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Full Length Research Paper

Antimycobacterial activity of *Usnea steineri* and its major constituent (+)-usnic acid

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The antimycobacterial activity of an extract of *Usnea steineri* and of its major constituent (+)-usnic acid was evaluated against *Mycobacterium tuberculosis* (ATCC 27294), *Mycobacterium kansasii* (ATCC 12478) and *Mycobacterium avium* (ATCC 15769). The acetone extract (ACE) of *U. steineri* displayed promising minimum inhibitory concentration (MIC) values of 32 µg/ml against *M. tuberculosis* and 62 µg/ml against both *M. kansasii* and *M. avium.* The isolated compound (+)-usnic acid was even more effective against the evaluated microorganisms with MIC values of 16 µg/ml against *M. avium* and 8 µg/ml against both *M. tuberculosis* and *M. kansasii*.

Key words: *Usnea*, usnic acid, antimycobacterial activity.

INTRODUCTION

Tuberculosis (TB) is a severe infectious disease caused by mycobacteria belonging to the *Mycobacterium tuberculosis* complex. According to the WHO, an estimated 1.7 million people died from TB in 2009. The highest number of deaths took place in the African Region (WHO, 2005). The chemotherapy of tuberculosis is based on the use of combined drug therapy including rifampicin, isoniazid and pyrazinamide. However, the incorrect use of medications and their prolonged administration, as well as their high cost and the countless side-effects have led to low compliance with the treatment. In other words, people abandon the treatment before being completely cured which culminates in resistant bacilli (Timmins and Deretic, 2006; Hardna et al., 2001).

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Abbreviations: ACE, Acetone extract; **MIC,** minimum inhibitory concentration.

There were an estimated 440,000 cases of multi-drug resistant TB (MDR-TB) in 2008 (WHO, 2005). In addition, the existence of MDR-TB reinforces the need for the development of novel safe and effective antimycobacterial pharmaceuticals. Over the last decade there has been intensification in the search for antibacterial compounds from natural sources, since the latter continue to be a major source of biologically active metabolites that may provide lead structures for the development of new drugs. Searching for these compounds among endophytic bacteria and lichens has proven effective since they represent biological associations that provide protection against competing organisms (Francolini et al., 2004). There appears to be a higher probability of discovering bioactive compounds from endophytic species (Strobel et al., 2004).

Usnic acid is a dibenzofuran unique to lichens. It is especially abundant in genera such as *Alectoria*, *Cladonia*, *Usnea*, *Lecanora*, *Ramalina* and *Evernia*. This acid displays a wide range of pharmacological activities including antibiotic, antiviral, antiprotozoal, anti-

inflammatory, analgesic and anti-cancer actions (Ingólfsdóttir, 2002; Cocchietto et al., 2002; Mayer et al., 2005). Considering the biological activities of usnic acid and as part of our ongoing work on the antimicrobial activity of natural products (Celotto et al., 2003; Cunha et al., 2007; Bernardes et al., 2010), the aim of the present work was to evaluate the antimycobacterial activity of an extract of *Usnea steineri* and of its major compound (+)-usnic acid.

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured on a JASCO polarimeter (P-2000 model). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ARX 400 spectrometer; samples were dissolved in methanol-d_6. Both analytical and semi-preparative high performance liquid chromatography (HPLC) analyses were carried out on a Shimadzu LC-6AD system equipped with a degasser DGU-20A5, a UV-VIS detector SPD-20A series with a CBM-20A module and a Reodyne manual injector. Analytical and semi-preparative separations were accomplished using Shimadzu Shim-pack ODS columns (250 × 4.6 mm and 250 × 20 mm, respectively) equipped with a pre-column of the same material. The mobile phase used for chromatographic purification was acetonitrile/water (77:23) with 0.1% AcOH, UV detection: 235 nm.

Plant material

U. steineri Zahlbr (Parmeliaceae) was collected in March 2010 in Reserva Jatai located in the city of Luis Antônio, state of São Paulo, Brazil. A voucher specimen was deposited in the Herbarium of Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo-USP (SPFR herbarium) under registration number 12661.

(+)-usnic acid isolation

U. steineri was dehydrated at 40°C, followed by pulverization (0.3 kg) and extraction with acetone by maceration (5 L, three days × 3) at room temperature. The solvent was removed under vacuum in a rotary evaporator yielding 18.4 g of the dry crude extract. An aliquot of the extract was analyzed by HPLC and usnic acid (Figure 1) was obtained as a yellow amorphous solid (R_t: 7.09 min), $[\alpha]_D^{22} = +0.410$ (c = 3 mg/ml, MeOH). Its chemical structure was confirmed by comparison of its ^1H and ^{13}C NMR data with those published in the literature (Ingólfsdóttir et al, 1998; Rashid et al., 1999).

Antimycobacterial activity

The antimycobacterial activity of the samples was assayed *in vitro* by the microdilution technique on a Resazurin microtiter assay plate (REMA) using a procedure adapted from Palomino et al. (2002) which allowed for determination of the minimum inhibitory concentration (MIC) against *Mycobacterium tuberculosis* H37Rv (ATCC 27294), *Mycobacterium kansasii* (ATCC 12478) and *Mycobacterium avium* (ATCC 15769). The compounds were dissolved in dimethylsulfoxide (DMSO) and serially diluted in Middlebrook 7H9 broth before inoculation. The concentrations of the tested compounds ranged from 1 to 2000 µg/ml while the final DMSO content in the assay was less than 0.3%. Isoniazid was used as the reference antibiotic drug and bioassays were performed in three independent experiments. In order to determine the MIC values for isoniazid, concentrations ranging from 5.00 to 0.01 µg/ml were employed. The visual MIC values were defined as the lowest drug concentration that inhibited bacterial growth and the values are expressed by the average of the assays.

RESULTS AND DISCUSSION

The lichen genus *Usnea* is widespread throughout the world and is known to elaborate a number of interesting metabolites (Muller, 2001). Some extracts and isolated compounds from *Usnea* species have been investigated for their biological properties (Ingólfsdóttir et al., 1998; Molnár and Farkas, 2010; Agar et al., 2011). However, this is the first time that *U. steineri* has been investigated. Usnic acid is the normal component of *Usnea* species and is one of the most common and abundant lichen metabolites. This natural compound exists as (+) and (−) enantiomers and has shown a great relevance in pharmacology and clinics. This compound acts as a strong inhibitor of Gram-positive bacteria (Vartia, 1973; Yamamoto et al., 1993) and of aerobic and anaerobic microorganisms (Lauterwein et al., 1995) and as a selective agent against races of *Streptococcus mutans* (Grasso et al., 1989) because of its capacity to modify the permeability of membranes, ATP production and enzymatic activity of bacteria (Vicente and Cifuentes, 1981; Cifuentes and Vicente, 1983). Non-compliance with the drugs prescribed for the treatment of tuberculosis and the side effects of the currently employed antituberculosis agents have led researchers to search for therapeutic alternatives. Over the last few years, countless investigators have concentrated their efforts on the examination of the activity of crude plant extracts and their fractions as well as the synthesis of novel compounds that can be potentially applied as antimicrobial agents (Okunade and Elvin-Lewis, 2004). Therefore, the aim of the present work was to evaluate the potential of a crude extract obtained from *U. steineri* and of its major constituent (+)-usnic acid (Figure 1) against three *Mycobacterium* species.

(+)-usnic acid is the most common constituent among *Usnea* species which in turn produce only one optical isomer (Marcano et al., 1999). Antimycobacterial activity results are summarized in the Table 1. The acetone extract (ACE) of *U. steineri* displayed promising MIC values especially against *M. tuberculosis* (32 µg/ml). The isolated compound (+)-usnic acid was even more effective against the evaluated microorganisms, yielding an MIC value of 16 µg/ml against *M. avium* and 8 µg/ml against both *M. tuberculosis* and *M. kansasii*. Our results are in agreement with data previously reported by Ingólfsdottir et al. (1998) who found high antimycobacterial activity for this compound against *M. aurum*, a non-pathogenic organism with a similar sensitivity profile similar to that of *M. tuberculosis*. In other studies in which

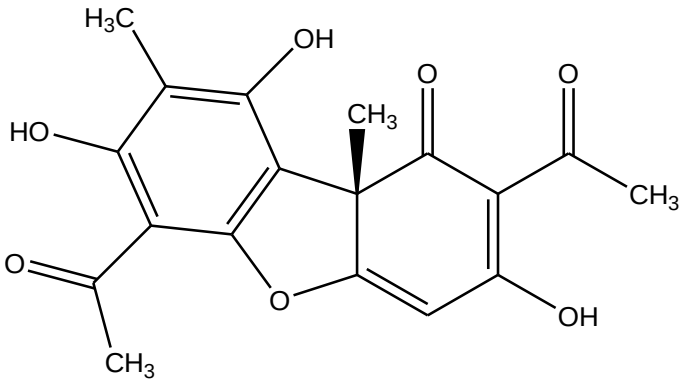

Figure 1. Chemical structure of (+)-usnic acid.

Table 1. Minimum inhibitory concentration (µg/ml) of the acetone crude extract of *Usnea steineri* (ACE) and of the isolated compound (+)-usnic acid against *M. tuberculosis, M. kansasii* and *M. avium.*

Sample	*M. tuberculosis* (ATCC 27294)	*M. kansasii* (ATCC 12478)	*M. avium* (ATCC 15769)
ACE[1]	32	62	62
UA[2]	8	8	16
Isoniazid[3]	0.03	0.05	1.0

[1] Acetone extract; [2] (+)-usnic acid; 3, standard antibiotic.

lichen compounds were screened for antimycobacterial activity, (+)-usnic acid was described to be active against *M. tuberculosis* (Stoll et al., 1950; Tosun et al., 2005; Honda et al., 2010). However, to our knowledge, this is the first time that (+)-usnic acid has been evaluated against *M. avium* and *M. kansasii*. In the present work, the microdilution technique on REMA was selected for determination of MIC values because it is a colorimetric technique that is easy to handle, provides better reproducibility and employs a reduced amount of natural compounds, not to mention that it enables one to test various compounds simultaneously. Today, the therapeutic arsenal for the treatment of tuberculosis is reduced and the treatment is long. This is a serious problem that has worsened over the last years because of the rise in the number of TB cases caused by resistant *Mycobacterium* strains (Okunade and Elvin-Lewis, 2004). Therefore, the search for new drugs from natural origin is urgent and extremely important.

Lipophilicity has been reported as an important parameter for antimycobacterial activity (Mallavadhani et al., 2004). Due to the fact that the cell wall of mycobacteria contain lipophilic substances such as mycolic acid, more lipophilic substances are likely to penetrate more easily into the cell (Palomino et al., 2002). Thus, the therapeutic antimicrobial activity of (+)-usnic acid has been related to its membrane uncoupling property (Cocchietto et al., 2002). The results obtained in this study suggest the potential application of (+)-usnic acid in the treatment of *Mycobacterium* species. Further investigations should be conducted in order to explore this application.

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