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Antimycobacterial and anti-inflammatory activities of metabolites from endophytic and soil fungi

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ABSTRACT

Background: Increased prevalence of high virulent strains of *Mycobacterium tuberculosis* and *M. kansasii* require the development of new antimycobacterial drugs safe and more effective. And, in this context, endophytic fungi have been known as prominent sources of bioactive compounds.
Purpose: To study the antimycobacterial and anti-inflammatory activities of six fungal metabolites: austdiol (**1**), austdiol diacetate (**2**), mycoleptone A (**3**) and eugenitin (**4**) isolated from cultures of endophyte *Mycoleptodiscus indicus*; emodin (**5**) from endophyte *Penicillium citrinum* and δ-lactam (**6**) from soil fungi *Humicola grisea.*
Methods: Antimycobacterial activity against *M. bovis* BCG, *M. tuberculosis* and *M. kansasii* strains was evaluated using MTT method to determine the MIC_{50}. The anti-inflammatory activity and cytotoxicity assay were carried out in LPS-stimulated RAW 264.7 macrophages.
Results: Emodin (**5**) was the most active compound against high virulent *M. tuberculosis* and *M. kansasii* strains exhibiting MIC_{50} of 8.2 ± 1.0 and 23.9 ± 0.9 μM respectively and against intracellular *M. tuberculosis* H37Rv growth (MIC_{50} of 6.5 ± 1.5 μM). Emodin inhibitory activity has been previously described only against extracellular *M. tuberculosis* strains. Azaphilones (**1** and **3**) also showed inhibitory activity against hypervirulent *M. tuberculosis* culture ($MIC_{50} \leq 40$ μM) and intracellular growth ($MIC_{50} \leq 30$ μM) whereas compounds **1** and **2** were active against high virulent *M. kansassi* strains ($MIC_{50} \leq 40$ μM). Anti-inflammatory activity to inhibit NO, TNF-α and IL-1β production on LPS-stimulated macrophages was observed notably for austdiol (**1**) and emodin (**5**) with $MIC_{50} \leq 10$ μM and good potential for compounds **2** and **3**.
Conclusion: Azaphilone compounds (**1, 2** and **3**) and emodin (**5**) exhibiting both activities, antimycobacterial and anti-inflammatory, being promising anti-TB agents for further investigations focusing on dual treatment of severe pulmonary TB and NMT infections associated to hyperinflammation.

Abbreviations

ADC	albumin dextrose complex
BCG	bacillus calmette-Guérin
DMEM-F12	dulbecco's modified eagle's medium
FBS	fetal bovine serium
HPLC	high liquid performance chromatography
IL-1 β	interleukin - 1β
iNOS	inducible nitric oxide synthase
L-NMMA	ng -methyl-l-arginine acetate salt
LPS	lipopolysaccharide
MDR	multidrug-resistant
MOI	multiplicity of infection
MS	mass spectrometry
MTT	3-4(4,5dimetiltiazol-2-il)-2,5-difenil tetrazol
NO	nitric oxide
NMT	nontuberculous mycobacteria
NMR	nuclear magnetic resonance

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PBS	phosphate buffered saline
TB	tuberculosis
TLC	thin layer chromatography
TNF-α	Tumor Necrosis Factor-alpha
CFU	colony forming unit
RIF	Rifampicin

Introduction

Mycobacterial infections have caused significant morbidity and mortality in humans for more than a century and remain a serious global health problem. These infections can be developed by a group of mycobacteria including *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), and nontuberculous mycobacteria (NTM), such as *M. kansasii*. One-fourth of the world population is estimated to have latent TB and 10-15% of these will go to develop active TB (WHO, 2020; Colangeli et al., 2020). In 2019, 10 million people fell ill with TB and 1.4 million died worldwide (WHO, 2020).

Mycobacterium tuberculosis mainly affects the lungs where immune recognition of the bacilli leads to development of a local inflammation with consequent influx of phagocytic cells as macrophages and neutrophils into the lung (Martino et al., 2019). Hypervirulent and resistant *M. tuberculosis* strains can induce excessive uncontrolled inflammation characterized by accumulation of myeloid leukocytes and exacerbated production of inflammatory mediators (NO, TNF-α and IL-1β) that drives to the development of pulmonary necrosis and consequent cavitation (Orme et al., 2015; Almeida et al., 2017). TB lung lesions result in loss of lung function and reduced antibiotic action on the bacilli (Muefong et al., 2020).

The burden of NTM disease has increased substantially in last decades (Champa et al., 2020). *M. kansasii* is one of the most pathogenic NTM, able to cause lung disease similar to TB in immunocompromised and immunocompetent adults (Bakuła et al., 2018; Goldenberg et al., 2020). Previous studies demonstrated formation of cavities in *M. kansasii* infection cases in humans (Bakuła et al., 2018; Goldenberg et al., 2020) and that highly virulent *M. kansasii* isolates can cause excessive and chronic inflammation in mice, reproducing *M. tuberculosis* necrotic pathology (Mussi et al., 2021).

Novel treatment strategies, such as host-directed therapies, target the host excessive inflammatory response in order to prevent or reduce lung necrotic lesions increasing the success of therapy against *M. tuberculosis* and NTM (Crilly et al., 2021; Muefong et al., 2020; Strong and Lee, 2021; Young et al., 2019; Zumla et al., 2015), which also require new antimycobacterial drugs safer and more effective (WHO, 2020). Thus, new compounds exhibiting both activities, antimycobacterial and anti-inflammatory, could provide relevant therapeutic advantage for severe mycobacterial pulmonary infections.

Interest in natural products with biological activity, especially the research for novel compounds, has stimulated studies involving microorganisms, like fungi, actinomycetes and bacteria. Plant endophytic fungi are microorganisms that colonize the internal tissues of the hosts for all or part of their lifecycles without causing apparent pathogenic symptoms (Song et al., 2017). They have been known as prominent sources of bioactive and new constituents such as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenols, and lactones (Yu et al., 2010; Zhang et al., 2006). These isolated metabolites showed different pharmacological activities, such as antioxidant, antiviral, antidiabetic, antibiotic, anticancer and immunosuppressive (Koul et al., 2016; Strobel et al., 2003). Thus, an increasing number of studies have been conducted on the isolation and screening of secondary metabolites from endophytic and soil fungi (Durand et al., 2019; Li et al., 2018).

Our group has been dedicated to the study of natural products of plant and microbial origin in order to search for new antimycobacterial agents (Araujo et al., 2021; Calixto et al., 2022). In this context, the present study was performed to evaluate the antimycobacterial and anti-inflammatory activity of six in-house microbial metabolites isolated from endophytic and soil fungi cultures, encouraged by our mentioned previous study (Calixto et al., 2022). The compounds evaluated were austdiol (**1**), austdiol diacetate (**2**), mycoleptone A (**3**), and eugenitin (**4**) isolated from cultures of endophyte *Mycoleptodiscus indicus*, emodin (**5**) from endophyte *Penicillium citrinum* and δ-lactam (**6**) from soil fungi *Humicola grisea*. Except for emodin, these compounds have never been evaluated for their extracellular antimycobacterial potential. And none of them intracellularly, in infected macrophages. Compounds **1-4** and **6** have their isolation and chemical characterization previously described (Andrioli et al., 2012. 2014).

Material and methods

General

H^1 NMR spectra were obtained in a Varian (400 MHz), using TMS as the internal standard and deuterated dimethylsulfoxide. Sephadex LH-20; CC: silica gel 60 (70–230 and 230–400 mesh) and TLC: silica gel plates F_{254} (0.25 mm in thickness). HPLC: high liquid performance chromatography Shimadzu Prominence LC-20A. LC-MS: liquid chromatography/mass spectrometry DIONEX DAD3000 (Thermo Fischer Cientific).

High-performance liquid chromatography coupled to diode array detector (HPLC-DAD) analysis

Pure compounds purity analyses were performed using a 250 × 4.6 mm PHENOMENEX® Luna - C18 5 µm column (SHIMADZU® LC-20AT). The adopted mobile phase was (A): ultrapure water and (B): methanol, with a gradient elution constituted by methanol from 5 to 100 % over 35 min, 100-100 % over 37 min, 100-5 % over 44 min, 5-5 % over 50 min, at a flow rate of 1 ml/min. The UV/DAD detection was monitored at 190-800 nm (Shimadzu SPD-M20A). The samples were injected at 1 mg/ml in methanol with a volume injection of 5 µL by an autosampler (Shimadzu SIL-20A).

Fungal material

The fungus isolated in this study was obtained as an endophyte from the plant *Ocotea notata*, belonging to the Lauraceae family. Two specimens of *O. notata* with a healthy appearance were collected from the National Park of Jurubatiba's Restinga (220' and 22023'S and 41015'S and 41045'S W) in July 2014. Then, the samples were taken to the laboratory and processed in 24 h. A voucher specimen of the plant (No. RFA38751) was identified and deposited at the Herbarium UFRJ-RJ. The isolate was identified as *Penicillium citrinum* based on sequence analysis of the ITS region of the rDNA (GenBank accession number GU220382.1). The soil fungi *Humicola grisea* var. *thermoidea* (Andrioli et al., 2012) and the endophytic fungi *Mycoleptodiscus indicus* (Andrioli et al., 2014) were isolated in our previous studies.

Cultivation, extraction and isolation

Penicillium citrinum was grown on potato dextrose agar plates for 7 days at 30°C. Then, 10 plugs were transferred to five Erlenmeyer flasks (500 ml), each containing 100 ml of potato dextrose broth prepared with distilled water. Flasks were shaken on a rotary shaker at 30°C and 120 rpm for 48 h. Next, 10 ml was transferred to each of 50 flasks containing 90 g of solid medium (rice-oat). These were grown for 60 days. On day 60, the mycelial mass was macerated with ethanol overnight and filtered. The filtrate was concentrated under vacuum to obtain a crude ethanol extract (29 g), which was partitioned with three times equal of hexane and ethyl acetate, respectively, yielding hexane (9 g) and ethyl acetate fractions (15 g). The EtOAc fraction was suspended in MeOH and submitted to chromatography on Sephadex LH-20 using MeOH as mobile phase to yield 16 fractions. The fraction 12 (235.1 mg) was

subjected to chromatography over a silica gel column (30 × 1.5 cm i.d.) using a hexane:ethyl acetate gradient to yield 44 fractions, which were combined on the basis of their TLC profiles (hexane−ethyl acetate (70:30, v/v) as eluent) into seven fractions: A (19 mg), B (33 mg), C (62 mg), D (27 mg), E (16 mg) F (12 mg), and G (8 mg). Fraction C (62 mg) yielded emodin (**5**, 45 mg), after crystallization in MeOH. Emodin (**5**) was characterized mainly by NMR and MS in accordance to the literature data (Dewi et al., 2008; Kurobane et al., 1979).

Compounds

The other microbial compounds evaluated to antimycobacterial and anti-inflammatory assays were obtained from previous studies of the group. From cultures of endophytic fungi *M. indicus* were obtained the compounds austdiol (**1**), austdiol diacetate (**2**), mycoleptone A (**3**) and eugenitin (**4**) (Andrioli et al., 2014), whereas a derivative δ-lactam (**6**) was obtained from cultures of the soil fungi *H. grisea* var. *thermoidea* (Andrioli et al., 2012).

Austdiol (**1**) – ((7R,8S)-7,8-dihydroxy-3,7-dimethyl-6-oxo-7,8-dihydro-6H-isochromene-5-carbaldehyde). Purity (HPLC): 99.9 %.

Austdiol diacetate (**2**) – 5-formyl-3,7-dimethyl-6-oxo-7,8-dihydro-6H-isochromene-7,8-diyl diacetate. Purity (HPLC): 96.4 %.

Mycoleptone A (**3**) – (7R,8S,7′R,8′S)-5-[(7′,8′-dihydroxy-3′,7′-dimethyl-6′-oxo-7′,8′-dihydro-6′H-isochromen-5′-yl)methyl]-7,8-dihydroxy-3,7-dimethyl-7,8-dihydro-6H-isochromen-6-one. Purity (HPLC): 96.8 %.

Eugenitin (**4**) – 5-hydroxy-7-methoxy-2,6-dimethyl-4H-chromen-4-one. Purity (HPLC): 99.6 %.

Emodin (**5**) – 1,3,8-trihydroxy-6-methyl-9,10-anthracenedione. Purity (HPLC): 96.7 %.

Δ-lactam derivative (**6**) – (3-(2-(4-hydroxyphenyl)-2-oxoethyl)-5,6-dihydropyridin-2(1H)-one). Purity (HPLC): 96.2 %.

Mycobacterial culture

In this study, we analyzed the potential of six fungal metabolites against avirulent *Mycobacterium bovis* Bacillus Calmette-Guérin (*M. bovis* BCG) strain Moreau, three strains of *M. tuberculosis*: the standard strain ATCC H37Rv and two clinical isolates obtained from Mozambique patients with pulmonary TB (*Beijing M. tuberculosis* isolates: M442 and M299) and against three strains of *M. kansasii*: the reference strain ATCC 12478 and two clinical isolates obtained from Brazilian patients with pulmonary infection (4404 and 8835). Genetic features and virulence pattern displayed for *M. tuberculosis* (Ribeiro et al., 2014) and *M. kansasii* (Machado et al., 2018; Mussi et al., 2021) strains used in this study were described previously. All strains were obtained from Laboratory of Molecular Biology Applicated to Mycobacteria, FIOCRUZ, Rio de Janeiro, Brazil. The vaccine strain and *M. tuberculosis*strains were grown in Middlebrook 7H9 medium (BD Difco, MD) added with 0.05% Tween 80 (oleic acid derived from Tween 80 provides a carbon source) and 10% ADC (albumin-dextrose-catalase enrichment – BD BBL) at 37 °C whereas *M. kansasii* strains were grown in the similar culture medium supplemented with 0.5% glycerol and 10% ADC, without Tween 80.

Antimycobacterial activity assay

The compounds (**1-6**) were diluted in DMSO (Sigma-Aldrich, St Louis, MO, USA) to obtain a stock concentration at 20 mM. Optical densities (OD) of the bacterial suspensions were measured at 600 nm (OD_{600}) by spectrophotometry and adjusted to 0.1. Fifty microliters of each bacterial suspension (1×10^7 CFU/ml) were inoculated into the 96-well plate and incubated in the presence of 50 μl of compounds at serially diluted at final concentrations of 0.8, 4, 20 and 100 μM for 5 days (*M. tuberculosis* and *M. kansasii*) and 7 days (*M. bovis*) at 37 °C. Briefly, 200 μl of sterile distilled water was distributed in the outer wells of the microplate (Falcon 3072, Becton Dickinson, Lincoln Park, NJ). Rifampicin (Sigma-Aldrich) was used as the reference drug at concentrations from 0.003 to 12 μM. After this incubation period, the MTT method described by Moodley et al. (2014) was utilized to quantify bacterial growth. Each experimental well was added of MTT solution (5 mg/ml, Sigma-Aldrich) for 3 h at 37 °C and then treated with 100 μl of lysis buffer (20% w/v SDS, 50% dimethylformamide—DMF in distilled water) for overnight at 37 °C. DMSO controls were included at concentration 0.5-2.5%. The optical densities were obtained at 570 nm for each treated suspension and compared to the growth of untreated culture (100%), used as control of the spontaneous growth of bacteria. MIC_{50} values were calculated by nonlinear regression analysis of log (concentration) response curves using GraphPad Prism 4 (GraphPad Software Inc., CA, USA) followed of the application of a sigmoidal dose-response variable slope curve fitting. MIC_{50} was defined as the lowest concentration of the compounds that produced a 50% inhibitory effect on growth of the *Mycobacterium* strains compared to spontaneous bacterial growth.

Cell culture and treatments

RAW 264.7 (ATCC, VA, USA - TIB-71) and J774A.1 (ATCC, VA, USA - TIB-67) murine macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM-F12) complemented with 10% fetal bovine serum (FBS) and gentamicin (50 μg/ml) at 37°C in 5% CO_2. Cells were plated (1×10^5 cells/ml) and incubated for 24 h at 37° C. After this period, the supernatant was removed and the cells were resuspended in a medium culture supplemented with 2% SFB containing LPS (*E. coli* 055: B5 - Sigma-Aldrich, 1 μg/ml) in the presence or absence of compounds (0.8, 4, 20 and 100 μM) for additional 24 h of incubation at 37°C in 5% CO_2.

Quantification of inflammatory mediators

The fresh culture supernatants were collected after 24 h LPS-stimulation and treatment following immediately for quantification of inflammatory mediators (NO, TNF-α e IL-1β). Nitric oxide production was assessed by the Griess method, which measures the formation of nitrite (NO^{2-}), one of the primary and stable metabolites of NO (Tsikas, 2005). A nonspecific NO synthase inhibitor, L-*N*MMA (3.2-80 μM, Sigma-Aldrich), was used as a positive control of NO inhibition. Commercial enzyme-linked immunosorbent (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to measure the levels of TNF-α and IL-1β, according to the manufacturer's instructions. Ibuprofen, a nonsteroidal anti-inflammatory drug (NSAID) was used as a positive control of TNF-α and IL-1β inhibition (3.2-25 μM). IC_{50} values were measured for each compound as described above for MIC_{50} and defined as the concentration of compound required for 50% inhibitory effect on the production of inflammatory mediators.

Macrophage viability assay

Cytotoxicity effects of six fungal metabolites were evaluated on the LPS-stimulated RAW 264.7 macrophages (5×10^5 cells/ml) treated with compounds at concentrations of 0.8, 4, 20 and 100 μM for 24 h at 37°C in 5% CO_2. Cell viability of macrophages culture was analyzed by MTT method described by Mosmann et al. (1983). After 24 h, 10 μl of MTT solution (5 mg/ml) was added to each well and incubated for 2 h at 37 °C in 5% CO_2. After incubation, the supernatant culture was removed and formazan crystals solubilized with acidic isopropanol. The OD was determined by spectrophotometry at a wavelength of 570 nm (BioTek Epoch, Santa Clara, CA, USA). The 50% cytotoxicity concentration (CC_{50}) was determined by logarithm regression analysis using GraphPad Prism 4 and defined as concentration that reduced the cell viability by 50% compared to controls without treatment. The selectivity index (SI) was determined by the CC_{50}/MIC_{50} ratio for each compound tested.

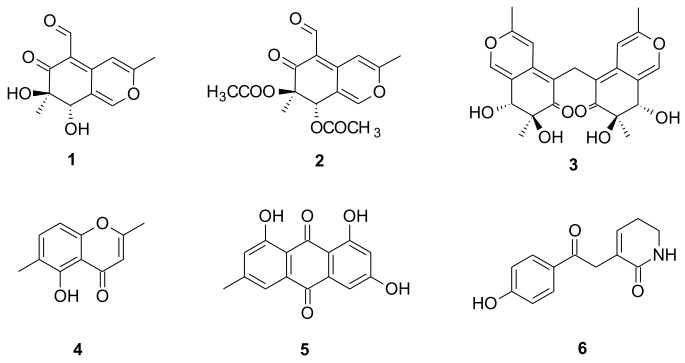

Fig. 1. Chemical structures of the six fungal metabolites from endophytes and soil fungi. (**1**) Austdiol; (**2**) Austdiol diacetate; (**3**) Mycoleptone A; (**4**) Eugenitin; (**5**) Emodin and (**6**) δ-lactam derivative.

Macrophage culture infection and quantification of intracellular Mycobacterium growth

RAW 264.7 macrophages were cultured and plated (5×10^5 cells/ml) according to described above for cell adherence and obtainment of a monolayer. After 24 h incubation at 37 °C in 5% CO_2, cells were resuspended in a complete DMEM-F12 medium supplemented with 2% FBS without antibiotics and infected with *M. tuberculosis* H37Rv at a multiplicity of infection (MOI) of 1:1 (bacteria/macrophage) for 3 h. The removal of extracellular non-phagocytosed bacteria was performed by washing twice with PBS. Subsequently, aliquots of the complete DMEM-F12 medium containing each compound separately (0.8, 4, 20 and 100 μM) were added to the infected cultures. After 4 days of incubation at 37 °C in 5% CO_2, the culture was lysed with 0.1% of saponin solution, collected, and serially diluted in PBS. Fifty microliters of each dilution were submitted to the CFU (colony-forming unit) test on Middlebrook 7H10 agar (BD Difco, MD) added with 0.5% glycerol and 10% OADC (oleic acid-albumin-dextrose-catalase enrichment – BD BBL). After 3-4 weeks at 37 °C, colonies were quantified and bacterial intracellular growth expressed in Log_{10}. CFU counts obtained on day 4 from infected macrophages culture was subtracted from CFU on day 1 for all conditions.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 4 software, differences among the experimental groups were considered significant for value of $p < 0.05$. A one-way analysis of variance (ANOVA) was employed to assess statistical significance of a single parameter when comparing multiple groups followed by Tukey's test. Data were expressed as the mean ± SD of three independent experiments.

Results and discussion

A series of microorganism-derived compounds were obtained from cultures of endophytic fungi *Mycoleptodiscus indicus*: austdiol (**1**), austdiol diacetate (**2**), mycoleptone A (**3**) and eugenitin (**4**); endophytic fungi *Penicillium citrinum*: emodin (**5**) and soil fungi *Humicola grisea* var. *thermoidea*: δ-lactam derivative (**6**) (Fig. 1).

The effect of compounds (**1-6**) in different concentrations (0.8; 4; 20 and 100 μM) on the viability of RAW 264.7 macrophages was evaluated by MTT method and results are presented in Table 1 as CC_{50} (μM). All the samples evaluated exhibited CC_{50} higher than 100 μM (Table 1). According to Fig. 2, all the compounds at concentrations of 0.8-20 μM displayed similar values to the negative control. Only at the higher concentration (100 μM), austdiol (**1**), mycoleptone A (**3**) and emodin (**5**) showed values significantly different of the untreated control exhibiting weak cytotoxicity, less than 30%. This analysis is an important parameter on natural product research to identify bioactive compounds without or low toxic effects to host cells, since one-third of the compounds developed have been discontinued due to toxicity (Hornberg et al., 2014).

In the face of the high mortality TB rate, emergence of drug-resistant *M. tuberculosis* strains and toxic side effects of the drugs used at least 6 months in the TB therapy, novel antimycobacterial drugs are important for improvement and success to TB treatment (WHO, 2020).

Thus, the compounds **1**- **6** were evaluated for their antimycobacterial

Table 1
Effects of fungal metabolites on viability RAW 264.7 macrophages and minimum inhibitory concentrations of these against growth of *M. bovis* BCG and *M. tuberculosis* strains in bacterial culture.

Compounds	RAW 264.7 CC_{50} (μM)	*M. bovis* BCG MIC_{50} (μM)	SI	*M. tuberculosis* MIC_{50} (μM)					
				H37Rv	SI	M442	SI	M299	SI
Austdiol (1)	**> 100**	11.7 ± 1.3	> 8.5	10.8 ± 1.3[a]	> 9.3	12.4 ± 1.0	> 8.1	33.4 ± 1.4	> 3.0
Austdiol diacetate (2)	**> 100**	23.4 ± 1.4	> 4.3	> 100	xx	> 100	> 1.0	93.4 ± 1.7	> 1.1
Mycoleptone A (3)	**> 100**	1.7 ± 0.2[a]	> 58.8	9.2 ± 1.2[a]	> 10.9	21.3 ± 1.8	> 4.7	37.4 ± 1.0	> 2.7
Eugenitin (4)	**> 100**	> 100	> 1.0	79.1 ± 1.1	> 1.26	81.2 ±1.2	> 1.2	> 100	> 1.0
Emodin (5)	**> 100**	0.4 ± 0.1[a]	> 250.0	5.0 ± 1.1	> 20	5.3 ± 1.4	> 18.9	8.2 ± 1.0	> 12.2
δ-Lactam derivative (6)	**> 100**	20.5 ± 1.3	> 4.9	> 100	> 1.0	> 100	> 1.0	> 100	> 1.0
Rifampicin[1]	**xx**	0.03 ± 0.01[a]	xx	0.2 ± 0.1	xx	0.2 ± 0.5	xx	1.2 ± 0.2	xx

[1] Standard antimycobacterial drug. Mean value ± SD (n = 3). Values in the same column with superscript letter (a) are significantly similar (p> 0.05).

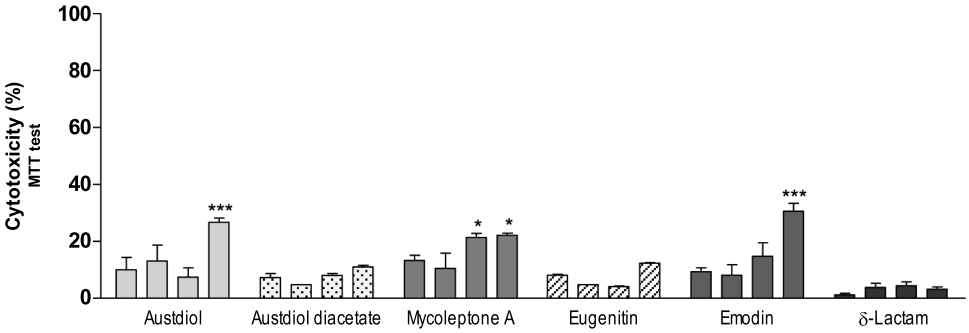

Fig. 2. Effect of the fungal metabolites on viability of RAW 264.7 macrophages by MTT method. Culture cells were treated with the compounds at 0.8, 4, 20, and 100 μM for 24 h. After, MTT solution was added for culture cells for 2 h and crystals obtained were diluted with acidic isopropanol. Untreated cells were used as a negative control (cytotoxicity 0%) and as positive control was used 1% Triton X-100 detergent-treated cells (cytotoxicity 100%). Arithmetic means ± standard deviation (n = 3). $p < 0.05$ (*) compared with the not treated cells.

Table 2
Minimum inhibitory concentrations of fungal metabolites against growth of *M. kansasii* strains in bacterial culture.

Compounds	*M. kansasii* MIC_{50} (μM)					
	12478	SI	4404	SI	8835	SI
Austdiol (1)	28.1 ± 1.1	> 3.6	32.4 ± 1.4	> 3.1	35.2 ± 1.1	> 2.8
Austdiol diacetate (2)	11.2 ± 1.0	> 8.9	9.1 ± 0.8[a]	> 11.0	39.7 ± 1.2	> 2.5
Mycoleptone A (3)	22.4 ± 1.3	> 4.5	41.9 ± 1.2	> 2.4	> 100	> 1.0
Eugenitin (4)	> 100	> 1.0	> 100	> 1.0	> 100	> 1.0
Emodin (5)	1.9 ± 1.1[a]	> 52.6	9.1 ± 1.0[a]	> 11.0	23.9 ± 0.9	> 4.2
δ-Lactam derivative (6)	54.5 ± 1.1	> 1.8	61.4 ± 1.0	> 1.6	> 100	> 1.0
Rifampicin[1]	0.05 ± 0.01[a]	xx	0.7 ± 0.1	xx	4.5 ± 1.0	xx

[1] Standard antimycobacterial drug. Mean value ± SD (n = 3). Values in the same column with superscript letter (a) are significantly similar (p> 0.05).

potential initially against vaccine strain, *M. bovis* BCG. Values of growth *Mycobacterium* inhibition were quantified e MIC_{50} values calculated are in Table 1. Initial *screening* of compounds on *M. bovis* BCG growth inhibition showed that mycoleptone A (**3**) and emodin (**5**) were the most potent compounds with lowest MIC_{50} (Table 1, p> 0.05). With exception of the eugenitin (**4**), that did not show inhibitory effect on *M. bovis* BCG growth, the other tested compounds exhibited high inhibitory effect presenting $MIC_{50} < 25\mu M$ and selectivity index (SI) ranged from 4.3-250 (Table 1).

Subsequently, we evaluated the compounds on the *M. tuberculosis* culture strains. For the assays were used the standard laboratory *M. tuberculosis* strain H37Rv and two clinical isolates of the Beijing *M. tuberculosis* lineage described by Ribeiro et al. (2014), the strain M442 (low virulence clinical isolate) and the strain M299 (highly virulent strain). The increased dissemination of the Beijing strains in the world has been demonstrated in epidemiological studies to be associated with drug resistance, elevated pathogenicity and disease outbreaks (Parwati et al., 2010; Ribeiro et al., 2014; Gupta et al., 2020).

The most active compounds against *M. tuberculosis* H37Rv were emodin (**5**) exhibiting MIC_{50} 5.0 ± 1.1 μM and azaphilone compounds, austdiol (**1**) and mycoleptone A (**3**) (MIC_{50} 10.8 ± 1.3 and 9.2 ± 1.3 μM, respectively). These compounds showed SI that ranged from 9.3 to 20, the last obtained by emodin (**5**) (Table 1).

The compounds emodin (**5**), austdiol (**1**) and mycoleptone A (**3**) also demonstrated high inhibitory activity against the growth of Beijing *M. tuberculosis* isolates (Table 1), highlighting the emodin (**5**) as most active compound. The compound **5** presented MIC_{50} value on the growth inhibition of M442 strain (5.3 ± 1.4 μM) similar to those observed against *M. tuberculosis* H37Rv and slightly less potential (p< 0.05) against M299 strain (8.2 ± 1.0 μM), although the SI was at least 12.1 (Table 1). Austdiol (**1**) maintained its activity profiles against the M442 strain (MIC_{50} 12.4 ± 1.0 μM) and was less potent against the clinical isolate M299, while the mycoleptone (**3**) exhibited lower inhibitory capacity against both strains, although both compounds had a MIC_{50} below 37 μM on the hypervirulent *M. tuberculosis*M299 culture. The compounds **2, 4** and **6** did not present inhibitory activity against *M. tuberculosis* strains. According to the Clinical and Laboratory Standards Institute (CLSI) criteria, compounds that show MIC≤ 25 μM and SI greater than 10 are considered relevant for the antimycobacterial activity and drug development process (Nguta et al., 2015).

Considering that *M. kansasii* is one of the human pathogenic NTM species most prevalent and frequently associated to chronic pulmonary disease similar to TB (Hoefsloot et al., 2013; Goldenberg et al., 2020), the antimycobacterial activity of the fungal compounds was initially evaluated against reference strain *M. kansasii* 12478. Emodin (**5**) and azaphilone compounds (**1, 2** and **3**) displayed high activity against *M. kansasii* 12478, highlighting emodin (**5**) with MIC_{50} value of 1.9 ± 1.1 μM and SI greater than 52.6 (Table 2).

In general, azaphilone compounds (**1, 2** and **3**) showed good ability to inhibit growth of *M. kansasii* 4404, the opposite of what was observed for the austdiol diacetate (**2**) against *M. tuberculosis* strains (Table 1). Austdiol diacetate (**2**) and emodin (**5**) were the most active on the growth inhibition of *M. kansasii* 4404 (Table 2), clinical isolate described by Mussi et al. (2021) as an intermediate virulent strain as well as the reference *M. kansasii* strain 12478. Both compounds (**2** and **5**) exhibited similar MIC_{50} value (p> 0.05) against *M. kansasii* 4404 and SI value greater than 11. The strain 8835, a high virulent strain (Mussi et al., 2021) proved to be more resistant to treatment with fungal compounds and with rifampicin. Emodin (**5**) exhibited the best growth inhibition potential of *M. kansasii* 8835 with MIC_{50} value of 23.9 ± 0.9 μM whereas austdiol (**1**) and austdiol diacetate (**2**) presented satisfactory activity against this *M. kansasii* strain with MIC_{50} less than 40 μM (Table 2). Hemtasin et al. (2016) evaluated antimycobacterial activity of fourteen azaphilone compounds, where isochromophilone VI and sclerotioramine, with 1,4 di-hydropiridine ring, displayed MIC_{50} values of 6.2 and 50.0 mg/ml against *M. tuberculosis* strain, while the other azaphilones with pyran ring (O replacing N) were inactive. Azaphilone compounds (**1, 2** and **3**) have been evaluated to several biological activities, in a previous work our group reported antileshmanial activity against *L. donovani* with LD_{50} of 20.5, 19.8 and 28.5 μM, respectively.

Emodin was the most active compound in antimycobacterial assay, considering *M. tuberculosis* and *M. kansasii*. Camacho-Corona et al. (2009) reported the bioevaluation of thirty-five plant-derived secondary metabolites and highlighted the quinone aloe-emodin from *Stephania dinklagei* with MIC value of 6.25 μg/ml against *M. tuberculosis* H37Rv. Dey et al. (2014) evaluated the antimycobacterial activity of quinonoids, including emodin, that displayed inhibitory activity against *M. tuberculosis* H37Ra (MIC 4 μg/ml) and MDR-*M. tuberculosis* strains (MIC 4-16 μg/ml) (Dey et al., 2014).

The compound selectivity to mycobacteria, showing non-toxic effects on macrophages, is an important issue regarding the search for a new antimycobacterial candidate. Emodin was able to act on the mycobacteria without showing cytotoxicity to macrophages, as showed above. However, another relevant selectivity issue, when considering an antitubercular application, is the selectivity to mycobacteria, showing no effects on the microbiota, due to the long-term TB treatment. In general, weak or absence of activity was observed for emodin against gram-negative bacteria: *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium*, and *Klebsiella pneumoniae*. And, emodin showed activity against some gram-positive bacteria: *Staphylococcus aureus, Streptococcus mutans, Bacillus cereus*, and *B. subtilis* (revised by Stompor-Gorący, 2021). Considering only the microbiota, detailed emodin activity was: against the gram-negative *E. coli*, MIC>0.5 mg/ml (Chukwujekwu et al., 2006), and against *S. mutans*, MIC > 2 mg/ml (Xu et al., 2014). In the present study, antimycobacterial activity of emodin ranges from MIC 0.4 to 24 μM (0.0001 to 0.0065 mg/ml), indicating, in comparison to the literature data regarding *E. coli* and *S. mutans*, that emodin is selective against mycobacteria, protecting microbiota.

Diverse factors contribute to hyperinflammation condition and necrotic lung pathology, frequent in severe pulmonary TB and NMT infections (Orme et al., 2015; Almeida et al., 2017; Young et al., 2019), such as virulence factors common to all mycobacteria, including *M. tuberculosis* and *M. kansasii* (Crilly et al., 2021; Strong and Lee, 2021) as well as the ability and immune status of the host to generate balanced and efficient inflammatory responses (Zumla et al., 2015). There is an urgent incentive to use the HDT approach, such as anti-inflammatory drugs in association with the use of antibiotics for these conditions in order to accelerate the clearance of *Mycobacterium* and reduce or prevent tissue damage (WHO, 2020).

The excessive and continuous production of inflammatory mediators (NO, TNF-α and IL-1β) and proteases mainly by activated macrophages

Table 3
Inhibitory effects of fungal compounds on production of NO, TNF-α and IL-1β by LPS-stimulated macrophages.

Compounds	IC_{50} (μM)		
	NO	TNF-α	IL-1β
Austdiol (1)	6.9 ± 1.2[a]	7.7 ± 1.0[a]	4.7 ± 1.2
Austdiol diacetate (2)	8.3 ± 1.3[a]	32.3 ± 1.2	20.3 ± 1.1
Mycoleptone A (3)	11.1 ± 1.2[a]	28.0 ± 1.2	11.3 ± 1.5[a]
Eugenitin (4)	≥100	≥100	≥100
Emodin (5)	7.8 ± 1.2[a]	5.5 ± 1.1[ab]	11.6 ± 1.3[a]
δ-Lactam derivative (6)	65.3 ± 1.1	≥100	≥100
L-NMMA[1]	57.3 ± 1.4	xx	xx
Ibuprofen [2]	xx	3.4 ± 0.8[b]	15.6 ± 1.2

[1] Nitric oxide synthase inhibitor
[2] NSAID; Mean value ± SD; n = 3; XX - not defined. Values in the same column with superscript letter (a,b) are significantly similar (p> 0.05).

and neutrophils against virulent *Mycobacterium* has been observed in the lungs of patients and in murine model of severe TB and NMT pulmonary infection (Ribeiro et al., 2014; Almeida et al., 2017; Mussi et al., 2021; Crilly et al., 2021; Strong and Lee, 2021) associated with extensive damage and loss lung function (Dorhoi et al., 2011). In this context, substances exhibiting antimycobacterial and anti-inflammatory properties can represent important alternatives for TB treatment. Thus, we evaluated the ability of fungal compounds to inhibit NO, TNF-α and IL-1β production by LPS-stimulated macrophages.

The results presented in Table 3 showed that with exception of eugenitin (**4**) and δ-lactam derivative (**6**), the other compounds (**1, 2, 3** and **5**) were able to inhibit the production of both inflammatory mediators evaluated. Azaphilone compounds (**1, 2** and **3**) and emodin (**5**) showed similar activity profiles (p> 0.05) on the inhibition of NO production by LPS-stimulated RAW 264.7 and at least 5-fold more potent than L-*N*MMA, a non-selective iNOS inhibitor used as positive control (IC_{50} of 57.3 ± 1.4 μM). Austdiol (**1**) and emodin (**5**) exhibited pronounced inhibitory activity on TNF-α production with IC_{50} values below 8 μM, with compound **5** presenting similar potential to ibuprofen (p< 0.05). Austdiol diacetate (**2**) and mycoleptone A (**3**) have also shown good capacity to inhibit this mediator (Table 3). Austdiol (**1**) stood out for the inhibition of IL-1β production by LPS-stimulated J774A.1 with IC_{50} of 4.7 ± 1.2 μM whereas mycoleptone A (**3**) and emodin (**5**) exhibited comparable inhibitory potential (p> 0.05). Both three compounds (**1, 3** and **5**) were more active than ibuprofen on the IL-1β inhibitory activity (Table 3).

Ma et al. (2014) showed in rat model of blast-induced traumatic brain injury that emodin could reduce brain damage in the treated animals possibly by inhibiting the iNOS expression and thus NO production. Anti-inflammatory effect of aloe-emodin on the suppression of NO production by LPS-stimulated RAW 264.7 macrophages was described by Park et al. (2009). Shrimali et al. (2013) reviewed the anti-inflammatory potential of emodin showing ability to suppress different LPS-induced inflammatory mediators, such as IL-1β, IL-6 and TNF-α due to inhibition of NF-κB activation.

Many azaphilones compounds exhibit bioactivities, including anti-inflammatory (Hsu et al., 2013). Tang et al. (2019) reported the inhibitory potential of nine azaphilone alkaloids isolated from *Penicillium sclerotiorum* on the NO production by LPS-stimulated RAW 264.7, whereas two azaphilone chermesinones isolated from *Penicillium chermesinum* were described by Zhang et al. (2021) as compounds that exhibited weak inhibitory activity in this same *in vitro* model. To the best of our knowledge, this is the first report demonstrating anti-inflammatory activity for azaphilones compounds (**1, 2** and **3**).

In order to provide further information about the antimycobacterial potential of the fungal compounds, we evaluated the ability of azaphilones compounds (**1, 2** and **3**) and emodin (**5**) to inhibit mycobacterial intracellular growth using *in vitro* model of macrophage RAW 264.7 infection, since these compounds exhibited antimycobacterial and anti-inflammatory activities. The development of new antimycobacterial drugs must consider the different physiological states of *Mycobacterium* and its microenvironments (Orme et al., 2015; Nguta et al., 2015).

As can be seen in Fig. 3, emodin (**5**) was the most active compound against intracellular *M. tuberculosis* H37Rv growth exhibiting MIC_{50} of 6.5 ± 1.5 μM. With exception of austdiol diacetate (**2**) that not presented inhibitory effect, austdiol (**1**) and mycoleptone A (**3**) showed ability to suppress *M. tuberculosis* growth in infected macrophages. Thus, compounds **1, 3** and **5** demonstrated notable inhibitory potential on *M. tuberculosis* growth in bacterial culture (Table 1) as well as intracellular efficacy (Fig. 3). These compounds are being described for the first time for this intracellular activity.

Conclusions

Bioassay evaluations displays that the azaphilone compounds (**1**and **3**) and the quinonoid emodin (**5**) showed notable activity against bacterial culture of *M. tuberculosis* H37Rv and clinical isolates, including hypervirulent *M. tuberculosis*, as well as intracellular efficacy to reduce *M. tuberculosis* growth in infected macrophages. Moreover, these compounds together with austidiol diacetate (**2**) also presented inhibitory potential against the reference strain *M. kansassi* 12478 and two isolates of intermediate and high virulence. Emodin (**5**) was the most active compound in antimycobacterial assay. In addition to the antimycobacterial property, azaphilone compounds (**1, 2** and **3**) and emodin (**5**) exhibited outstanding anti-inflammatory potential on LPS-stimulated macrophages. These results revealed the *dual* potential, antimycobacterial and anti-inflammatory, of microbial metabolites

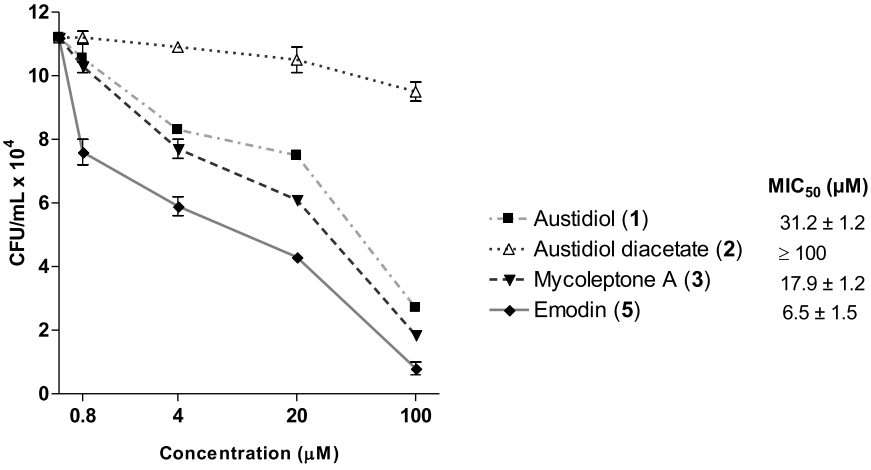

Fig. 3. Effect of fungal compounds on intracellular M. tuberculosis H37Rv growth in macrophages. RAW 264.7 macrophages were infected with M. tuberculosis H37Rv at a MOI of 1:1 (bacterium: macrophage) and treated for 4 days with the compounds (0.8, 4, 20 and 100 μM). On day 4, cells were lysed and suspension plated in Middlebrook 7H10 agar. After 21 days, bacterial colonies were counted (CFU test). MIC values are reported as mean ± SD. Mean value of each group were compared to mean value of the positive control (untreated infected macrophages, 11.2 ± 1.0 CFU/mL x 104).

which may be promising candidates for the development of new anti-TB drugs aimed at the treatment of severe mycobacterial lung diseases caused by tuberculosis or MNT infections associated with excessive inflammation. In addition, fungal compounds are natural products rich in chemistry diversity and biological activities and this study reinforces the importance of the investigation of metabolites from microorganisms in the search for lead compounds.

Conflicts of interest

The authors declare no conflict of interest.

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