyranoside (**4**) [13], quadranoside III (**5**) [14], rosmarinic acid (**6**) [15], caffeic acid (**7**) [16], and luteolin (**8**) [17] (*Fig. 3*).

Compounds **1**–**8** were tested for antioxidant, antimicrobial, antimalarial and cytotoxic activities. Compounds **6**–**8** showed prominent antioxidant activities at 1.0, 3.1, and 3.7 μg/ml, respectively (positive control vitamin C, 1.02 μg/ml; *Table 2*). In an antimicrobial assay, all compounds were evaluated against four fungal strains, *Candida albicans*, *C. glabrata*, *C. krusei*, and *Aspergillus fumigatus*, and four bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacte-*



1 **2**

Fig. 2. *^{1}H,^{1}H-DQF-COSY* (—) *Correlations, and HMBCs* (H → C) *and HMQCs of compounds* **1** *and* **2**

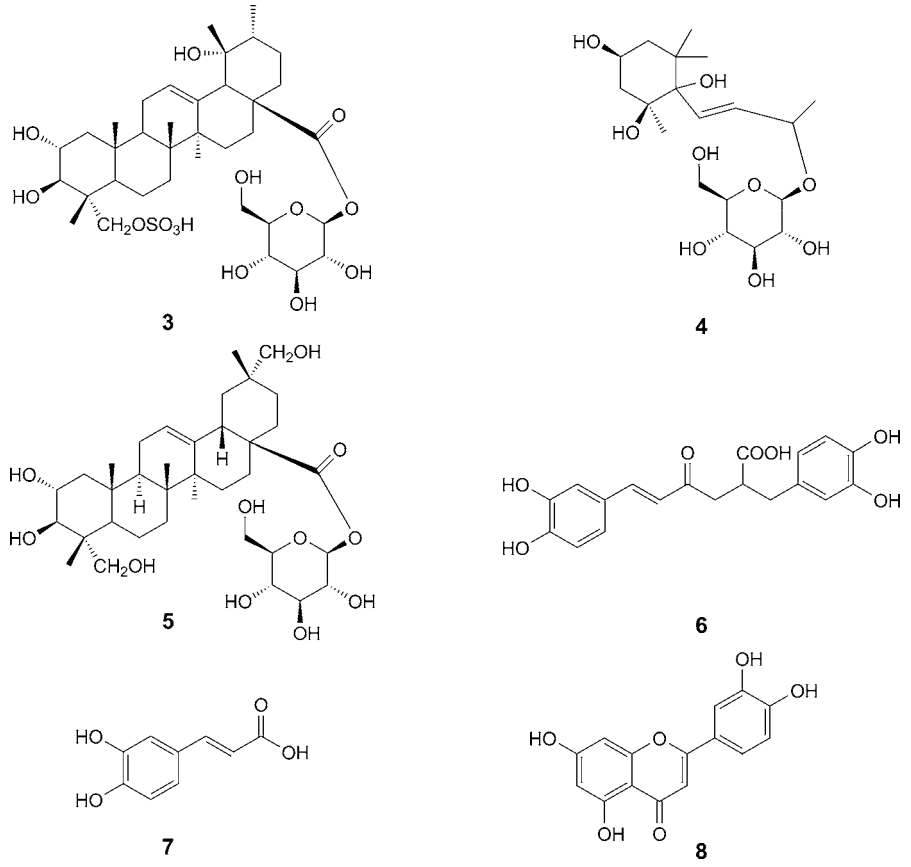


Fig. 3. *Structures of compounds* **3**–**8**

rium intracellulare (*Table 3*). Compounds **6**–**8** showed inhibitions at *MIC* values of 1.5, 3.4, and 2.1 μg/ml against *E. coli*, *P. aeruginosa*, and *C. krusei*, respectively. In the antimalarial assay, most of the compounds did not show significant results; only compound **4** and **8** displayed some activity at 16.2 and 14.3 μg/ml, respectively,

Table 2. *Antioxidant Activities of Compounds* **1**–**8**

Compound	IC_{50}[a]) [μg/ml]	Compound	IC_{50} [μg/ml]
1	32.2	**5**	NA[b])
2	26.4	**6**	1.0
3	22.5	**7**	3.1
4	17.1	**8**	3.7
Vitamin C[c])	1.02		

[a]) IC_{50}, the concentration that results in 50% inhibition. [b]) NA, No action until 40 μg/ml. [c]) Positive control.

Table 3. *Antimicrobial Activities of Compounds* **1**–**8**

Compound	*MIC*[a]) [μg/ml]	Compound	*MIC* [μg/ml]
1	41.2	**5**	NA[b])
2	32.4	**6**	1.5
3	29.7	**7**	3.4
4	NA	**8**	2.1
Ciprofloxacin[c])	0.98		

[a]) *MIC*, Minimum inhibitory concentration (the lowest concentration that allows no detectable growth). [b]) NA, No action until 50 μg/ml. [c]) Positive control.

Table 4. *Antimalarial* (*in vitro*) *Activities of Compounds* **1**–**8**

Compound	IC_{50}[a]) [μg/ml]	Compound	IC_{50} [μg/ml]
1	NA[b])	**5**	NA
2	NA	**6**	41.2
3	NA	**7**	35.5
4	16.2	**8**	14.3
Chloroquine[c])	2.50		

[a]) IC_{50}, the concentration that results in 50% inhibition. [b]) NA, No action until 90 [μg/ml]. [c]) Positive control.

Table 5. *Cytotoxic Activities of Compounds* **1**–**8**

Compound	IC_{50}[a]) [μg/ml]	Compound	IC_{50} [μg/ml]
1	NA[b])	**5**	NA
2	NA	**6**	34.6
3	NA	**7**	41.1
4	NA	**8**	62.4
Doxorubicin[c])	0.90		

[a]) IC_{50}, the concentration that results in 50% inhibition. [b]) NA, No action until 100 [μg/ml]. [c]) Positive control.

compared with the positive control chloroquine 2.50 μg/ml (*Table 4*). None of the compounds displayed cytotoxic activity, only **6** at a concentration of 34.6 μg/ml showed some inhibition compared with the positive control 0.90 μg/ml (*Table 5*).

Conclusions. – We described the chemical profiling (isolation and essential-oil analyses) of leaf margins of lemon balm, the hydroalcoholic extract of the leaf margins, which led to the isolation of two sulfated ursane-type triterpenes, one sulfated oleanane-type triterpene, one oleanane triterpene, α-ionol derivative, rosmarinic acid, caffeic acid, and luteolin. The isolation of such diverse natural products from lemon balm supports the biogenesis of isoprenoid, phenylpropanoid, and shikimic acid pathway in genus *Melissa*. This is the first report on chemical investigation and essential-oils composition of leaf margins only of Lemon balm.

M. A. T. thanks the *Council of Scientific and Industrial Research* (CSIR), New Delhi, India, for providing fellowship as Pool Officer under the Grant No. IA-27401.

Experimental Part

General. Sugar samples and *p*-anisaldehyde were purchased from *Sigma–Aldrich* (St. Lousis, MO). The solvents (*Fisherbrand*) used for HPLC and other chromatographic procedures were of HPLC and certified grades, resp. TLC: Aluminium backed plates precoated with silica gel F_{254} (20 × 20 cm, 200 μm, 60 Å; *Merck*); visualization by spraying with *p*-anisaldehyde (0.5 ml in 50 ml glacial AcOH) reagent, followed by heating. Gravity column chromatography (CC): silica gel (SiO_2, 40 μm for flash chromatography, 60 Å; *J. T. Baker*), reversed phase *RP-C18* silica (*Polarbond*, *J. T. Baker*), and *Sephadex LH-20* (*Sigma*); *Sep-Pak* cartridges *C-18* 60 ml, 10 g) were purchased from *Supelco*. HPLC: *Waters Alliance 2695*, equipped with 996 photodiode array detector (*Waters Corp.*, Milford, MA), computerized data station (*Waters Empower-2* software), and *Luna C-18* column (150 × 4.6 mm, 5 μm particle size; *Phenomenex Inc.*, Torrance, CA) connected with a 2-cm *LC-18* guard column (*Phenomenex Inc.*). Optical rotations: *Rudolph Research Analytical Autopol IV* automatic polarimeter at r.t. IR Spectra: *Bruker Tensor 27* spectrophotometer; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Varian AS 500* NMR spectrometer; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI-MS and HR-ESI-MS: *Agilent Series 1100 SL* mass spectrometer; in *m*/*z*. GC/MS: *HP 6890* series GC, equipped with a split/splitless cap. injector, a *HP 6890* series injector autosampler, and an *Agilent DB-5* ms column (30 m × 0.25 mm × 0.25 μm). The GC was interfaced to a *HP 5973* quadrupole mass-selective detector through a transfer line set at 280°. The injector temp. was 250°, and 1-μl injections were performed in split (1:10) mode. Column flow was set at a constant pressure 30 psi, with an initial flow of 1.0 ml/min, using He as carrier gas. The oven temp. was raised from 50 to 350° (hold 8.5 min) at a rate of 20°/min, for a total run time of 25 min. The filament was operated at 70 eV, with an emission current of 35 μA. The multiplier voltage was automatically set to 2240 V. The ion source and quadrupole temp. were 230 and 150°, resp. The acquisition range was *m*/*z* 30–800 at 1.95 scan/s, starting 3.5 min after injection.

Plant Material. The leaves of lemon balm were collected from Bonera Pulwama (Kashmir Valley) and identified by the taxonomist at Centre for Biodiversity and Taxonomy Biodiversity (CBT), University of Kashmir, Srinagar India. A voucher specimen of the plant was deposited.

Extraction and Isolation. The fresh leaves of Lemon balm were taken and leaf margins were separated from the main leaf and allowed to shade-dry. The dried leaf margins (454.0 g) were extracted with EtOH/H_2O 70:30, (3 × 2 l) in a *Soxhlet*-type apparatus to obtain an extract. The extract was dried on a rotary evaporator to provide 42.2 g of residue. The residue was suspended in hexanes to remove low-polarity lipophilic constituents. The remaining residue (35.0 g) was partitioned between ^{i}PrOH and H_2O, to obtain an ^{i}PrOH-soluble portion (21.9 g). An aliquot of ^{i}PrOH extract (15.0 g) was submitted to vacuum liquid chromatography (VLC; SiO_2 (40 μm, 220 g; through gravity column (120 × 3.5 cm); an isocratic system of mobile phase, $CHCl_3$/MeOH/H_2O 65:35:10 (lower layer)) to yield *Frs. 1–18*. CC of *Fr. 4* (4 g) (silica gel (100 × 2.5 cm; 150 g; mesh 200–400); $CHCl_3$/MeOH/H_2O 65:35:10 (lower layer)) afforded five subfractions, *Frs. 4a–4e*. The *Fr. 4b* (512.5 mg) after CC (silica gel (25 × 0.3 cm; 60 g; 40 μm) afforded **6** (66.2 mg), *Fr. 4c* (667.2 mg) after same repetitive chromatography afforded **7** (12.4 mg). *Fr. 9* (3.0 g) was subjected to CC (*Sephadex LH-20* (60 × 1 cm; 80 g)) to afford three fractions, *Frs. 9a–9c*. *Fr. 9b* (1.42 g) was purified by CC (*Sephadex LH-20* (30 × 0.5 cm) to afford **8** (7.2 mg). *Fr. 15* (2.50 g) was subjected to MPLC (*Biotage*) to give ten subfractions, *Frs. 15a–15j*. *Fr. 15f* (902.0 mg) was subjected to RP-HPLC (*RP-C8* cartridge; MeOH/MeCN 90:10, isocratic) to furnish **3** (4.2 mg) and **2** (6.7 mg). RP-HPLC (*RP-C18*; MeOH/H_2O 80:20) of *Fr. 16* (811.2 mg), afforded six subfractions, *Frs. 16a–16f*. *Fr. 16b* (21.2 mg) after crystallization afforded **5** (6.5 mg), and *Fr. 16c* gave **1** (5.2 mg) after repeated crystallization. *Fr. 18* (1.2 g) was subjected to CC (*RP-C18*; H_2O/MeOH 60:40) to provide three subfractions, *Frs. 18a–18c*. *Fr. 18b* was submitted to HPLC (*RP-C18*) to yield **4** (8.9 mg).

The essential oil of lemon balm was obtained by the hydrodistillation of fresh plant material (leaf margins) in a *Clevenger*-type apparatus for 4 h. The sample afforded white viscous oil with characteristic lemon-scented flavor (yield 0.10%). The oil was dried (Na_2SO_4) and placed in a refrigerator at low temp. to preserve it from artefaction (*Table 6*).

Table 6. *Composition of the Leaf* (margins) *Essential Oils of Lemon Balm* (*Melissa officinalis* L.)

Constituent	*RI*[a])	%	Identification[a])
α-Thujene	926	3.4	MS, *RI*
α-Pinene	932	2.1	MS, *RI*
Camphene	936	0.03	MS, *RI*
Sabinene	942	2.3	MS, *RI*
Linalool	947	0.02	MS, *RI*
β-Pinene	984	11.2	MS, *RI*
Myrcene	1005	0.34	MS, *RI*
α-Phellandrene	1009	1.2	MS, *RI*
Citronellal	1079	4.8	MS, *RI*
Citranellol	1087	3.1	MS, *RI*
α-Terpinene	1092	1.0	MS, *RI*
p-Cymene	1098	0.01	MS, *RI*
Limonene	1128	0.5	MS, *RI*
Geraniol	1145	0.05	MS, *RI*
1,8-Cineole	1151	0.8	MS, *RI*
(*Z*)-β-Ocimene	1197	0.7	MS, *RI*
Neral	1213	0.008	MS, *RI*
Benzeneacetaldehyde	1268	0.001	MS, *RI*
γ-Terpinene	1392	10.3	MS, *RI*
cis-Sabinene hydrate	1415	1.2	MS, *RI*
Geranic acid	1431	1.3	MS, *RI*
Linalool	1447	0.04	MS, *RI*
trans-Sabinene hydrate	1461	0.02	MS, *RI*
β-Caryophyllene	1537	3.4	MS, *RI*
trans-Pinocarveol	1542	0.7	MS, *RI*
trans-Verbenol	1571	0.006	MS, *RI*
Geranylacetate	1627	2.03	MS, *RI*
Pinocarvone	1634	3.7	MS, *RI*
p-Mentha-1,5-dien-8-ol	1688	0.002	MS, *RI*
Terpinen-4-ol	1722	8.7	MS, *RI*
α-Humulene	1743	0.2	MS, *RI*
Myrtenal	1779	0.001	MS, *RI*
γ-Cadinene	1856	0.01	MS, *RI*
Bornyl acetate	1872	1.3	MS, *RI*
Thymol	1897	2.5	MS, *RI*
Humulene oxide	1899	0.002	MS, *RI*
Carvacrol	1910	0.06	MS, *RI*
(*E*)-β-Damascenone	1912	0.05	MS, *RI*
β-Bourbonene	1918	1.2	MS, *RI*
(*E*)-Caryophyllene	1951	3.1	MS, *RI*
α-Humulene	1967	0.2	MS, *RI*
Germacrene D	1989	2.7	MS, *RI*
(*E*)-β-Ionone	2021	0.7	MS, *RI*
Bicyclogermacrene	2053	1.1	MS, *RI*
trans-β-Guaiene	2063	0.07	MS, *RI*
β-Bisabolene	2113	4.9	MS, *RI*
Caryophyllene oxide	2178	12.5	MS, *RI*
Caryophyllenol II	2185	1.2	MS, *RI*
(Hexahydrofarnesyl)acetone	2191	0.2	MS, *RI*
Manoyl oxide	2229	0.002	MS, *RI*
Total		98.952	

[a]) *RI*, Relative retention indices relative to C_9–C_{23} alkanes on the *BP-5* column. GC/MS Identification based on comparison of mass spectra.

*3,23-Disulfate of 2α,3β-23,29-Tetrahydroxyolean-12-ene-28-oic Acid 28-*O*-β-*D*-Glucopyranoside* (=*1-*O*-[(2α,3β)-2,29-Dihydroxy-28-oxo-3,23-bis(sulfooxy)olean-12-en-28-yl]-β-*D*-glucopyranose*; **1**). Amorphous powder (5.2 mg). $[\alpha]_D^{20} = -19.7$ ($c = 0.10$, MeOH). IR (KBr): 3412 (OH), 1741 (COO), 1265 (S–O). ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 827.1723 ($[M+H]^+$, $C_{36}H_{59}O_{17}S_2^+$; calc. 827.3194), 747.3678 ($[M-SO_3+H]^+$), 667.5633 ($[M-2\,SO_3+H]^+$), 505.4880 ($[M-2\,SO_3-\text{sugar}+H]^+$).

*23-Sulfate of 2α,23-Dihydroxyurs-12-ene-28-oic Acid 3-*O*-β-*D*-Glucopyranoside* (=*(2α,3β)-3-(β-*D*-Glucopyranosyloxy)-2-hydroxy-23-(sulfooxy)urs-12-en-28-oic Acid*; **2**). White powder (6.7 mg). $[\alpha]_D^{20} = -10.2$ ($c = 0.10$, MeOH). IR (KBr): 3423 (OH), 1711 (CO) 1259 (S–O). ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 731.2157 ($[M+H]^+$, $C_{36}H_{59}O_{13}S^+$; calc. 731.3676), 651.4112 ($[M-SO_3+H]^+$), 489.3279 ($[M-SO_3-\text{sugar}+H]^+$).

Sugar Analysis. Compounds **1** and **2** (1 mg) were hydrolyzed with 1N HCl (2 ml) for 3 h at 90°. The mixture was cooled, neutralized, and partitioned between AcOEt (3 ml) and H_2O (3 ml). The aq. layer was treated with $NaBH_4$ (3 mg) at r.t. for 3 h, and excess of $NaBH_4$ was removed by glacial AcOH. The residue was dissolved in pyridine (0.5 ml), and 0.1M L-cysteine methyl ester hydrochloride in pyridine (1 ml) was added. The mixture was heated at 70° for 1 h. An equal volume of Ac_2O was added with heating continued for another h. Acetylated thiazolidine derivatives were subjected to GC analysis (conditions: a *ThermoQuest Trace 2000* GC; column, *Phenomenex DB-5* (30 m × 0.25 mm × 0.25 μm); carrier gas, He; injection temp., 250°, detection temp., 270°; column temp., 100° (1 min), 20°/min to 300° (30 min). The configurations were determined by comparing their retention times (t_R 11.3 min (D-glucose; major isomer), 11.9 min (D-glucose; major isomer)) with those of acetylated thiazolidine derivatives prepared in a similar way starting from standard sugars.

Compounds **1** and **2** (1 mg each) were hydrolyzed with 1N HCl (3 ml) for 4 h at 95°. The mixture was cooled, neutralized, and partitioned between AcOEt (3 ml) and H_2O (3 ml). The aq. layer was analyzed by TLC ($CHCl_3$/MeOH/H_2O 65 : 35 : 10) and comparison with authentic samples of D-glucose. The spots were visualized by spraying with *p*-anisaldehyde/H_2SO_4, followed by heating. The sugars obtained on hydrolysis showed R_f values comparable to those of D-glucose (R_f 0.48).

Sulfate Detection. Compounds **1**–**3** (each 1 mg) were refluxed with 10% HCl (3 ml) for 5 h, and then extracted with $CHCl_3$. An aliquot of the aq. layer of each was treated with 70% $BaCl_2$ to give a white precipitate of $BaSO_4$ [9].

Biological Assays. Antioxidant Activity. Antioxidant activities of the compounds were evaluated by the DCFH-DA (2,7-dichlorofluorescin diacetate) method [18]. Myelomonocytic HL-60 cells (1×10^6 cells/ml; ATCC, Manassas, VA, USA) were suspended in *RPMI-1640* medium with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). The cell suspension (100 μl) was added to the wells of a 96-well plate. After treatment with different concentrations of the test compounds for 30 min, cells were stimulated with 100 ng/ml phorbol 12-myristate- 13-acetate (PMA; *Sigma*) for 30 min. DCFH-DA (*Molecular Probes*, *Invitrogen Corp. Carlsbad*, CA, USA, 5 μg/ml) was added, and cells were further incubated for 15 min. Levels of fluorescent DCF (produced by ROS-catalyzed oxidation of DCFH) were measured on a *PolarStar* with excitation wavelength at 485 nm and emission wavelength at 530 nm. Antioxidant activities of test samples were determined in terms of % decrease in DCF production compared to that of the control. Vitamin C (*Sigma*) was used as the positive control in each assay. The IC_{50} values were obtained from dose curves generated by plotting % DCF production *vs.* test concentrations.

Antimicrobial Activity. Assays were performed as described in [19].

Antimalarial Activity. Assays were performed as described in [20].

Cytotoxicity. Assays were performed as described in [21].

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Received January 1, 2014