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Short communication

In vitro growth inhibition and bactericidal activity of spathulenol against drug-resistant clinical isolates of *Mycobacterium tuberculosis*

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ABSTRACT

Spathulenol was isolated from an extract of *Azorella compacta* Phil., Apiaceae, by various chromatographic method; identification of the chemical structure was confirmed by comparing its spectroscopic data with those reported in the literature. The anti-*Mycobacterium tuberculosis* activity of spathulenol was evaluated on MDR, pre-XDR, and XDR clinical isolates of *M. tuberculosis*, as well as on the reference susceptible strain H37Rv and its cytotoxic activity was evaluated on the Vero Cell Line. The anti-*M. tuberculosis* activity of spathulenol was twice as potent against the MDR, pre-XDR, and XDR clinical isolates (6.25 μg/ml) than on the susceptible H37Rv strain (12.5 μg/ml). Additionally, the anti-*M. tuberculosis* activity shown by spathulenol was established as bactericidal on drug-resistant and susceptible strains of *M. tuberculosis*. Finally, cytotoxic activity on the Vero cell line (CC_{50} = 95.7 μg/ml) indicated that spathulenol is a selective anti-*M. tuberculosis* compound, with a selective index of 15.31 against drug-resistant clinical isolates of *M. tuberculosis*.

Introduction

The spread of drug-resistant tuberculosis (TB) is a major threat to global TB control. In 2017, Multi-Drug-Resistant (MDR, resistant to at least Isoniazid and Rifampin) *Mycobacterium tuberculosis* strains caused an estimated of 230,000 deaths globally. Moreover, 22% and 8% of MDR-*Mycobacterium tuberculosis* strains were also pre-eXtensively-Drug-Resistant (pre-XDR, MDR plus resistant to one Fluoroquinolone or one of three injectable second-line drugs) and XDR (MDR plus resistant to one Fluoroquinolone and one of three injectable second-line drugs), respectively (WHO, 2018). Patients infected with drug-resistant strains of *M. tuberculosis* have to endure longer treatments (24 months or longer), severe adverse effects, and high cost, with a low possibility of being cured (Quan et al., 2017). Hence, the search for new anti-TB drugs that are fast-acting, and highly effective against drug-resistant *M. tuberculosis* strains, is a priority.

In the search for novel anti-TB drugs, natural products have played an important role in maintaining human health for thousands of years (Bernardini et al., 2018). *Azorella compacta* Phill., commonly known as "llareta", is a green, compact, resinous cushion shrub of the Apiaceae family growing in the high Andes of southern Peru and Bolivia, northeastern Chile, and northwestern Argentina. This medicinal plant has been traditionally employed to treat colds, pain, diabetes, asthma, bronchitis, womb ailments, gastric disorders, backache, wounds, and altitude sickness (Wickens, 1995). We have previously reported on the anti-*M. tuberculosis* activity of a number of natural azorellane and mulinane diterpenoids isolated from this medicinal plant (Molina-Salinas et al., 2010); as part of our continuing search for natural products with anti-*M. tuberculosis* activity, we wish to report here the *in vitro* growth inhibition and bactericidal activity of spathulenol, isolated from *A. compacta*.

Materials and methods

Vacuum Liquid Chromatography (VLC) and Column Chromatography (CC) were carried out using TLC-grade (GF_{254}, Sigma–Aldrich) and 70–230 mesh (Sigma–Aldrich) silica gel. Thin Layer

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Table 1
Anti-*Mycobacterium tuberculosis* activity of spathulenol.

Organism	Drug resistance profile	Spathulenol (μg/ml)	
		MIC	MBC
M. tuberculosis MDR	(STR, INH, RIF, EMB, PZA)	6.25	6.25
M. tuberculosis Pre-XDR	(STR, INH, RIF, PZA, LVX, OFX)	6.25	6.25
M. tuberculosis XDR	(STR, INH, RIF, PZA, AMK, KAN, LVX, OFX)	6.25	6.25
M. tuberculosis H37Rv	–	12.50	12.50

STR, Streptomycin; INH, Isoniazid; RIF, Rifampin; EMB, Ethambutol; PZA, Pyrazinamide; LVX, Levofloxacin; OFX, Ofloxacin; AMK, Amikacin; KAN, Kanamycin; CZM, Clofazimine; MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration. Positive controls: *M. tuberculosis* MDR (OFX, MIC = 0.50 μg/ml),*M. tuberculosis* pre-XDR (CZM, MIC = 0.50 μg/ml), *M. tuberculosis* XDR (CZM, MIC = 0.50 μg/ml); *M. tuberculosis* H37Rv (RIF, MIC = 0.06 μg/ml).

Chromatography (TLC) was performed on precoated aluminum silica gel plates (60°A F_{254}, Merck, 0.2-mm in thickness). TLC plates were first observed under Ultraviolet (UV) light (254 and 350 nm) and the components were visualized by spraying the plate with phosphomolybdic acid reagent [250 ml of 5% H_2SO_4, 10 g of phosphomolybdic acid, and 1.25 g of cerium (IV) sulfate hydrate (99%)], followed by heating for 5 min at 105 °C. InfraRed (IR) spectra were on a Nicolet Magna 750 (FTIR) Thermo Scientific spectrometer. Gas Chromatography-Mass Spectrometry (GC-MS) analyses were carried out in an Agilent Technologies 6890 N Gas Chromatograph coupled to a 5975B Mass Spectrometer. Nuclear Magnetic Resonance (NMR) spectra were recorded in $CDCl_3$ using a Brucker Advanced Ultra Shield 400 (400 MHz) spectrometer, with TMS as an internal standard.

Whole plants of *Azorella compacta* Phil., Apiaceae, were collected in northern Chile. The voucher specimen of the sample (Azc150411-14) has been preserved at the Natural Products Laboratory of the Universidad de Antofagasta, Antofagasta, Chile.

The hexane extract (27 g) of *A. compacta* was subjected to VLC over silica gel using a gradient elution with mixtures of hexane ethyl acetate (EtOAc, 100:0-85:15). Fraction 3 (953.2 mg) was further purified by CC on silica gel, using a gradient elution with mixtures of hexane and EtOAc (100:0-70:30) to yield pure spathulenol (192.7 mg), identified by comparing its spectroscopic data with those reported in the literature (Inagake and Abe, 1985).

In vitro anti-*M. tuberculosis* activity using the modified Microplate Alamar Blue Assay (MABA) was carried out as previously described (Molina-Salinas et al., 2006) on four strains of *M. tuberculosis*: three clinical isolates resistant to first-line drugs, first- and second-line drugs, and a drug-susceptible laboratory reference strain (H37Rv, ATCC 27294). Spathulenol was dissolved with dimethyl sulfoxide (DMSO) and tested using a concentration range of 100 to 1.56 μg/ml. The results were reported as Minimum Inhibitory Concentration (MIC). Rifampin, Ofloxacin, or Clofazimine were included as positive controls. All evaluations were carried out in triplicate. Spathulenol was also tested for mycobactericidal effect following the procedure previously described (Molina-Salinas et al., 2006).

The *in vitro* cytotoxic assay on Vero Cells (ATCC CCL-8) was evaluated using the Sulforhodamine B (SRB) method (Skehan et al., 1990). Spathulenol was dissolved with DMSO and tested using a concentration range of 200 to 6.25 μg/ml. The results were expressed as the concentration of product that killed 50% of the cells (CC_{50}). Docetaxel and untreated cells were used as positive and negative controls, respectively. All evaluations were performed in triplicate, and CC_{50} values were calculated using GraphPad Prism ver. 5 software.

Results and discussion

Purification of the extract of *A. compacta* yielded a pure metabolite that showed fifteen carbon signals in its ^{13}C-NMR spectrum, suggesting a sesquiterpenoid structure. The ^{1}H-NMR spectrum demonstrated the characteristic signals of protons in a cyclopropane ring at δ 0.46 (dd, J = 11.3, 9.5 Hz, H-6) and 0.71 (m, H-7) and of protons in exocyclic double-bond signals at δ 4.67 (s, H-14a) and 4.69 (s, H-14b). On the basis of this spectroscopic data, and by comparing these data with those reported in the literature, the purified sesquiterpene was identified as spathulenol (Inagake and Abe, 1985), previously reported as a component of the volatile oils (VO) of *Campomanesia* spp. (Limberger et al., 2001), and also as main component (38%) of VO of *Azorella trifurcata*, which showed antimicrobial activity on *Pseudomonas* spp. and *Staphylococcus* spp. (Lopez et al., 2018). Similarly, the VO from *Salvia cassia* containing 3.1% of spathulenol also demonstrated activity against some Gram-positive cocci and Gram-negative bacilli (Utsukarci et al., 2019). Finally, spathulenol isolated from *Helichrysum amorginum* exhibited weak activity on *Staphylococcus* spp. (Chinou et al., 2004), while a concentrated fraction from the aerial parts of *Salvia mirzagannii* containing 62% of spathulenol displayed a immunomodulatory effect (Ziaei et al., 2011).

The testing of spathulenol against different clinical isolates of drug-resistant and a susceptible *M. tuberculosis* strains showed that spathulenol was twice as potent against the MDR, pre-XDR, and XDR clinical isolates (MIC and MBC = 6.25 μg/ml) as the susceptible H37Rv (MIC and MBC = 12.5 μg/ml) *M. tuberculosis* strain (Table 1). These results identify this sesquiterpene as an anti-*M. tuberculosis* active metabolite according to reports in the literature, where a MIC ≤ 64 μg/ml is considered promising activity for a pure product (Cantrell et al., 2001). Further evaluation of spathulenol, the MBC were equal to MIC values in all four *M. tuberculosis* strains, suggesting that its anti-*M. tuberculosis* activity is bactericidal, which is desirable to reduce the risk of developing resistance in *M. tuberculosis* strains; spathulenol exhibited antimycobacterial activity on all drug-resistant clinical isolates, suggesting that its target in *M. tuberculosis* could be different from the current anti-TB drugs. Testing of spathulenol for its cytotoxic activity on primate cells revealed a CC_{50} of 95.69 μg/ml (positive control docetaxel CC_{50} = 1.68 μg/ml) and Selective Indexes (SI) of 15.31 and 7.67 for the drug-resistant clinical isolates of *M. tuberculosis* and the susceptible *M. tuberculosis* reference strain, respectively. SI to drug-resistant clinical isolates were considerably higher than 10, considered as being of interest to the pharmaceutical industry (Vonthron-Sénécheau et al., 2003).

do Nascimento et al. (2018) reported weak activity in spathulenol isolated from *Psidium guineense* expressed as the concentration that inhibits the growth of 90% of *M. tuberculosis* H37Rv (IC_{90} = 231.9 μg/ml) using the fluorometric Resazurin Microtiter Assay Plate (REMA) method (do Nascimento et al., 2018). Our studies by colorimetric MABA showed that spathulenol is active on the same susceptible reference strain *M. tuberculosis* H37Rv (MIC = 12.50 μg/ml). On the addition of two microassays utilized to evaluate the inhibitory effect of spathulenol, it is important to highlight that the working *M. tuberculosis* inoculum for REMA (1.5×10^7 CFU/ml)(Palomino et al., 2002) is higher than for MABA (6×10^6 CFU/ml) (Molina-Salinas et al., 2006). According to Jaki et al. (2008), a common finding in the literature on natural products

is that for the same compound had reported inconsistent values of its biological activity, which could be due to the unique specificity of each individual assay and to variability in the performance of the bioassays (Jaki et al., 2008). One example is the case of anti-*M. tuberculosis* activity of ursolic acid on H37Rv using MABA, which has reported different values of MIC: 8 (Woldemichael et al., 2003), 31 (Gu et al., 2004), and 65 (Jaki et al., 2008) μg/ml.

Authors' contributions

GMMS and LMPR contributed to conceptualization of the study; AHUC, LMPR and GMMS contributed to formal analysis; JB and GMMS contributed to funding acquisition;All authors contribute to investigation and methodology; GMMS contributed to project administration; JB, LAL and GMMS contributed to resources; AHUC and GMMS contributed to writing the original draft and all authors writing - review & editing the manuscript.

Ethical disclosures

Protection of human and animal subjects

The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data

The authors declare that no patient data appear in this article.

Right to privacy and informed consent

The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

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