Structural Elucidation and Antimicrobial Characterization of Novel Diterpenoids from *Fabiana densa* var. *ramulosa*

Deborah Quaglio, Silvia Corradi, Silvia Erazo, Valeria Vergine, Simone Berardozzi, Fabio Sciubba, Floriana Cappiello, Maria Elisa Crestoni, Fiorentina Ascenzioni, Francesco Imperi, Bruno Delle Monache, Mattia Mori, Maria Rosa Loffredo, Francesca Ghirga, Bruno Casciaro, Bruno Botta, and Maria Luisa Mangoni

**ABSTRACT:** Novel diterpenoids were isolated from the extracts of *Fabiana densa* var. *ramulosa* and found to display a selective activity against Gram-positive bacterial strains with negligible cytotoxicity toward human keratinocytes. This study highlighted the role played by the acidic group at C18 of the tetracyclic ent-beyerene scaffold for antibacterial effects and how the length and flexibility of the alkyl chain between the two carbonyl groups are crucial factors to increase the antimicrobial activity of the molecules, supporting the development of natural products from *F. densa* and their derivatives for treatment of microbial infections.

**KEYWORDS:** Natural products, plant secondary metabolites, diterpenes, Gram-positive bacteria, antimicrobial activity, cytotoxicity

Nowadays, a significant portion of the commercial drugs occurs in nature or is derived from natural products by means of chemical transformations or de novo synthesis. One of the difficulties in developing new drugs is the decline of natural products research, which started near the end of the 20th century, in favor of the development of enormous synthetic libraries of small molecules by combinatorial approaches. Nowadays, this trend is changing and a renewed interest in nature as a source of hit and lead compounds for drug discovery is emerging. Particularly, we are entering a New Golden Age of natural products drug discovery. However, a tremendous wide discrepancy between their historical significance and their occurrence in modern drug development still exists. Recently, a predictive structural and biodiversity-based analysis assessed that taxon’s abundance considerably affects the development of a natural product into a drug, and most plants of interest today are usually indigenous only to biodiversity-rich countries especially in the tropics and subtropics. In addition, the isolation of bioactive chemical constituents faces a number of technical challenges including the variability of the source material, the difficulty in isolating the active constituents, and the costs of collection and storage. A unique in-house library of about 1 000 natural compounds, mostly isolated from several plants used in traditional medicine of South America and collected over the years, is available at the Organic Chemistry Laboratory of the Department of Chemistry and Technology of Drugs (Sapienza University of Rome, Italy). Besides natural products from different classes, the library has been recently enlarged with natural products from commercially available sources and synthetic and semisynthetic derivatives. All components of our collection are incorporated into a virtual library, showing a satisfactory chemical diversity. Therefore, this in-house library offers a unique chance to identify unexpected new scaffolds for the development of therapeutically relevant molecules. Furthermore, it has been successfully screened in...
silico and in vitro for the identification of hit and lead compounds in recent early stage drug discovery projects. In order to further enrich the library with novel natural products, we investigated the Fabiana densa Remy var. ramulosa Wedd., a native shrub of Chile commonly called “checal”, “tolilla” or “tola-checal”. The genus Fabiana (Solanaceae family) grows in South America, especially along arid mountainous area between 16° and 51° latitude and between 1 000 and 4 900 m over sea level, featuring needlelike leaves and profuse tiny tubular flowers. This genus comprises 15 species: ten are present in Argentina, seven in Chile, four in Bolivia and one in Perú. However, the latest revision of the vascular flora of Chile indicates a total of eight species of the Fabiana genus, including F. densa J. Remy.12 Fabiana spp. extracts have long been employed as fuel (firewood), as incense, and as ash in many rituals and in traditional South American medicine, but there is no evidence to validate their folk use. A moderated diuretic activity of F. patagonica extract was reported by Alvarez et al., and it was suggested to be associated with the presence of oleanolic acid, which was isolated as the major metabolite.13 The antimicrobial activity of tinctures and aqueous extracts of Argentinian species occurring in the highlands (including F. bryoides, F. densa, and F. punensis) was reported against a panel of sensitive and multidrug-resistant Gram-positive and Gram-negative bacteria. The effectiveness of these plants as inhibitors of inflammatory mediators along with their free radical scavenging properties and genotoxic effects were investigated by Cuello and colleagues by studying the aqueous and ethanolic extracts of four Fabiana species growing in mountainous area of Argentina (F. bryoides, F. punensis, F. densa, and F. patagonica). An integrative overview of the traditional uses, chemistry, bioactivity, and chemical profiling of the crude F. imbricate, the most common species in central Chile, was recently provided. Notably, so far no studies have been reported on Fabiana densa Remy var. ramulosa, except for the work of Erazo and co-workers. This research group described the isolation of two diterpenoids, i.e., the succinoyl and the oxaloyl esters of the ent-beyer-15-en-18-ol which was isolated as the major metabolite. The antimicrobial activity of tinctures and aqueous extracts of Argentinian species occurring in the highlands (including F. bryoides, F. densa, and F. punensis) was reported against a panel of sensitive and multidrug-resistant Gram-positive and Gram-negative bacteria. The effectiveness of these plants as inhibitors of inflammatory mediators along with their free radical scavenging properties and genotoxic effects were investigated by Cuello and colleagues by studying the aqueous and ethanolic extracts of four Fabiana species growing in mountainous area of Argentina (F. bryoides, F. punensis, F. densa, and F. patagonica). An integrative overview of the traditional uses, chemistry, bioactivity, and chemical profiling of the crude F. imbricate, the most common species in central Chile, was recently provided. Notably, so far no studies have been reported on Fabiana densa Remy var. ramulosa, except for the work of Erazo and co-workers. This research group described the isolation of two diterpenoids, i.e., the succinoyl and the oxaloyl esters of the ent-beyer-15-en-18-ol (previously identified as Baccharis tola). The succeoletic activity of these compounds was evaluated against a panel of Gram-negative and Gram-positive bacteria, respectively (Figures S4 and S8). Accordingly, the 1H NMR spectral data of compound 4 revealed a multiplet centered at 2.67 ppm integrated for four protons, which was attributed to H-22 and H-23 of the succinate side chain (Figure 1). Comparing the 13C NMR spectra, the only difference was the presence of signals at 158.65 and 157.77 ppm in the case of 2 and 177.44 and 172.28 ppm in the case of 4, attributed to the oxalate and succinate carbonyl groups, respectively (Figures S4 and S8). Accordingly, the 1H NMR spectral data of compound 3 was almost coincident with those of the ent-beyerene scaffold of 1, 2, and 4, except for the signals (a pair of doublets) of H-18a and H-18b which were shifted at 3.98 and 3.75 ppm and a singlet at 3.45 ppm integrated for two protons, which was attributed to the protons of the malonate side chain (Figure 1). Comparison of the 13C NMR spectral data of 3 with those of alcohol diterpene 1 evidenced two characteristic signals at 138.65 and 137.77 ppm, respectively, which were attributed to the olefinic carbonyl groups of the malonate side chain (Figure 1).
169.30 and 167.81 ppm, which were assigned to the carbonyl groups at C-23 and C-21 of the malonate function, respectively (Figure S6). Taking into account the spectral information on 2, 3, and 4, structural elucidation of the new compounds 5, 6, and 7 was carried out. With respect to compound 5, the NMR spectral data were coincident with those of oxaloyl ester 2 (Figure 1), except for the signal at 158.08 ppm in the 13C NMR spectrum which was assigned to the equivalent carbonyl groups at C-21 and C-21′ of the oxalate bridge, thus suggesting a dimeric structure (Figure S10). NMR structural elucidation was confirmed by the ESI-high-resolution mass spectrum, which revealed the diagnostic peak at 653.45408 m/z attributed to the [M + Na]+ sodium adduct. From a visual inspection of the 6 and 7 1H NMR spectra, the integrals of signals located in the region from 2.5 to 6.0 ppm suggested dimeric structures featuring two diterpene moieties linked by an intramolecular malonate and succinate bridge, respectively (Figure 1). With regard to the product 6, the 1H NMR spectral data were almost coincident with those of the malonoyl ester 3 (Figure 1). However, the singlet of the protons H-22, belonging to the intramolecular malonate bridge, was integrated in a 1:1 ratio with respect to the characteristic signals of the diterpene scaffold. The dimeric structure was confirmed by the 13C NMR, which showed only one signal at 166.84 ppm for both the carbonyl groups at C-21 and C-21′ of the malonate bridge (Figure S12), and by the ESI-high-resolution mass spectrum which revealed the diagnostic peak at 667.46911 m/z attributed to the [M + Na]+ sodium adduct. Regarding dimer 7, a comparison of its NMR spectral data with those of the monomeric diterpene 4 was revealed by the following points. In the proton spectrum, a singlet centered at 2.66 ppm integrated for four protons was assigned to the equivalent protons H-22 and H-22′ (Figure 1). In addition, this signal was integrated in 1:2 ratio with the characteristic signals of diterpene scaffold, suggesting a dimeric structure also for 7. Only one signal at 172.45 ppm for both the carbonyl groups at C-21 and C-21′ of the succinate bridge in the carbon spectrum (Figure S14) and a diagnostic peak at 681.48580 m/z in the ESI-high-resolution mass spectrum, which was attributed to the [M + Na]+ sodium adduct, confirmed the dimeric structure. The pentacyclic triterpenes 8 and 9 are widely distributed in the plant kingdom. In particular, triterpene 8 was isolated in high yields from the Chilean medicinal plants belonging to the Fabiana genus: Fabiana imbricata R. et P. and F. patagonica Specie. In this work, triterpenes 8 and 9 were isolated for the first time as constituents of F. densa var. ramulosa. Chemical identity of these compounds was confirmed by NMR (Figures S15−S18) and ESI-MS experiments and proved to be in agreement with the literature data.\(^\text{20,21}\)

**Semisynthesis of Diterpene Esters 2, 3, and 4.** In order to obtain the monomeric diterpenes in higher yield, the alcohol 1 was used as a starting material. In particular, the esterification of diterpene 1 with the corresponding acyl chloride was carried out in Et2O at reflux for 30 min (Scheme 1). The corresponding oxaloyl 2, malonoyl 3, and succinoyl 4 esters were obtained in 68%, 70%, and 80% yields, respectively. The semisynthetic approach, based on the employment of 1 as a suitable platform featuring the ent-beyerene scaffold, provided a mild and cost-effective procedure to prepare several diterpene derivatives.

**Antimicrobial Activity.** The newly identified molecules from F. densa var. ramulosa (compounds 3, 5, 6, 7) as well as the diterpenoids previously isolated from this plant species (compounds 1, 2, and 4) were analyzed for their capability to inhibit the growth of a panel of Gram-negative and Gram-positive bacterial reference strains. Ursolic and oleanolic acids (compounds 8 and 9), already identified in other plant species and largely investigated for their antimicrobial activity,\(^\text{8,22,23}\) were not studied in this work. As reported in Figure 2, compound 1 resulted to be inactive against all the tested bacterial strains. In comparison, compound 2 displayed a selective activity against Gram-positive bacterial strains, causing approximately 50% inhibition of growth of Staphylococcus epidermidis ATCC 12228 and Bacillus thuringensis B15 at the highest concentration used (64 μM), while compound 4...
induced more than 80% inhibition of \textit{S. epidermidis} and \textit{Bacillus megaterium} Bm11 growth, and complete inhibition of bacterial growth in the case of \textit{B. thuringensis}. A weaker effect (\sim 40% growth inhibition) was instead exhibited by the two compounds (2 and 4) against \textit{S. aureus} ATCC 25923. Interestingly, the new diterpene, i.e., the malonyl ester (compound 3), which was tested for the first time in this study, was found to be active against all the Gram-positive species, although the inhibitory effect was stronger against \textit{Bacillus spp.} (>70% inhibition at 64 \textmu M). In contrast, the dimeric compounds 5, 6, and 7 were devoid of efficacy against all the tested microorganisms and therefore are not shown.

\textbf{Cytotoxicity.} On the basis of the results obtained from the antimicrobial assays, we studied the effect of compounds 1, 2, 3, and 4 on the viability of mammalian cells that can be easily infected and colonized by \textit{Staphylococcus spp.}, such as keratinocytes. \cite{24,25} Therefore, human immortalized keratinocytes (HaCaT cells) were incubated for 24 h with the selected molecules at a concentration range from 16 to 64 \textmu M, and the percentage of metabolically active cells was evaluated by the 3-
(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Figure 3).

Figure 3. Effect of compounds 1, 2, 3, and 4 at different concentrations, on the viability of HaCaT cells. The percentages of metabolically active cells, calculated with respect to the vehicle-treated cells, are the mean of three independent experiments ± SEM.

Compound 1 which was inactive against microbial strains (Figure 2) was also harmless to HaCaT cells at all concentrations tested. In parallel, compounds 2 and 4 caused about 36% and 20% reduction of cell viability at the highest concentration of 64 μM. Interestingly, after treatment with compound 3, the reduction in the percentage of metabolically active cells at 64 μM was only 14%, suggesting this compound as the less toxic molecule among those showing significant antimicrobial activities.

Conclusions. Based on previous evidence on the antimicrobial activity of noncommon diterpenes from Fabiana densa var. ramulosa, we performed a more comprehensive study of this plant species. In this work, we described the isolation and structural elucidation of novel chemical constituents of F. densa and, among them, three dimeric diterpenes and a new diterpenoid (3) were identified and reported for the first time. Interestingly, a thorough characterization of their antimicrobial and cytotoxic activities indicated diterpene 3 as the less toxic molecule among those showing significant antimicrobial activities, whereas the dimeric diterpenes were not active toward any of the tested microorganisms. These results indicated a key role played by the acidic group at C18 of the tetracyclic ent-beyerene scaffold for the antibacterial effects and highlighted how the length and flexibility of the alkyl chain between the two carbonyl groups are crucial factors for the biological activity of the molecule. Importantly, these studies should assist the optimization of new diterpene-based drugs for the development of new anti-infective agents.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications Web site. The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00605.

Chemistry experimental section and the biological assays (PDF)

AUTHOR INFORMATION

Corresponding Authors
Francesca Ghirga — Center For Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, 00161 Rome, Italy; orcid.org/0000-0002-5591-5190; Email: francesca.ghirga@iit.it

Bruno Casciaro — Center For Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, 00161 Rome, Italy; Email: bruno.casciaro@iit.it

Authors
Deborah Quaglio — Department of Chemistry and Technology of Drugs, "Department of Excellence 2018–2022", Sapienza University of Rome, 00185 Rome, Italy
Silvia Corradi — Department of Chemistry and Technology of Drugs, "Department of Excellence 2018–2022", Sapienza University of Rome, 00185 Rome, Italy; Center For Life Nano Science@Sapienza, Istituto Italiano di Tecnologia, 00161 Rome, Italy
Silvia Erazo — Department of Pharmacological and Toxicological Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile
Valeria Vergine — Department of Chemistry and Technology of Drugs, "Department of Excellence 2018–2022", Sapienza University of Rome, 00185 Rome, Italy
Simone Berardozzi — Department of Chemistry and Applied Biosciences, ETH Zurich, 8092 Zurich, Switzerland
Fabio Sciuuba — Department of Chemistry, Sapienza University of Rome, 00185 Rome, Italy
Floriana Cappiello — Department of Biochemical Sciences, Laboratory Affiliated to Pasteur Italia-Fondazione Cenci Bolognetti, Sapienza University of Rome, 00185 Rome, Italy
Maria Elisa Crestoni — Department of Chemistry and Technology of Drugs, "Department of Excellence 2018–2022", Sapienza University of Rome, 00185 Rome, Italy
Maria Rosalba Cremona — Department of Chemistry, Sapienza University of Rome, 00185 Rome, Italy
Floriana Cappiello — Department of Biochemical Sciences, Laboratory Affiliated to Pasteur Italia-Fondazione Cenci Bolognetti, Sapienza University of Rome, 00185 Rome, Italy
Maria Elisa Crestoni — Department of Chemistry and Technology of Drugs, "Department of Excellence 2018–2022", Sapienza University of Rome, 00185 Rome, Italy
Francesco Imperi — Department of Sciences, Roma Tre University, 00154 Rome, Italy
Mattia Legnani — Centro Chimica dei Recettori, C.N.R., 00168 Roma, Italy
Mattia Mori — Department of Biotechnology, Chemistry and Pharmacy, "Department of Excellence 2018–2022", University of Siena, S3100 Siena, Italy; orcid.org/0000-0003-3299-1254
Maria Constanza Mariani — Department of Biochemical Sciences, Laboratory Affiliated to Pasteur Italia-Fondazione Cenci Bolognetti, Sapienza University of Rome, 00185 Rome, Italy
Bruno Bottà — Department of Chemistry and Technology of Drugs, "Department of Excellence 2018–2022", Sapienza University of Rome, 00185 Rome, Italy; orcid.org/0000-0017-8707-4335
Maria Luisa Mangoni — Department of Biochemical Sciences, Laboratory Affiliated to Pasteur Italia-Fondazione Cenci Bolognetti, Sapienza University of Rome, 00185 Rome, Italy; orcid.org/0000-0002-5991-5868

Complete contact information is available at:
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Author Contributions
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Notes
The authors declare no competing financial interest.
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