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Isoprenoid Glycosides from *Liriosma ovata*

Patrizia Picerno, Teresa Mencherini, Luca Rastrelli, Annalisa Piccinelli, and Rita Aquino*

Dipartimento di Scienze Farmaceutiche, University of Salerno, Via Ponte Don Melillo, 84084, Fisciano, Salerno, Italy

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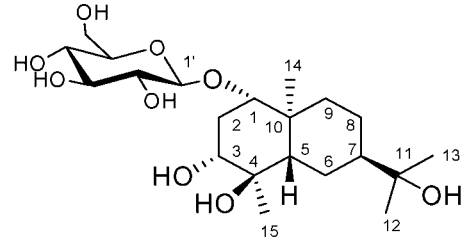
A methanol extract of the dried bark of *Liriosma ovata* afforded two new isoprenoid glycosides, a eudesmane sesquiterpene, 1α(*β*-D-glucopyranosyloxy)eudesma-3α,4*β*, 11-triol (**1**), and a C_{13}-norisoprenoid, 3α,6α-dihydroxymegastigman-7-en-9-one 3-*O*-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**2**), as well as a known constituent, isopropyl-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside (**3**). The structures of **1** and **2** were determined using spectroscopic methods. The methanol extract had a significant topical anti-inflammatory activity (ID_{50}, dose inducing 50% edema inhibition = 780 $\mu g/cm^2$) about 8 times less than that of indomethacin (ID_{50}, 93 $\mu g/cm^2$) assayed by the croton oil ear test in mice. In addition, the chloroform extract and compounds **1** and **2** showed weak antimicrobial activity against S*taphylococcus aureus* (MIC = 5.1, 2.1, and 4.0 mg/mL, respectively) using a broth microdilution assay.

Liriosma ovata Miers [Olacaceae; syn. *Dulacia inopiflora* (Miers), Kuntze],[1] commonly known by the trivial name "muira puama", is a small tree native to the Brazilian Amazon and other parts of northern Brazil.[2] Indigenous tribes in Brazil use the infusion of the roots and bark for treating sexual debility and impotency, neuromuscular problems, rheumatism, and cardiac and gastrointestinal asthenia, and to prevent baldness.[3] It is also used externally in baths and massages for treating skin diseases.[3] The use of the extracts of *L. ovata* as an antioxidant pharmaceutical preparation is reported in some patents.[4,5] Two closely related species, *Ptychopetalum olacoides* and *P. uncinatum* (Olacaceae), also known as "muira puama", are used interchangeably with *L. ovata* as "potency wood", but their active constituents (free long-chain fatty acids, sterols, coumarins, alkaloids, essential oil) have been studied.[4,5] For the title plant, *L. ovata*, no previous phytochemical work has been reported in the literature. Thus, we describe herein the isolation and characterization of two new isoprenoid glycosides (**1** and **2**) from a methanol extract of the bark of *L. ovata*. Their structures were elucidated by spectroscopic methods including 1D (^1H and ^{13}C) and 2D (DFQ-COSY, HSQC, and HMBC) NMR experiments as well as HRESIMS analysis. An in vivo topical antinflammatory assay was carried out on three crude extracts of *L. ovata.* In addition in vitro antimicrobial activity was evaluated for the methanol and chloroform extracts and on compounds **1**, **2**, and **3**.



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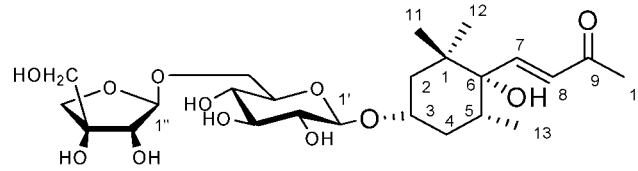

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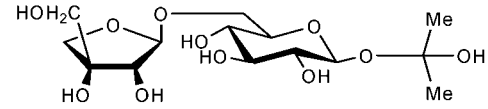

3

The dried bark of *L. ovata* was extracted sequentially with solvents of increasing polarity, using *n*-hexane, chloroform, and methanol. The dried methanol extract was then partitioned between water and *n*-BuOH. The lyophilized *n*-BuOH-soluble extract was separated by gel filtration on a Sephadex LH-20 column and by RP-HPLC, yielding, as major constituents, a new eudesmane sesquiterpene (**1**), a new C_{13}-norisoprenoid (**2**), and a known constituent, isopropyl-*β*-D-apiofuranosyl-(1→6)-*β*-D-glucopyranoside, identified by ESIMS and NMR spectroscopy and by comparison with data reported in the literature.[6]

The positive-ion HRESIMS of compound **1** exhibited a molecular ion at *m/z* 457.1282 $[M + Na]^+$, corresponding to the molecular formula $C_{21}H_{38}O_9$. The MS/MS showed a prominent fragment at *m/z* 277.117 $[(M + Na) - 162 - H_2O]^+$, which was consistent with the loss of a hexose moiety from an oxygenated sesquiterpene. The ^1H and ^{13}C NMR spectra indicated compound **1** to be a glycosylated tetrahydroxy-eudesmane derivative (Table 1).[7–10] The ^1H NMR spectrum displayed signals for four tertiary methyl groups at δ_H 0.90, 1.04, and 1.18 (×2), in the aliphatic region, and for two oxymethines at δ_H 3.49 (1H, dd, J = 11.8 and 4.4 Hz) and 3.40 (1H, dd, J = 12.3 and 3.9 Hz), for which coupling constants suggested an axial configuration. In the ^{13}C NMR spectrum, the 15 signals for the aglycone were ascribable to four methyls, four methylenes (δ_C 22.0, 23.2, 33.7, and 41.6), two methines (δ_C 50.6 and 51.5), two oxymethines (δ_C 77.7 and 84.1), a quaternary carbon (δ_C 39.7), and two oxygenated quaternary carbons (δ_C 73.0 and 76.6). Two out of the four methyls (δ_C 27.0 and 27.3) were assigned to an oxygenated isopropyl group (carbinol signal at δ_C 73.0) and one (δ_C 16.4) to a methylcarbinol unit (carbinol signal at δ_C 76.6), with the fourth (δ_C 14.6) being angular. In the homonuclear $^1H-^1H$ COSY spectrum, the oxymethine signal at δ_H 3.49 (H-1) was coupled with a methylene signal (δ_H 1.64, H-2a and 2.06, H-2b), which was further coupled with the oxymethine signal centered at δ_H 3.40 (H-3). A further sequence was seen starting from the methylene signal at δ_H 1.15 (H-5) to the H_2-6 (δ_H 1.64), H-7 (δ_H 1.30), H-8a (δ_H 1.19), H-8b (δ_H 1.98), H-9a (δ_H 1.09), and H-9b (δ_H 2.06) resonances. Analysis of the HSQC and HMBC correlations confirmed these assignments and suggested **1** to be a 1,3,4,11-eudesmane-tetrol[7–9] glycosylated at C-1 (δ_C 84.1).[8] Ad-

* To whom correspondence should be addressed. Tel: ++39 089 969737. Fax: ++39 089 969602. E-mail: aquinorp@unisa.it.

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

	1		**2**	
position	δ_C	δ_H ($J_{HH\ in\ Hz}$)[b]	δ_C	δ_H ($J_{HH\ in\ Hz}$)[b]
1	84.1	3.49, dd (11.8, 4.4)	40.9	
2	33.7	1.64, ddd (12.3, 12.3, 11.8)	42.5	1.74, t (12.1, 3.6)
		2.06, ddd (12.3, 4.4, 3.9)		1.61, ddd (12.1, 3.6, 2.0)
3	77.7	3.40, dd (12.3, 3.9)	75.7	3.96, tt (12.1, 3.6)
4	76.6		37.9	1.92, m (12.1)
				1.52, q (12.1, 3.6)
5	51.5	1.15, dd (9.0, 3.9)	35.3	2.18, m (12.1, 6.9, 3.6)
6	23.2	1.64, ddd (12.7, 9.0, 3.9)	79.0	
7	50.6	1.30, br, m	154.5	6.92, d (16.5)
8	22.0	1.98, br (12.3, 3.9)	131.4	6.37, d (16.5)
		1.19, br		
9	41.6	1.09, br	201.1	
		2.06, dt (12.7, 5.1)		
10	39.7		27.4	2.31, s
11	73.0		25.1	1.08, s
12	27.3	1.18, s	25.9	0.90, s
13	27.0	1.18, s	16.4	0.81, d (6.86)
14	14.6	0.90, s		
15	16.4	1.04, s		
Glc				
1′	101.8	4.31, d (7.5)	102.8	4.38, d (7.5)
2′	74.9	3.14, dd (8.5, 7.5)	75.0	3.14, dd (8.5, 7.5)
3′	77.8	3.35, t (8.5)	77.9	3.30, t (8.5)
4′	71.6	3.28, t (8.5)	71.8	3.30, t (8.5)
5′	78.0	3.26, m	78.0	3.35, m
6′	62.9	3.66, dd (12.0, 4.5), 3.87, dd (12.0, 3.0)	68.6	3.63, dd (12.0, 4.5), 4.00, dd (12.0, 3.0)
Api				
1″			110.9	5.06, d (2)
2″			78.0	3.92, d (2)
3″			80.6	
4″			75.1	4.00, d (10), 3.79 d (10)
5″			65.6	3.61, s

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

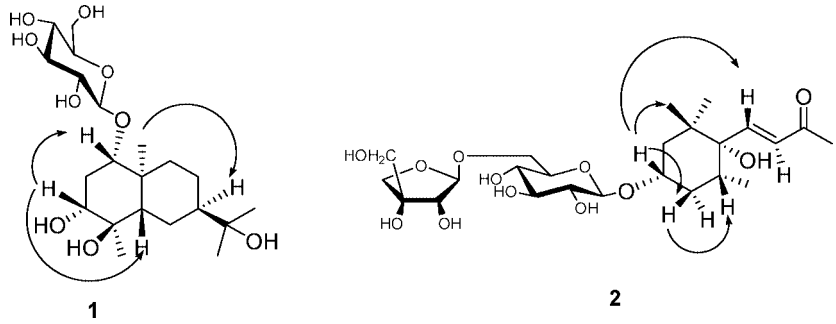

Figure 1. Correlations observed in the ROESY spectra of compounds **1** and **2**.

ditionally, evidence for the structural determination of compound **1** was provided from the HMBC spectrum. Thus, two methyl signals of the isopropyl group (δ_H 1.18), displaying a ^{2}J correlation to the oxygenated quaternary carbon signal at δ_C 73.0 (C-11), showed a ^{3}J coupling to the methine carbon signal at δ_C 50.6 (C-7), which confirmed that they are geminal and that the isopropyl group could be placed at C-7. The signal for the angular methyl (δ_H 14.6), showing a ^{2}J coupling to the quaternary C-10 and ^{3}J couplings to the C-1, C-5, and C-9 signals, was assigned to Me-14. The remaining methyl signal of the methylcarbinol unit at δ_H 1.04 was assigned to Me-15, exhibiting a ^{2}J coupling to the oxygenated quaternary carbon at C-4 (δ_C 76.6), and ^{3}J interactions with C-5 (δ_C 51.5) and the oxymethine at C-3 (δ_C 77.7). The relative configuration at C-1, C-3, C-5, and C-7 was deduced as H-1β, H-3β, H-5β, and H-7α from the observed coupling constants of the relative hydrogen signals (Table 1) and confirmed from the results of the ROESY spectrum showing correlations between H-1β and H-3β and H-5β, as well as between the Me-14α, Me-15α, and H-7α signals (Figure 1). The absence of any ROE correlation between H-5 and Me-14 supported a *trans* ring A/B junction.[10,11] The ^{1}H NMR spectrum for the sugar portion of compound **1** showed an anomeric proton signal at δ_H 4.31 (H-1′, d, J = 7.5 Hz). On the basis of the ^{1}H and ^{13}C NMR data, the sugar unit was identified as a β-glucopyranosyl unit. In the HMBC spectrum, the anomeric proton signals at δ_H 4.31 of the glucopyranosyl unit showed a ^{3}J correlation to C-1 at δ_C 84.1, which confirmed an ether linkage between C-1 and the glucosyl unit. The configuration of the glucose unit was determined to be D after hydrolysis of **1** with 1 N HCl and GC analysis. On the basis of these data, the structure of **1** was determined as 1α-(β-D-glucopyranosyloxy)eudesma-3α,4β,11-triol.

The positive-ion ESIMS of compound **2** gave a molecular ion at m/z 543 $[M + Na]^+$, corresponding to the molecular formula $C_{24}H_{40}O_{12}$. The MS/MS showed a prominent fragment at m/z 411 $[(M + Na) - 132]^+$, cleavage of a terminal pentose unit, and a fragment at m/z 335 ($132 + 162 + H_2O + Na^+$) ascribable to a disaccharide unit (pentose plus hexose unit). Compound **2** exhibited a UV absorption maximum at 279 nm, indicative of a conjugated enone structure. The ^{13}C NMR spectrum (Table 1) showed, in

Table 2. Antimicrobial Activity of *L. ovata* Crude Extracts and Compounds **1**, **2**, and **3**

	chloroform		methanol		**1**		**2**		**3**	
microrganism	MIC[a]	MBC[a]	MIC[a]	MBC[a]	MIC[a]	MBC[a]	MIC[a]	MBC[a]	MIC[a]	MBC[a]
S. aureus ATCC 6538	5	20	20	NA[b]	2	4	4	NA[b]	NA[b]	NA[b]
S. epidermis ATCC 12228	20	20	20	NA[b]						
B. spizienii ATCC 6633	NA[b]	NA[b]	20	NA[b]						
P. aeruginosa ATCC 9027	20[b]	NA[b]	NA[b]	NA[b]	4	4	NA[b]	NA[b]	NA[b]	NA[b]
E. coli ATCC 8739	NA[b]	NA[b]	20	NA[b]						
C. albicans ATCC 10231	NA[b]	NA[b]	20	NA[b]						
A. niger ATCC 16404	NA[b]	NA[b]	20	NA[b]						

[a] Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values are expressed as mg/mL. [b] NA = not active (MIC > 20 mg/mL).

addition to the signals due to a disaccharide moiety, 13 signals that consisted of a carbomethoxy group (δ_C 201.1 and 27.4), three methyl groups (δ_C 25.9, 25.1, and 16.4), two methylenes (δ_C 37.9 and 42.5), a methine (δ_C 35.3), a quaternary carbon (δ_C 40.9), an oxymethine group (δ_C 75.7), an oxygenated quaternary carbon (δ_C 79.0), and a one disubstituted *trans* double bond (δ_C 154.5 and 131.4). These data suggested that compound **2** is a glycoside with a C-13 norisoprenoid structure also known as a megastigmane or a dihydro-β-ionone derivative.[12–15] From the inspection of the ^{1}H NMR spectrum, the double bond (δ_H 6.37 and 6.92, each H, d, J = 16.5 Hz) was conjugated with the keto group [(δ_H 2.31 (3H, s)] and adjacent to a quaternary carbon atom, leading to the structure elucidation of the side chain of the megastigmane skeleton. Additional evidence was provided by the ^{1}H−^{1}H COSY spectrum, starting from the methylene proton signals at C-2 (H-2b, δ_H 1.74 and H-2a, δ_H 1.6), in which cross-peaks were followed to signals at δ_H 3.96 (H-3), 1.92 (H-4b), 1.52 (H-4a), 2.18 (H-5), and 0.81 (Me-13). Thus, the secondary alcoholic group was located at C-3, and H-3 could be assigned an axial (β) orientation as shown by its coupling constants (tt, J = 12.1 and 3.6 Hz). The remaining quaternary carbons at δ_C 79.0 (oxygenated carbon) and 40.9 were located at C-6 and C-1 from the HMBC correlation observed between these carbons and the *gem*-dimethyl group (Me-11, δ_H 1.08; Me-12, δ_H 0.90). HMBC and HSQC experiments were used to complete the assignments of the NMR data of the aglycon of compound **2** (Table 1), which were the same as those of (3*S*,5*R*,6*S*)-3,6-dihydroxymegastigman-7-en-9-one.[14,15] The relative configuration in **2** at C-3, C-4, C-5, and C-7, deduced from the observed coupling constants of the H-3 and H-5 (β, axial) signals (Table 1), was confirmed by the correlation observed between H-3β, H-5β, H-7β, and Me-11 and between H-4β, H-5β, and H-7β in the ROESY spectrum (Figure 1), indicating that the side-chain is in the equatorial orientation. For the sugar moiety, the ^{1}H NMR spectrum of compound **2** showed the presence of two anomeric proton signals at δ_H 5.06 (H-1″, d, J = 2.0 Hz) and δ_H 4.38 (H-1′, d, J = 7.5 Hz). The 1D TOCSY and ^{1}H−^{1}H COSY experiments allowed the assignment of all proton resonances to a β-apiofuranosyl[13,16] and a β-glucopyranosyl[13,16] unit in **2**. The assignments of the corresponding carbons, using the HSQC spectrum, indicated that the apiofuranosyl is the terminal unit and glucopyranosyl is substituted at C-6. The relative positions of the glucopyranosyl and apiofuranosyl units were established unambiguously by HMBC correlations observed between the anomeric proton signal at δ_H 4.38 (H-1′, glucopyranosyl) and C-3 (δ_C 75.7) of the aglycon and between the anomeric proton signal at δ_H 5.06 (H-1″, apiofuranosyl unit) and C-6′ (δ_C 68.6) of the glucopyranosyl unit. The configurations of the sugar units were determined as D-glucose and D-apiose, after hydrolysis of **2** with 1 N HCl and GC analysis. Considering the optical rotations and the very similar NMR data, **2** was assigned with the same absolute configuration as that of (−)-boscialin.[17] Therefore, the structure of **2** was determined as 3α,6α-dihydroxymegastigman-7-en-9-one 3-*O*-D-apiofuranosyl-(1→6)-β-D-glucopyranoside, a new megastigmane glycoside.

Anti-inflammatory activity has been reported for megastigmane[18] and antimicrobial effects for eudesmane sesquiterpenes,[17–21] so extracts and compounds from *L. ovata* were screened for these activities. Each of the prepared extracts from *L. ovata* was submitted to the croton oil mouse ear test, at a dose of 300 μg/cm^2, to evaluate their topical anti-inflammatory effects. All three extracts exhibited some anti-inflammatory activity, inducing 36% (hexane), 39% (chloroform), and 30% (methanol) edema inhibition. The dose−activity relationship of the methanol extract was further investigated in comparison to that of a positive control, indomethacin. This extract induced dose-dependent edema inhibition, showing a potency about eight times less than that of indomethacin, with ID_{50} values (the dose inducing 50% edema inhibition) of 780 and 93 μg/cm^2, respectively.

The extracts, at a concentration ranging from 20 to 0.04 mg/mL, and compounds **1**, **2**, and **3**, at a concentration ranging from 4 to 0.008 mg/mL, were tested for antimicrobial activities by a broth microdilution method, and the results are expressed as MIC (minimum inhibitory concentration) values and as MBC (minimal bactericide concentration) values (Table 2). *Staphylococcus aureus* was more sensitive to both the chloroform extract (MIC 5.0 mg/mL) and compounds **1** and **2** (MIC 2 and 4 mg/mL, respectively) than the other bacteria. A total growth inhibition of *S. aureus*, expressed as MBC (20 mg/mL for the chlorform extract and 4 mg/mL for compound **1**, respectively), was observed for the incubation times assayed, suggesting a bactericidal effect. These results are in accord with previous papers that have shown that eudesmane derivatives, tested by the agar diffusion method, are a class of natural plant products that possess weak but interesting antimicrobial activity,[17–20] attributed to the presence of the α,β-unsaturated keto and hydroxyl groups.[20]

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. UV spectra were recorded on a UV-2101PC UV/vis scanning spectrophotometer (Shimadzu Italia srl, Milan, Italy). For NMR experiments, a Bruker DRX-600 NMR spectrometer at 300 K was used, operating at 599.2 MHz for ^{1}H and at 150.9 MHz for ^{13}C; 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 Hz; 2D NMR experiments were carried out using the conventional pulse sequences as described in the literature.[22,23] ESIMS were recorded using a Finnigan LC-Q Deca instrument (Thermoquest, San Jose, CA) equipped with Xcalibur software (capillary temperature was 220 °C). Exact masses (HR-ESIMS) were measured by a Q-TOF Premier (Waters) triple-quadrupole orthogonal time-of-flight (TOF) instrument equipped with 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.

Plant Material. A commercial sample of bark of *Liriosma ovata* Miers was supplied in October 2006 by the Commercio Alternative s.c.a.r.l., Ferrara, Italy. A voucher sample (CIF L.O.1) was deposited at the Herbarium of the Department of Pharmaceutical Sciences, University of Salerno.

Extraction and Isolation. The dried bark (500 g) was defatted at room temperature with *n*-hexane (dried extract, 450 mg) and $CHCl_3$

(dried extract, 2.7 g) and then extracted by maceration at room temperature with MeOH to give 10 g of residue. This was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH-soluble portion (7.8 g). An aliquot (5 g) of the *n*-BuOH extract was chromatographed over a Sephadex LH-20 (1 m × 3 cm i.d.) column (Pharmacia, Uppsala, Sweden) using MeOH as eluent at a flow rate of 0.5 mL/min. Fractions (8 mL each) were collected and checked by TLC [silica 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 pooled, giving four major fractions (I−IV), which were further purified by RP-HPLC on a C_{18} μ-Bondapack column (30 cm × 7.8 mm i.d.) (flow rate of 1.8 mL/min), using MeOH−H_2O (3:7) as the eluent. Fractions II (474 mg) and III (416 mg) yielded pure compounds **1** (10 mg, t_R = 16 min), **2** (8 mg, t_R = 21 min), and **3** (74 mg, t_R = 8 min).

Compound 1: pale yellow, amorphous powder; $[\alpha]^{31}_D$ +14.40 (*c* 0.27, MeOH); ^{1}H (CD_3OD, 600 MHz) and ^{13}C (CD_3OD, 150.9 MHz) NMR data, see Table 1; HRESIMS *m/z* 457.1282 (calcd for $C_{21}H_{38}O_9Na$, 457.1321).

Compound 2: pale yellow, amorphous powder; $[\alpha]^{31}_D$ +14.60 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ϵ) 279 (2.37) nm; ^{1}H (CD_3OD, 600 MHz) and ^{13}C (CD_3OD, 150.9 MHz) NMR data, see Table 1; HRESIMS *m/z* 543.1752 (calcd for $C_{24}H_{40}O_{12}Na$, 543.1790); ESIMS (positive-ion mode) *m/z* 543 $(M + Na)^+$, *m/z* 411 $[(M + Na) - 132]^+$, *m/z* 335 $[(132 + 162 + H_2O + Na)]^+$.

Compound 3: ^{1}H and ^{13}C NMR data were consistent with those previously reported;[6] ESIMS *m/z* 355 $[M + H]^+$, *m/z* 353 $[M - H]^-$.

Acid Hydrolysis of Compounds 1 and 2. A solution (0.8 mg each) of **1** and **2** in 1 N HCl was stirred at 80 °C for 4 h, and after cooling, it was 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 by 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 both 200 °C. 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 of the hydrolysates of both compounds were detected at 14.72 min (D-glucose) and 11.90 min (D-apiose). Retention times of authentic samples, after treated in the same way with 1-(trimethylsilyl)imidazole in pyridine, were 14.71 min (D-glucose) and 11.90 min (D-apiose).

In Vivo Topical Anti-inflammatory Activity. Croton oil and indomethacin were purchased from Sigma (St. Louis, MO). Ketamine hydrochloride was obtained from Virbac Srl (Milan, Italy). Male CD-1 mice (28–32 g) were from Harlan-Italy, Udine, Italy. The topical anti-inflammatory activity was evaluated as inhibition of the croton-oil-induced ear edema in mice.[24,25] All experiments complied with the Italian D.L. n. 116 of January 27, 1992, and the associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609 ECC). The vehicles used were acetone for hexane, chloroform extracts and its control and 42% aqueous EtOH (v/v) for croton oil, methanol extract and its control. The edematous response was measured by weight difference between two plugs (6 mm, diameter) of the treated (right) and the untreated (left) ears. The anti-inflammatory activity was expressed as percentage of the edema reduction in treated mice, compared with the control mice. As a reference, the nonsteroidal anti-inflammatory drug (NSAID) indomethacin was used.

Statistical Analysis. Edema was expressed as mean ± standard error of the mean. Edema values were analyzed by one-way analysis of variance followed by Dunnett's test for multiple comparison of unpaired data, with $p < 0.05$ considered as significant. The ID_{50} value (dose giving 50% edema inhibition) was calculated by graphic interpolation of the dose–effect curve.

In Vitro Antimicrobial Activity. The chloroform and methanol extracts and compounds **1**, **2**, and **3** from *L. ovata* were tested for antimicrobial activity using the broth microdilution method in 96-multiwell microtiter plates, in duplicate, as reported by Koneman (1995)[26] and Camporese (1997)[27] and recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2001).[28] The following microorganisms from American Type Culture Collection (ATCC) were utilized: Gram-positive bacteria (*Bacillus spizizenii* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228), Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739), a yeast (*Candida albicans* ATCC 10231), and a mold (*Aspergillus niger* ATCC 16404). Dried extracts or compounds were dissolved in dimethyl sulfoxide (DMSO) and then in Tryptone Soya broth. The antimicrobial activity was evaluated as previously reported.[29,30] The MIC is the lowest concentration of extract or compound at which microbial growth was inhibited after 24 h. The minimum bactericide concentration (MBC) is the lowest concentration of 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 on agar plates a portion of the broth culture, where MIC values were previously defined.[30,31] A blank control was taken using DMSO alone. 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, were also 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*).

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