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Synergistic antimycobacterial activities of sesquiterpene lactones from *Laurus* spp.

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***Objectives*: The aim of this study was to determine the antimycobacterial potential of laurel oil, its fractions and its two sesquiterpene lactones against several mycobacterial strains and clinical isolates, and to establish the possibility of occurrence of some synergistic effects between those lactones using a modification of the fluorometric Alamar Blue microassay (FMABA).**

***Methods*: The *in vitro* antimycobacterial activity of whole oil and its fractions and pure active compounds were determined by FMABA. A bioassay-guided fractionation of the traditional preparation of laurel oil from Madeira Islands was performed, yielding pure compounds chemically identified by standard procedures. Synergism of pure compounds was established by *X*/*Y* quotient analysis adapted to FMABA.**

***Results*: Sesquiterpene lactones, costunolide and dehydrocostuslactone, were the compounds responsible for the antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv with MICs of 6.25 and 12.5 mg/L, respectively. Antimycobacterial activity against drug-resistant *M. tuberculosis* clinical isolates was better for the mixture than for pure compounds.**

***Conclusions*: Both lactones presented synergistic activity, i.e. analysis of relative fluorescence units presented an *X*/*Y* value <0.5 at a concentration of 1/8 MIC of each compound in the combination. Establishment of synergism by FMABA represents another application of the microplate Alamar Blue assay.**

Keywords: costunolide, dehydrocostuslactone, Alamar Blue, synergism

Introduction

The resurgence of tuberculosis (TB) is one of the most serious public-health challenges of the 21st century. Despite continued control efforts, TB remains a leading cause of illness and death worldwide. The recent rise of TB is associated with the emergence of the human immunodeficiency virus (HIV) and the rapid spread of multidrug-resistant TB strains. There is therefore a requirement for new classes of antibacterial compounds that have activity against these strains. Naturally occurring pure compounds as well as extracts from higher and lower forms of plants and terrestrial and marine organisms have indicated that inhibitory activity against *Mycobacterium tuberculosis* is widespread in nature.[1] Preparations of plants containing sesquiterpene lactones are often used in traditional medicine.[2] These compounds are starting to receive more attention regarding their potential biological activities, since there has been solid evidence that they possess anti-inflammatory, anti-tumour and antimicrobial activities.[3–5]

Laurel oil, the oil expressed from the ripe fruit of *Laurus novocanariensis* (the *Laurus* subspecies found in the Madeira archipelago) has been used for centuries in traditional medicine. It is externally applied to treat skin infections and as an anti-rheumatic and vulnerary. It is internally taken as a blood depurative, stomachic and haemostatic. Folk medicine recommends laurel oil for the treatment of influenza and other respiratory symptoms as well as for apoplexy and constipation.[6] The chemical characterization of the whole oil was recently published, and its sesquiterpene lactone content was quantitatively determined, with average values of 3.8% costunolide and 1.5% dehydrocostuslactone.[7,8]

To our knowledge, laurel oil has not previously been studied for its potential as an anti-TB herbal medicine. In this study, we analysed the antimycobacterial potential of laurel oil, its

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fractions and its two lactones against several mycobacterial strains and clinical isolates.

Materials and methods

Plant material

Laurel oil is a viscous dark green fluid with a very strong and characteristic aroma. Oil samples were obtained from local producers, and production was monitored to assure authenticity. Fractionation by different methods was used to separate the various families of compounds.

Fractionation of laurel oil by liquid–liquid partition

The separation of laurel oil into essential oil (EO), lipids and lactones is fully described in Castilho *et al.*[7] Briefly, volatile components were removed by hydrodistillation of the oil in a Clevenger-type apparatus to yield the EO and an odourless residue containing the lipids and other non-volatile compounds. The residue was partitioned between *n*-hexane and methanol to separate the lipid fraction (LF) from the more polar compounds, including the lactones. The methanolic fraction (MF) containing mainly the lactones costunolide (**1**) and dehydrocostuslactone (**2**) was further fractioned by column chromatography; **1** and **2** (Figure 1) were identified by their mass spectra, and proton and carbon NMR spectra and their purity established as >95% by gas chromatography.

Mycobacterium strains and clinical isolates

The *Mycobacterium* species, *Mycobacterium tuberculosis* H37Rv (ATCC 27294), H37Rv isoniazid-resistant (ATCC 35822), H37Rv rifampicin-resistant (ATCC 35838) and H37Rv ethambutol-resistant (ATCC 35837), were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Three drug-resistant pulmonary isolates of *M. tuberculosis* were obtained from patients from different hospitals in Mexico. The drug-resistant isolates were selected based on their drug susceptibility patterns to the antimycobacterial drugs determined by the Alamar Blue microplate method. Three non-tuberculous mycobacteria clinical isolates (*Mycobacterium avium*, *Mycobacterium chelonae* and *Mycobacterium fortuitum*) and the *Mycobacterium smegmatis* (mc^2) reference strain were also employed.

Growth conditions and inoculum preparation

Reference strains and clinical isolates were cultured at 37°C in Middlebrook 7H9 broth (BBL) supplemented with 0.2% glycerol and 10% OADC enrichment (oleic acid/albumin/dextrose/catalase; Difco) until log-phase growth was achieved. Inocula for the fluorometric assays were prepared by diluting log-phase growth cultures with sterile Middlebrook 7H9 broth to turbidities equivalent to that of a no. 1 McFarland standard and then further diluted 1:10 for tuberculous mycobacteria and 1:50 for non-tuberculous mycobacteria.

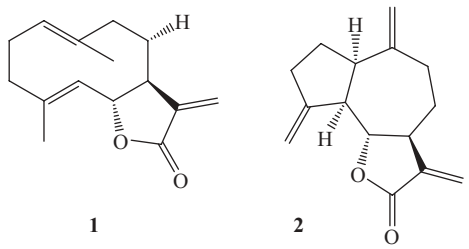

Figure 1. Costunolide **1** and dehydrocostuslactone **2**; 3.8% and 1.5% of Madeira laurel oil, respectively.[8]

Antimycobacterial activity determination by fluorometric microplate Alamar Blue assay

The methodology was fully described in Jimenez-Arellanes *et al.*,[9] but some modifications were made in the present study. Briefly, stock solutions of whole oil and EO, LF and MF were prepared in DMSO at a concentration of 20 g/L; pure compounds **1** and **2** were dissolved in DMSO at a concentration of 5 g/L under sterile conditions and stored at −70°C until use. Serial dilutions of each fraction or compound were prepared; final testing concentrations ranged from 200 to 50 mg/L for whole oil, 200 to 12.5 mg/L for fractions and 50 to 1.75 mg/L for pure compounds. Duplicates of each sample were made per plate, and each experiment was repeated at least twice. Bacterial suspension (100 μL) was added to test wells and to controls. A 1:10 diluted control was included in each plate representing the growth of 10% of the bacterial population tested (10% control). Plates were incubated at 37°C. After 5 days of incubation, the plates were developed by adding to each well 20 μL of Alamar Blue solution (Trek Diagnostics, Westlake, OH) and incubating for 24 h. Fluorescence was measured in a plate fluorometer (Fluoroskan Ascent FL, Thermo, Finland) at an excitation wavelength of 490 nm and an emission wavelength of 540 nm, and relative fluorescence units (rfu) were recorded. MIC was defined as the lowest drug concentration that presented rfu values lower that those presented by the 10% growth control. There was always a correlation between fluorometric and visual observations, i.e. pink wells presented high rfu values.

Determination of synergistic antimycobacterial activity by fluorometric Alamar Blue microassay

Synergistic antimycobacterial activity was evaluated against *M. tuberculosis* H37Rv, by a modification of the fluorometric Alamar Blue microassay, using combinations of the two pure lactones **1** and **2**, at one-half, one-fourth and one-eighth of the MIC previously determined in the antimycobacterial assay described earlier. The assay was performed in 96-well microplates (Costar). Briefly, working solutions of both compounds were prepared in 7H9 broth, at a concentration corresponding to four times the MIC when 1/2 of MIC combination was analysed; for 1/4 and 1/8 MICs combinations, compound solutions were prepare at two times and one time the corresponding MICs. At least two wells per combination were used.

Working solutions of costunolide (50 μL) and dehydrocostuslactone (50 μL) were added simultaneously to the well and mixed thoroughly, and then 100 μL of the bacterial suspension (adjusted to a turbidity equivalent to that of a no. 1 McFarland standard and diluted 1:10) was added. Controls for each pure compound were obtained by adding 50 μL of the corresponding working solution and 50 μL of 7H9 broth, and finally adding 100 μL of the same adjusted bacterial suspension. To check bacterial growth, controls free of drug were also obtained by adding

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100 μL of culture medium to the well and then 100 μL of adjusted bacterial suspension. Plates were sealed and incubated for 5 days at 37°C; after this period, 20 μL of Alamar Blue solution was added to the wells and reincubated overnight at 37°C. Finally, rfu were measured in the plate fluorometer.

Results

The whole laurel oil without any further preparation (i.e. the herbal remedy, as usually sold to patients in local pharmacies) was tested by the Alamar Blue microassay against *M. tuberculosis* H37Rv, showing a considerable activity that reached an MIC <50 mg/L (Figure 2a). These results moved us to perform its separation into families of compounds.

From the oil, three main preparations were obtained, each containing different types of compounds: EO consisted predominately of non-oxygenated mono and sesquiterpenes; LF presented mostly triglycerides and small amounts of sterols; and MF is composed mostly of sesquiterpene lactones. The antimycobacterial activity of these preparations is presented in Table 1; it is evident that the anti-TB activity of the oil resides

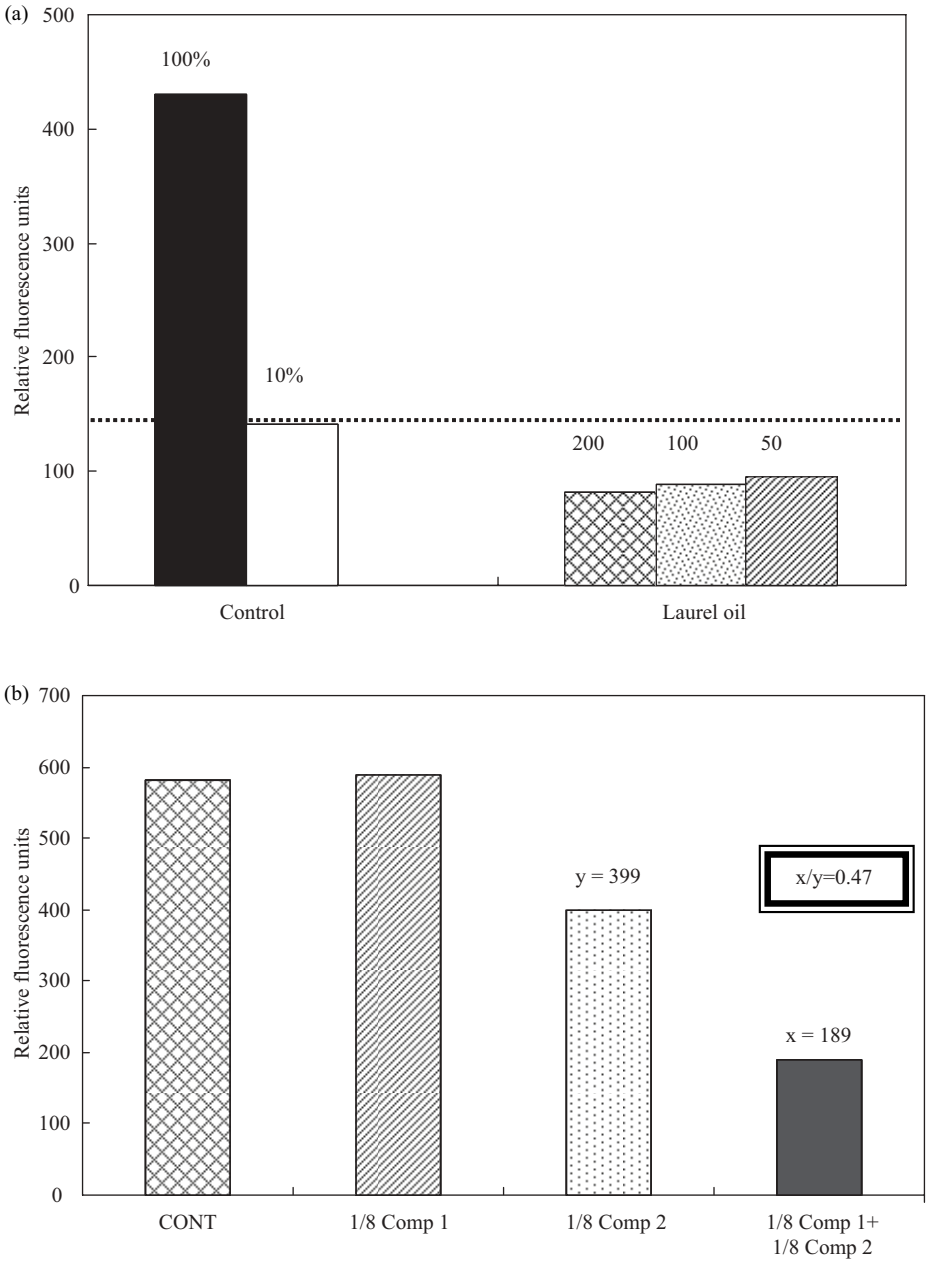

Figure 2. (a) Activity of laurel oil against *M. tuberculosis* H37Rv. Antimycobacterial activity was determined by fluorometric Alamar Blue assay at three concentrations of laurel oil (200, 100 and 50 mg/L). 10% bacterial growth was used to determine minimal inhibitory concentration (in this case, MIC was $\leq$50 mg/L). (b) Synergism determination by fluorometric Alamar Blue assay. Costunolide **1** (Comp 1) and dehydrocostuslactone **2** (Comp 2) were tested alone and in combination at 1/8 of their MIC against *M. tuberculosis* H37Rv; the value of the X/Y quotient denotes a synergistic combination. CONT indicates bacterial growth free of testing compounds.

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Table 1. Antimycobacterial activity of laurel oil fractions and pure compounds

Strain drug-resistance pattern	MICs of fraction/compound (mg/L)				
	EO	LF	MF	1	2
H37Rv pan-susceptible	150	200	50	12.5	6.25
H37Rv-INH-r isoniazid-resistant	150	200	3.25	12.5	12.5
H37Rv-RIF-r rifampicin-resistant	150	100	25	6.25	12.5
H37Rv-STR-r streptomycin-resistant	75	200	25	25	50
H37Rv-EMB-r ethambutol-resistant	>150	>200	12.5	12.5	6.25
Clinical isolate MMDO isoniazid- and ethambutol-resistant	>150	>200	25	25	50
Clinical isolate SIN4 isoniazid-, rifampicin-, streptomycin- and ethambutol-resistant	150	>200	50	25	50
Clinical isolate MTY 147 isoniazid- and rifampicin-resistant	150	>200	25	50	25

EO, FL and MF = laurel oil fractions; 1 = costunolide; 2 = dehydrocostuslactone.

mainly in the MF. MICs for the different strains and isolates analysed ranged from 3.25 to 50 mg/L. However, none of the preparations was active against the group of non-tuberculous mycobacteria studied (MICs >200 mg/L). From the active MF, costunolide **1** and dehydrocostuslactone **2** were isolated, and their antimycobacterial activities were also determined (Table 1).

The activity against H37Rv-INH-r, a strain resistant only to isoniazid was the most interesting result (Table 1), not only because the MF presented the highest activity, but because the pure compounds obtained from it (**1** and **2**) were less active than the whole fraction, hence the awareness of a possible synergistic effect between those lactones.

Determination of pharmacological interactions was performed following *X*/*Y* quotient analysis, as described by other authors.[10,11] *X* represents the rfu value obtained with the combination of both compounds, and *Y* is the rfu value of the compound that presented the lowest rfu value, when alone. Synergy was considered when the *X*/*Y* value was <0.5, additive activity when *X*/*Y* was >0.5 and <1.0, no activity when *X*/*Y* was 1–2, and antagonism when *X*/*Y* was >2.

In order to establish a synergistic effect between **1** and **2**, a simple determination was made based on the fluorometric microplate Alamar Blue assay principles as described in the Materials and methods section. The *X*/*Y* quotient was calculated based on the rfu values recorded. *X* was the value obtained with both compounds, and *Y* was the value of compound **2** (the lower rfu value of the compounds tested alone). Synergism, considered when the *X*/*Y* quotient was <0.5, was present up to the combination containing 1/8 MIC of each compound (Figure 2b).

Discussion

Sesquiterpenes are C-15 terpenoids that occur in nature as hydrocarbons or oxygenated forms such as alcohols, ketones, aldehydes, acids or lactones. The majority of the more than 4000 known different lactone structures have a guaiane, eudesmane or germacrane framework. Costunolide (Figure 1) is structurally the simplest of all germacranolides, and it is generally accepted as the parent compound of the three mentioned types of sesquiterpene lactones.[12]

Costunolide **1** and dehydrocostuslactone **2** were found as major components of the MF of Madeira *Laurus* oil[8] and have been suggested to possess various biological activities.[3–5] In the present work, we confirm that both lactones presented an important inhibitory action against both drug-susceptible and drug-resistant *M. tuberculosis* at MICs as low as 6.25 mg/L (range 50–6.25 mg/L). This activity corresponds to the ranges previously described by Cantrell *et al.* for sesquiterpene lactones of the germacranolide, guaianolide and eudesmanolide type.[13] Costunolide was reported to have an MIC of 32 mg/L against *M. tuberculosis* H37Rv, and dehydrocostuslactone (MIC of 2 mg/L) was reported as the most active compound of the series of guaianolides screened against *M. tuberculosis*.[14] Both previous determinations were performed using the radiorespirometric BACTEC system, and here by the Alamar Blue microassay, confirming the usefulness of the Alamar Blue microassay and its correlation with other established standard determinations. The significant activity of dehydrocostuslactone has been attributed to its high lipophilicity since introduction of hydroxyl groups to different positions of the guaianolide skeleton significantly reduced the antimycobacterial activity;[14] molecules with increased polarity have been reported to present a reduced transport through the outer lipid layer of the mycobacteria and, consequently, lower activity towards the mycobacteria. However, lipophilicity may not be high enough to allow the molecule to cross the cell walls of the non-tuberculous mycobacteria studied, since no activity was observed against this group. Additional studies are needed to confirm this hypothesis.

An important conclusion from our study is the fact that further steps of purification of extracts and fractions end up in

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a loss of biological activity, indicating that synergistic activities may occur between components. We approached this possibility by establishing a new application of the Alamar Blue microplate assay, the determination of synergism by the establishment of *X*/*Y* quotient analysis.[10–11] Fluorometric Alamar Blue determinations are quantitative as they are measured in relative fluorescence units. So, in accordance with previously used *X*/*Y* quotient analysis with radiometric units obtained with the BACTEC system (radiometric units, e.g. growth index), we adopted this procedure by substituting radiometric units by fluorometric units. Our results clearly showed the usefulness of the approach by establishing that compounds **1** and **2** had a synergistic effect, up to the combination containing 1/8 MIC of each compound.

Such compounds having antimicrobial properties in addition to their other pre-designated pharmacological actions are currently referred to as 'non-antibiotics'. Most antimycobacterial non-antibiotics reported so far have shown *in vitro* MIC values ranging from 10 to 25 mg/L, which seem to be in accordance with those of sesquiterpene lactones **1** and **2**.[15]

Costunolide and dehydrocostuslactone, being non-steroidal anti-inflammatory compounds, with modest *in vitro* antimycobacterial potential that is well enhanced when the two compounds appear together, as in Madeira laurel oil, raise the possibility that laurel oil might be used as an adjuvant to current regimens used for the management of newly diagnosed TB patients. In future work, we aim to obtain enriched fractions with mixtures of these compounds with a synergistic effect, avoiding laborious purification steps, enhancing yield and reducing costs, thereby making a new generation of potential non-antibiotic antitubercular mixtures.

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Transparency declarations

None to declare.

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