New Labdene Diterpenes from *Eupatorium glutinosum*

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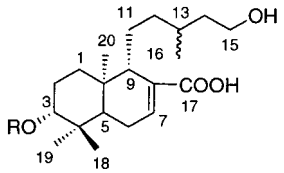
The new diterpene glucoside 3,15-dihydroxy-*ent*-labd-7-en-17-oic acid 3-*O*-*β*-D-glucoside (**1**) and its aglycone (**2**) have been isolated from *Eupatorium glutinosum*. The structures were determined by IR, one- and two-dimensional NMR, high-resolution mass spectrometry, chemical transformations, and comparison of spectroscopic data with closely related diterpenes. Crude extracts showed antimicrobial and cytotoxic activities, but compounds **1** and **2** showed only antimicrobial activity. These results support the vernacular medicinal use of the plant as an antimicrobial.

The genus *Eupatorium* belongs to the Eupatorieae, one of the 13 tribes of the Asteraceae.[1,2] Plants of the genus *Eupatorium* have been used for their medicinal properties for many decades. A number of bioactive natural products have been reported in extracts of *Eupatorium* species, and these could be promising bioresources for the preparation of drugs and value-added products.[3,4]

Eupatorium glutinosum has been used for a long time in traditional medicine. It is found growing at an altitude of about 3000 m in Ecuador and Peru. A decoction of leaves and twigs of the plant has been used as an astringent, antirheumatic, and antimicrobial and to cure stomach ulcers, diarrhea, and headaches.[5] However, no phytochemical or pharmacological studies on *E. glutinosum* (Lam.) have been reported. *E. glutinosum* was collected in Ecuador and investigated as part of our ongoing chemical and pharmacological studies of Ecuadorian plants used in folk medicine.[6,7] The aim of this study was to find novel chemical compounds having anticancer or antimicrobial activity.

Guided by an assay for inhibition of microbial activity, an EtOAc extract of the leaves and twigs of *E. glutinosum* was fractionated by CC, preparative TLC, and Sephadex LH-20. The fractionation procedure resulted in the isolation of a new labdene diterpene glucoside (**1**) and its aglycone (**2**).



1. R = Glucosyl
2. R = H

The high-resolution mass spectrum of **1** gave an $[M + H]^+$ ion at *m/z* 501.3067 corresponding to a molecular formula $C_{26}H_{45}O_9$. The ^{1}H NMR spectrum showed the presence of three tertiary methyl groups at *δ* 0.89, 1.09, 1.17 and one secondary methyl group at *δ* 1.02. The downfield shift of the olefinic proton at *δ* 6.94 indicated an unsaturated acid. The coupling constant, $J = 7.8$ Hz, of the signal resulting from the anomeric proton at *δ* 4.96 indicated the glucosidic linkage to have *β* configuration. The ^{13}C NMR spectrum showed 26 carbon signals, six from a glucopyranosyl group and 20 from the diterpene moiety (four methyl groups, seven methylenes, four methines, two quaternary carbon atoms, two olefinic carbon atoms, and one carboxyl group). The location of the glucoside linkage at C-3 was confirmed by the downfield shift from *δ* 78.1 in the aglycone to *δ* 89.0 in the glucoside. The HMBC showed significant correlation of the olefinic proton at *δ* 6.94 with the COOH group at *δ* 171.6, confirming the presence of the unsaturated acid fragment. The data from one- and two-dimensional NMR and multiple bond correlation spectroscopy established the structure of **1** as 3,15-dihydroxy-*ent*-labd-7-ene-17-oic acid-3-*O*-D-glucoside. The stereochemistry at C-13 was not determined. The ^{1}H and ^{13}C NMR data of **2** were very similar to those of **1**, apart from the absence of resonances from the glucose unit. The COSY, ^{13}C NMR (DEPT sequence), NOESY, HMQC, and HMBC spectra proved **2** to be 3,15-dihydroxy-*ent*-labd-7-en-17-oic acid. The optical rotation, with negative values, suggested an *ent*-labdene nucleus.[8]

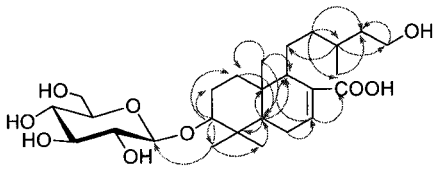


Figure 1. Selected HMBC correlations of **1**.

The antimicrobial activities of compounds **1** and **2** were assessed employing Gram-positive and -negative bacteria. At the dose of 10 *μ*g per disk, **2** exhibited inhibitory activity against *Staphylococcus aureus* (IAM 1011) and *Bacillus cereus* (IAM 1069) (both Gram-positive) and *Pseudomonas aeruginosa* (IAM 1275) and *Escherichia coli* (IAM 1268) (both Gram-negative) with MICs of 256, 128, 64, and 128 *μ*g/mL, respectively, while **1** exhibited inhibitory activity against only *E. coli* and *P. aeruginosa* with MICs of 128 and 256 *μ*g/mL, respectively. This could at least support the vernacular medicinal use of *E. glutinosum* as an antimicrobial. Compounds **1** and **2** showed no cytotoxicity toward Sarcoma 180; however, the original EtOAc extract was active ($IC_{50} = 2$ *μ*g/mL).

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Experimental Section

General Experimental Procedures. IR spectra were recorded on a JASCO Model A-202 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on JEOL JNM ALPHA and GX-400 NMR spectrometers. High-resolution mass spectra were obtained on a Hitachi M-80 B GC−MS spectrometer operating at the ionization energy of 70 eV or on a JEOL JMS-700 spectrometer. Optical rotations were recorded on a JASCO DIP-360 digital polarimeter.

Plant Material. Leaves and twigs of *E. glutinosum* was collected in 1990 in the Provincia del Napo, Ecuador, dried, ground, and kept in the dark at room temperature. A voucher specimen, F. G. 491, is kept in the Herbario Economico, Escuela Politecnica National, Ouito, Ecuador.

Extraction and Isolation. The plant material, 1.15 kg, was extracted with light petroleum (40−60 °C) at room temperature three times with occasional stirring and filtered. The macerate was then extracted three times with MeOH for 7 days each time. The extracts were combined and evaporated in vacuo to give 53.1 g (light petroleum) and 149.9 g (MeOH) of gelatinous and oily materials, respectively. The MeOH extract was partitioned between EtOAc and H_2O to give 43.6 g of an EtOAc extract and 104.4 g of the H_2O extract after freeze-drying. An insoluble residue, 1.9 g, was discarded.

The EtOAc extract (42.5 g) was adsorbed onto silica gel (50 g) and chromatographed on a silica gel (780 g) column eluted with continuous hexane−CH_2Cl_2 and CH_2Cl_2−MeOH gradients. The eluted fractions were evaluated by TLC to give 19 main fractions. Fraction 6 (2.1 g) was purified over a Sephadex LH-20 column eluted with $CHCl_3$−MeOH (8:2), which afforded 24 mg of compound **2**. Fraction 15 (1.6 g) was chromatographed over Sephadex LH-20 eluted with $CHCl_3$−MeOH (7:3) and further separated by preparative TLC using $CHCl_3$−MeOH−H_2O (7:3:0.5) as eluent to yield 85 mg of compound **1**.

3,15-Dihydroxy-*ent*-labd-7-en-17-oic acid-3-*O*-*β*-D-glucoside (1): $[\alpha]^{22}_D$ −37.2° (*c* 0.7, MeOH); UV (MeOH) λ_{max} (log ϵ) 221 (5.10), 232 (4.95); IR (KBr) ν_{max} cm^{-1} 3600−2600, 1687, 1638, 1509, 1386, 1363, 1258, 1160, 1074, 1034, 626; ^{1}H NMR (C_6D_6) δ 6.94 (1H, brq, J = 3.4 Hz, H-7), 4.96 (1H, d, J = 7.8 Hz, H-1′), 4.56, 4.42 (2H, dd, J = 2.4, 5.4, 11.7 Hz, H-6′), 4.24−4.00 (2H, dd, J = 2.4, 5.4, 11.7 Hz, H-3′,4′,5′), 3.93 (2H, m, H-15), 3.45 (1H, dd, J = 7.3, 8.8 Hz, H-3), 2.29, 1.80 (2H, m, H-2), 2.25 (1H, m, H-9), 2.12, 1.39 (2H, m, H-12), 2.07 (2H, m, H-6), 1.78, 1.14 (2H, m, H-1), 1.91, 1.61 (2H, m, H-14), 1.83 (1H, m, H-13), 1.81, 1.55 (2H, m, H-11), 1.23 (1H, m, H-5), 1.00 (3H, d, J = 6.8 Hz, H-16), 1.30, 1.06, 0.81 (9H, s, H-18, 19, 20, respectively); ^{13}C NMR (C_6D_6) δ 171.6 (C-17), 137.4 (C-8), 135.4 (C-7), 106.1 (C-1′), 89.0 (C-3), 78.8 (C-3′), 78.3 (C-5′), 75.8 (C-2′), 71.8 (C-4′), 63.0 (C-6′), 60.4 (C-15), 51.7 (C-9), 49.8 (C-5), 40.8 (C-14), 39.3 (C-4), 39.2 (C-12), 37.9 (C-1), 36.8 (C-10), 31.2 (C-13), 28.2 (C-18), 26.7 (C-2), 26.2 (C-11), 23.8 (C-6), 16.6 (C-19), 14.6 (C-20); FABMS *m*/*z* 499 [M − H]$^-$ (100), 471 (8), 417 (5), 355 (13), 337 (27), 307 (13), 283 (5), 225 (7), 163 (18), 89 (28); HRFABMS *m*/*z* 501.3067 (calcd for $C_{26}H_{45}O_9$, 501.3061).

3,15-Dihydroxy-*ent*-labd-7-en-17-oic acid (2): $[\alpha]^{22}_D$ −44.9° (*c* 0.3, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 224 (4.61), 233 (4.81); IR (KBr) ν_{max} cm^{-1} 3600−2600, 1689, 1643, 1437 1385, 1382, 1258, 1021, 953, 708; ^{1}H NMR (C_6D_6) δ 6.98 (1H, brq, J = 3.8 Hz, H-7), 3.94 (2H, m, H-15), 3.46 (1H, dd, J = 7.3, 8.8 Hz, H-3), 2.31 (1H, m, H-9), 2.13 (2H, m, H-6), 2.13, 1.45 (2H, m, H-12), 1.95, 1.61 (2H, m, H-14), 1.92, 1.24 (2H, m, H-1), 1.90, 1.60 (2H, m, H-11), 1.85 (2H, m, H-2, 13), 1.24 (1H, m, H-5), 1.03 (3H, d, J = 6.8 Hz, H-16), 1.17, 1.09, 0.89 (9H, s, H-18, 19, 20, respectively); ^{13}C NMR (C_6D_6) δ 171.7 (C-17), 137.4 (C-8), 135.8 (C-7), 78.1 (C-3), 60.4 (C-15), 51.8 (C-9), 49.6 (C-5), 40.8 (C-14), 39.2 (C-12), 39.1 (C-4), 38.2 (C-1), 37.2 (C-10), 31.1 (C-13), 28.6 (C-18), 28.2 (C-2), 26.2 (C-11), 24.0 (C-6), 16.0 (C-19), 14.7 (C-20); FABMS *m*/*z* 337 [M − H]$^-$ (100), 335 (12), 291 (13), 275 (22), 247 (5), 181 (14), 155 (8), 137 (4); HRFABMS *m*/*z* 339.2538 (calcd for $C_{20}H_{35}O_4$, 339.2531).

Acid Hydrolysis of 1. Compound **1** (30 mg) was refluxed for 75 min in 4 M HCl−dioxane (20 mL). The acid hydrolysate was concentrated, extracted with $CHCl_3$, and purified by PTLC using EtOAc−hexane 60:40 as eluent, yielding the liberated aglycone, compound **2**. The acidic mother liquor was neutralized with Na_2CO_3, filtered, and evaporated to dryness for examination of the sugar moiety, which proved to be glucose by detection on paper chromatography and TLC [EtOAc−2-propanol−H_2O (65:23:12)], sprayed with freshly prepared anisaldhyde−H_2SO_4 reagent, followed by heating.

Antimicrobial Assay. Antimicrobial activity was determined using the agar overlay method.[9] Melted soft nutrient agar (3 mL, 0.7%) at 40 °C with 50−100 μL of the test bacteria grown in nutrient broth was poured over 20 mL of 1.5% nutrient agar plates. Samples of the extracts and of the pure compounds (10 μL) were deposited over solidified agar, and after 18 h of incubation at 37 °C, the inhibition zone diameter was determined. Aliquots of known antibiotics (chloramphenicol and tetracycline) and methanol as the solvent control were also analyzed. The minimal inhibitory concentrations (MICs) were determined by applying to the agar plates 10 μL of methanolic solutions of the samples, starting with a maximum concentration of 1024 μg/mL, and then reducing it by successive 2-fold dilutions of that stock solution. MIC determinations were carried out in five independent experiments, and MICs were expressed as the lowest concentration inhibiting bacterial growth. Samples that showed no antimicrobial activity at concentrations of 1024 μg/mL were considered inactive.

Cytotoxicity Assay. The in vitro cytotoxicity assay was carried out according to the procedures by Carmichael et al.[10]

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Supporting Information Available: Tables 1 and 2 containing detailed NMR data including DEPT, COSY, HMBC, HMQC, and NOSEY are available free of charge via the Internet at http://pubs.acs.org.

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