Antibacterial Diterpenoids from *Fabiana densa* var. *ramulosa*

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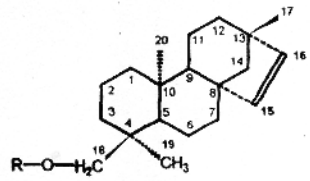
Abstract

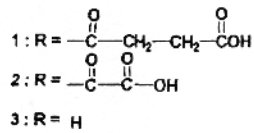
A biologically monitored fractionation of the resinous exudate of *Fabiana densa* Remy var. *ramulosa* Wedd. led to the isolation of the two new diterpenes: *ent*-beyer-15-en-18-*O*-succinate and *ent*-beyer-15-en-18-*O*-oxalate as the unique compounds responsible for the observed antibacterial activity of this extract. Their structures were determined by 1D and 2D NMR spectroscopy.

Fabiana densa Remy var. *ramulosa* Wedd., Solanaceae, common name "checal", "tolilla", "tola-checal" [1] is a native shrub of Chile [2] that grows from the "Altiplano" region to the Puna [3]. The resinous exudate from the leaves is used in traditional medicine to immobilize fractured extremities [4], the infusion for the cough and illness of the lungs [5]. No studies have been carried out on this plant. We report here the isolation and structure elucidation of two new diterpenoids as constituents of *F. densa* and the antimicrobial activity of these compounds detected by *means of the bioautographic techniques. The aims of this study* were to establish scientific basis for future uses in Chilean traditional medicine.

Compounds **1**, $C_{24}H_{36}O_4$ and **2**, $C_{22}H_{32}O_4$ exhibited in the ^{1}H-NMR ($CDCl_3$) spectra signals for two coupled olefinic protons (δ = 5.67–5.66 d and 5.45 d), and for three methyl groups, and similar ^{13}C-NMR parameters. The only difference was the presence of signals (δ_C = 174.0 and 172.1) in **2**, and (δ_C = 177.4, 172.1, 29.0 and 28.9; δ_H 2.68, four protons) in **1** which were attributed to oxalate and succinate side chains, respectively. The complete assignments were confirmed by selective INEPT and NOE difference experiments.

Alkaline hydrolysis of both compounds gave compound **3** which was identified as *ent*-beyer-15-en-18-ol previously reported from *Baccharis tola* [6], therefore **1** and **2** are the succinoyl and the oxaloyl esters of **3**, respectively. Malonate and carbonate esters of sesquiterpenoids have been reported from *Fabiana imbricata*, therefore the occurrence of succinoyl and oxaloyl esters in *Fabiana densa* is not unexpected. [7,8]





All the extracts exhibited antimicrobial activity, the resinous exudate being the most active. The sensitive species were *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Salmonella aviatum*. No antimicrobial activity against *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Saccharomyces cerevisiae* was detected.

ent-Beyer-15-en-18-*O*-succinate (**1**) and *ent*-beyer-15-en-18-*O*-oxalate (**2**) are more active against Gram positive and less active against Gram negative bacteria (Table **1**). In these compounds, the presence of the succinoyl and oxaloyl residues seems to be essential for the activity; *ent*-beyer-15-en-18-ol (**3**) obtained by hydrolysis showed no antimicrobial effect against any of the microorganisms tested. Minimal inhibitory concentration of *ent*-beyer-15-en-18-*O*-succinate (**1**) against *S. aureus* was <10 µg/ml and for *ent*-beyer-15-en-18-*O*-oxalate (**2**) 60 µg/ml, while MIC for the resinous exudate extract is 200 µg/ml (Table **2**). MIC for Ampicillin as reference antibiotic is 5 µg/ml.

The antimicrobial properties found in this studys, could explain the use of *F. densa* in traditional medicine. In particular, activity against *S. aureus*, a bacterial pathogen which exhibits high levels of antibiotic resistance and is responsible for superinfection of

Table **1** Antimicrobial activity of compounds **1** and **2**

Microorganisms	*1*	*2*
S. aureus	+	+
B. subtilis	+	+
E. coli	+	-
S. aviatum	+	-

+ Antibacterial activity – No antibacterial activity.

Table **2** Minimum inhibitory concentration for *S. aureus*

Tested material[a]	*MIC/µg/ml*
Compound **1**	<10
Compound **2**	60
Resinous exudate	200
Ampicillin[b]	5

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the respiratory tract following viral infections [11], explains use of this plant to treat coughs and illness of the lungs [5].

Materials and Methods

Aerial parts of *F. densa* were collected in the Lauca National Park, I Region, Chile, and identified by the botanist Eliana Belmonte. A voucher specimen is kept at the Herbarium of the School of Chemistry and Pharmacy (SQF Nº 20918), University of Chile.

Air-dried, ground leaves (650 g) were sequentially extracted at room temperature with dichloromethane by immersion for the extraction of the resinous exudate (105.5 g), then with *n*-hexane, dichloromethane and methanol, yielding 23.5 g, 26.5 g and 37.5 g of extract respectively; which were used for the evaluation of the antimicrobial activity by a bioautography agar overlay bioassay, in order to the isolate the active compounds [9]. This resinous exudate (105.5 g) as the most active was subjected to column chromatography over silica gel 60 G and eluted with dichloromethane followed by increasing percentages of ethyl acetate , obtaining an active fraction (1 g) constituted of a mixture of two compounds eluted with CH_2Cl_2 : EtOAc (9 : 1) in fractions 50 – 62 of 200 ml. This fraction was subjected to a second column chromatography on silica gel with CH_2Cl_2 : EtOAc (98 : 2), in fractions 72 – 114 affording compound **1** (70 mg; 0.011 %) while compound **2** (150 mg; 0.023 %) was obtained by successive elution with: CH_2CL_2 : EtOAc (1 : 1) in fractions 127 – 132 of 30 mL Both were crystallised from CH_2Cl_2 with drops of methanol.

ent-*Beyer-15-en-18*-O-*succinate* (**1**): $C_{24}H_{36}O_4$, m.p. 107 – 108 °C, $[\alpha]_D^{20}$: + 14.6° (*c* 0.025, $CHCl_3$), R_f: 0.5 in silica gel with CH_2Cl_2 : EtOAc (8.5 : 1.5). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 5.67 (H-15; d, *J* = 5.6 Hz), 5.45 (H-16; d, *J* = 5.6 Hz), 3.88 (H-18a; d, *J* = 10.8 Hz), 3.68 (H-18b; d, *J* = 10.8 Hz), 2.68 (4H, -CH_2CH_2-, m), 1.00 (Me-17, s), 0.84 (Me-19, s), 0.78 (Me-20, s). ^{1}H-NMR (CD_3OD), δ = 5.71 (H-15; d, *J* = 5.7 Hz), 5.44 (H-16; d, *J* = 5.7 Hz), 3.90 (H-18a; d, *J* = 10.8 Hz), 3.63 (H-18b; d, *J* = 10.8 Hz), 2.60 (4H, s), 0.98 (Me-17, s), 0.86 (Me-19, s), 0.82 (Me-20, s). ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 38.6 (C-1), 17.8 (C-2), 35.9 (C-3), 37.2 (C-4), 49.9 (C-5), 20.2 (C-6), 36.9 (C-7), 48.9 (C-8), 92.8 (C-9), 36.6 (C-10), 20.1 (C-11), 33.1 (C-12), 43.6 (C-13), 61.1 (C-14), 135.1 (C-15), 136.4 (C-16), 24.9 (C-17), 73.5 (C-18), 17.6 (C-19), 15.5 (C-20) 172.1 (C-1´), 28.9 (C-2´), 29.0 (C-3´), 177.4 (C-4´). Long-range HETCOR connectivities: C-1, C-5 and C-9 with δ = 0.78 (Me-20); C-4 and C-18 with δ = 0.84 (Me-19); C-12, C-13 and C-16 with δ = 1.00 (Me-17); C-1´ and C-4´ with δ 2.68 (-CH_2-CH_2-).

Difference NOE experiments: the selective irradiation at δ = 5.68 (H-15) enhanced the signal at δ = 0.78 (Me-20) and *vice versa*; the irradiation at δ = 5.46 (H-16) gave a response for Me-17 (δ = 1.00) and *vice versa*; irradiation at δ = 3.88 (H-18a) or at δ = 3.68 (H-18b) enhanced the signal of Me-19 (δ = 0.84).

ent-*Beyer-15-en-18*-O-*oxalate* (**2**): $C_{22}H_{32}O_4$, m.p. 169 – 170 °C, $[\alpha]_D^{20}$: + 10°(*c* 0.025, $CHCl_3$) , R_f: 0.7 in silica gel with EtOAc: CH_2Cl_2 (7:3), Detection with anisaldehyde-H_2SO_4 giving deep pink colour and purple Liebermann-Burchard reagent respectively with compounds **1** and **2**. ^{1}H-NMR ($CDCl_3$): δ = 5.66 (H-15; d, *J* = 5.7 Hz), 5.45 (H-16; d, *J* = 5.7 Hz), 3.79 (H-18a), 3.36 (H-18b), 0.99 (Me-17, s), 0.84 (Me-19, s), 0.77 (Me-20, s). ^{1}H-NMR (CD_3OD): δ = 5.71 (H-15; d, *J* = 5.7 Hz), 5.43 (H-16; d, *J* = 5.7 Hz), 3.95 (H-18a; d, *J* = 10.9 Hz), 3.69 (H-18b; d, *J* = 10.9 Hz), 0.97 (Me-17, s), 0.87 (Me-19, s), 0.82 (Me-20, s). ^{13}C-NMR ($CDCl_3$): δ = 38.6 (C-1), 17.8 (C-2), 36.0 (C-3), 37.2 (C-4), 50.3 (C-5), 20.2 (C-6), 37.0 (C-7), 48.9 (C-8), 52.7 (C-9), 36.4 (C-10), 20.2 (C-11), 33.1 (C-12), 43.6 (C-13), 61.1 (C-14), 135.1 (C-15), 136.5 (C-16), 24.9 (C-17), 74.6 (C-18), 17.6 (C-19), 15.5 (C-20), 172.1 (C-1´), 174.0 (C-2´).

Hydrolysis of compounds **1** *and* **2:** Compounds **1** or **2** (30 mg) were dissolved in 1 % NaOH in ethanol and left at room temperature overnight. After neutralization and evaporation the raw product was purified on silica gel column eluted with hexane: CH_2Cl_2 (9 : 1) to give compound **3**, mp 111 – 112 °C. $[\alpha]_D^{20}$: + 29.5 (*c* 0.025, $CHCl_3$). The IR and NMR data are identical to those of *ent*-beyer- 15-en-18-ol (5).

Antimicrobial activity: The antimicrobial activity of the resinous exudate and compounds **1** and **2** were determined against *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (clinical isolate), *Salmonella aviatum* (ATCC 2228), *Pseudomonas aeruginosa* (ATCC 14207), *Staphylococcus aureus* (ATCC 6538P), *Micrococcus flavus* (ATCC 10290), *Bacillus subtilis* (ATCC 6633) and *Saccharomyces cerevisiae.*

The extracts were dissolved in DMSO. Dilutions of 100 and 200 µg/ml were added to a fixed volume of Plate Count Agar (PCA). They were then superficially inoculated with a single line of an overnight culture of the different microorganisms and incubated at 37 °C for 24 h. Results were recorded as growth or growth inhibition at each extract concentration [10]. The MIC was determined for the active compounds **1** and **2**, which showed antibacterial activity in the plate assay. The turbidimetric method was used with serial dilutions of the extract in 4 ml of the Plate Count Broth or Tryptic Soy Broth. Both media were used to assay the MIC of the active compound against *S. aureus* [11].

Bioautographic agar overlay assay in TLC of the resinous exudate and compounds **1** and **2** was carried out on silica gel 60G F_{254} glass and developed with $CHCl_3$: EtOAc (8.5 : 1.5) as solvent, [9].

Acknowledgements

The authors are grateful to the CEPEDEQ-Faculty of Chemical and Pharmaceutical Sciences, University of Chile and The Andrew W. Mellon Foundation for financial supports.

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