Introduction

Tuberculosis is a severe infectious disease caused by mycobacteria belonging to the *Mycobacterium tuberculosis* complex. According to WHO, tuberculosis affects nearly 30% of the world’s population and is responsible for 3 million deaths worldwide each year, mainly in developing countries (Raviglione, 2003; WHO, 2003). The chemotherapy of tuberculosis has been based on the use of combined drug therapy with rifampicin, isoniazid, and pyrazinamide. However, the incorrect use and long drug administration, as well as the high cost and the countless side-effects have led people to abandon the treatment before being completely cured, leading to resistant bacilli (Timmins and Deretic, 2006; Hardna *et al*., 2001). In addition, the existence of drug-resistant tuberculosis reinforces the need to develop new safe and effective antimycobacterial drugs. Although several research programs have focused on various strategies to control tuberculosis, drug discovery has been one of the main areas of concentrated effort (Okunade and Elvin-Lewis, 2004; Raviglione, 2003; Collins and Franzblau, 1997).

In the last decade there has been intensification in the search for antibacterial compounds from natural sources, mainly from plants, which continue to be a major source of biologically active metabolites that may provide lead structures for the development of new drugs (Copp, 2003; Okunade and Elvin-Lewis, 2004; Da Silva Filho *et al*., 2004a, 2008a; Pontin *et al*., 2008). Lignans, one of the oldest classes of natural products, have attracted much interest over the years on account of their broad range of biological activities, including antileishmanial (Da Silva Filho *et al*., 2008b), trypanocidal (Da Silva Filho *et al*., 2004b; Souza *et al*., 2005), and anti-inflammatory (Silva *et al*., 2005; Souza *et al*., 2004; Da Silva Filho *et al*., 2004c) activities. Cubebin, a dibenzylbutyrolactolic lignan, and its semi-synthetic derivatives have been investigated for their antiprotozoal and antibacterial activities (Andrade *et al*., 2009; Souza *et al*., 2005). Recently, it has been reported that dibenzylbutyrolactone lignans, obtained from cubebin, display significant antibacterial activity against oral pathogens (Silva *et al*., 2007).

Considering the antibacterial activity of dibenzylbutyrolactone lignans and as part of our works...
on the antimicrobial activity of medicinal plants and natural products (Da Silva Filho et al., 2008a; Andrade et al., 2008; Jorge et al., 2008; Oliveira et al., 2007; Scalon et al., 2007), the aim of the present work was to evaluate the antimycobacterial activity of several lignans obtained from cubebin, which have not yet been described.

Experimental

General experimental procedures

Optical rotations were measured at 589 nm on a Schmidt-Haensch polartronic HHS polarimeter using an 1.0-cm cell. IR spectra were recorded on a Nicolet FT-IR Protegé 520 instrument. NMR spectra were recorded on a Bruker ARX 400 spectrometer; samples were dissolved in CDCl₃, and the spectra were calibrated at δ 7.26 (¹H) or δ 77.0 (¹³C) of the solvent signals; the splitting patterns are as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet. The reaction was monitored by thin layer chromatography (TLC); the developed chromatograms were observed under ultraviolet light (254 – 265 nm). A Shimadzu HPLC unit, bearing an LC-10ADVP pump, SPD-M10A VP arrangement of the diode detector and a DGU-14A Shimadzu degasifier (Tokyo, Japan), was used for purity determination. Analysis on a C-18 CLC-ODS Shimadzu column using a gradient of MeOH/H₂O (1:1) to MeOH (100%) within 30 min was performed. The solvents used were generally distilled and dried before use.

(-)-Cubebin (1) isolation

Powdered seeds of Piper cubeba L., bought from the market, were exhaustively extracted by maceration with 96% ethanol. The concentrated crude extract was partitioned between n-hexane and methanol/water (9:1), furnishing 430 g of the dried methanol/water fraction, which was submitted to repeated column chromatography over 1.0 kg silica gel (12 × 120 cm column). Elution with increasing portions of n-hexane, dichloromethane, and ethyl acetate yielded 6 fractions. The cubebin-rich fractions [hexane/dichloromethane (1:1) and 100% dichloromethane] were submitted to repeated crystallization from n-hexane/acetone to furnish crystalline cubebin (37 g). Its chemical structure was confirmed by comparison of its ¹H NMR and IR data with those published in the literature (Koul et al., 1988; Souza et al., 2004). The purity of 1 was estimated to be 99% by both HPLC and spectral data analysis.

Preparation of hinokinin (2)

A solution of 50 mg of 1 (1.40 mmol) in 10 mL dichloromethane was treated with 2 equivalents of pyridinium chlorochromate (PCC), and the reaction mixture was stirred for 12 h. The solvent was removed in vacuo, and the residue was chromatographed on a silica gel column eluted with ethyl acetate/n-hexane (80:20 v/v), affording 4.926 g (98%) of the product as an oil; [α]ᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦᵦ㎡⁺ −30° (26 °C) (c 0.99, CHCl₃) (Souza et al., 2004, 2005).

Preparation of (-)-O-acetyl-cubebin (3)

(-)-O-acetyl-cubebin was prepared by reacting 50 mg of 1 (1.25 mmol) with 3 mL acetic anhydride in 0.8 mL dry pyridine at room temperature (r.t.). At the end of the reaction, the mixture was poured into a flask containing toluene and evaporated under reduced pressure, to eliminate pyridine. Dichloromethane was then added, and the mixture was evaporated under reduced pressure to eliminate toluene residue. This process furnished a yellow oil containing 85% (34.25 mg/0.09 mmol) stoichiometric yield of the product, which was purified over a silica gel column using n-hexane/ethyl acetate (3:2). Its purity was estimated to be 98% by both HPLC and spectral data analysis (Souza et al., 2004, 2005).

Preparation of (-)-O-methyl-cubebin (4)

A solution of 50 mg of 1 (1.35 mmol) in 5 mL THF was added to a suspension of NaH (1 g washed with n-hexane, free of paraffin grease) in dried THF (50 mL), and the mixture was stirred for 30 min at room temperature. Then, methyl iodide (1 mL) was added, and the mixture was stirred overnight under N₂ atmosphere. Excess NaH was decomposed by addition of aqueous methanol (1:1). Diluted HCl was added, and the medium was partitioned three times with ethyl acetate (3 × 30 mL). The organic phase was neutralized with 5% NaHCO₃ (2 × 20 mL), 10% NaCl (3 × 20 mL), and 5% NaHCO₃ (2 × 20 mL), dried over MgSO₄, and filtered. The solvent was evaporated under reduced pressure, yielding a brown residue that was purified by silica gel column chromatography using n-hexane/ethyl acetate (4:1) as eluent, furnishing a colourless oil with
92% (46.0 mg/0.12 mmol) stoichiometric yield. Its purity was estimated to be 98% by both HPLC and spectral data analysis (Souza et al., 2004, 2005).

Preparation of (-)-O-(N,N-dimethylamine-ethyl)-cubebin (5)

60 mg (0.14 mmol) of 1 in 1 mL ethanol was added to a solution of sodium ethoxide (5 mL ethanol, 2 Eq Na+) and stirred under reflux for 2 h. After that, 24 mg (0.22 mmol) dimethylethylammonium chloride was added, the reaction was monitored by TLC, and the reflux was carried out for additional 6 h. At the end of the reaction, 5 mL water were added, the phases were separated, and the organic phase was extracted with ethyl acetate (3 × 10 mL). The organic phase was washed with 10% NaCl aqueous solution and its purity was estimated to be 76% by both HPLC and spectral data analysis (Souza et al., 2004, 2005).

Preparation of (-)-6,6′-dinitrohinokinin (6)

A solution of 200 mg (0.45 mmol) hinokinin (2) in 30 mL chloroform (−6 °C) was added to 6 equivalents of HNO3. The reaction mixture was stirred in 30 mL chloroform (−6 °C) was added to 6 equivalents of HNO3. The reaction mixture was stirred in 30 mL chloroform (−6 °C). The organic phase was extracted with 10% NaCl aqueous solution (3 × 10 mL), dried over MgSO4, and filtered. The organic extracts were combined, and the aqueous phase was extracted several times with methylene chloride. The product was obtained as dark yellow oil, and the residue was purified by chromatography on a silica gel column eluted with methylene chloride. The purity was estimated to be 98% by both HPLC and spectral data analysis (Souza et al., 2004, 2005).

Antimycobacterial activity

The antimycobacterial activity of compounds 1–6 was assayed in vitro by the microdilution technique on a Resazurin microtiter assay (REMA) plate, 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), M. kansasii (ATCC 12478), and M. 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 15 to 2000 μg/mL, while the final DMSO content in the assay was less than 0.3%. Rifampicin was used as the reference antibiotic drug, and bioassays were performed in three independent experiments. 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

A number of efficacious antitubercular agents were discovered in the late 1940 s and 1950 s, and the last to be introduced was rifampicin, in the 1960 s (Grassi and Peona, 1995; Sepkowitz et al., 1995). These agents have reasonable efficacy but, when they are employed in combination, drug resistance is expected to develop.

There have been a number of practical obstacles to the development of new antimycobacterial agents, among them a lack of economic incentive due to the predominance of the disease in the developing world. The very slow growth and highly contagious nature of M. tuberculosis have also served to discourage the drug discovery effort.

Recent studies have shown that hinokinin displays antitumoural activity, without cytotoxicity or mutagenicity (Medola et al., 2007). Therefore, the aim of the present work was to evaluate the potential of (-)-cubebin (1), hinokinin (2), and their semi-synthetic derivatives 3–6 (Fig. 1) against three species of Mycobacterium.

Non-compliance with the drugs prescribed for the treatment of tuberculosis and 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).

Compounds 1–6 were screened for their antimycobacterial activities by using the minimal inhibitory concentration (MIC) method; the results are shown in Table I. Screening of the antimycobacterial activity was undertaken using rifampicin...
as the reference drug (Collins and Franzblau, 1997; Palomino et al., 2002).

(-)-Cubebin (1) did not display any activity against the investigated strains. Hinokinin (2) was moderately active against *M. tuberculosis*, with a MIC value equal to 62.5 μg/mL. Compound 3, whose lactol group is acetylated, displayed activity against *M. tuberculosis* (MIC = 125 μg/mL) and *M. avium* (MIC = 62.5 μg/mL). The best result was achieved with compound 4, whose lac-

![Chemical structures and conditions of the reactions.](image)

**Fig. 1.** Chemical structures and conditions of the reactions. (a) Acetic anhydride, pyridine, r.t., 24 h (85%). (b) Dimethylethylammonium chloride, EtONa, dry THF, r.t., N₂ atmosphere, 6 h (76%). (c) Methyl iodide, NaH, dry THF, r.t., N₂ atmosphere, 6 h (92%). (d) PCC (pyridinium chlorochromate) in dry methylene chloride (98%), 24 h, in an ice bath with continuous stirring. (e) HNO₃, chloroform, –6 °C, 2 h (88%).

**Table I. Minimal inhibitory concentration (MIC) of 1–6 against *M. tuberculosis*, *M. kansasii*, and *M. avium*.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC [μg/mL]</th>
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<tbody>
<tr>
<td></td>
<td><em>M. tuberculosis</em> (ATCC 27294)</td>
</tr>
<tr>
<td>1</td>
<td>500</td>
</tr>
<tr>
<td>2</td>
<td>62.5</td>
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<tr>
<td>3</td>
<td>125</td>
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<tr>
<td>4</td>
<td>250</td>
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<td>5</td>
<td>250</td>
</tr>
<tr>
<td>6</td>
<td>1000</td>
</tr>
<tr>
<td>Rifampicin*</td>
<td>0.031</td>
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</tbody>
</table>

* Standard antibiotic.
tol group is methylated, leading to a MIC value equal to 31.25 μg/mL against \textit{M. avium}. The other compounds were not active against any of the studied mycobacteria. In the case of the lactol-containing compounds evaluated here, it seems to be essential that the lactol group is absent, and the substituent of this group should be small, as in the case of 3 and 4. \textit{M. kansasii} (ATCC 12478) was the most resistant mycobacterium concerning the evaluated compounds, with MIC values varying between 1000 and 2000 μg/mL.

In the present work, the microdilution technique on a REMA plate was selected for determination of the MIC values because it is a colorimetric technique that is easy to handle, it provides good reproducibility, and it employs a reduced amount of natural compounds, not to mention that it enables one to test various compounds simultaneously. The MIC value is defined as the lowest drug concentration that effects an inhibition of ≥90% relative to untreated cultures.

To sum up, cubebin and hinokinin semi-synthetic derivatives were prepared and evaluated for their antymycobacterial activity. Some derivatives were active against \textit{M. tuberculosis} and \textit{M. avium}, suggesting that this class of compounds may lead to a new generation of antituberculosis agents without the cytotoxicity displayed by the currently used antituberculosis drugs. Further studies on the structure-activity relationship should also furnish the promising results. Today, the therapeutic arsenal for the treatment of tuberculosis is reduced, and the treatment is long. This is a serious problem that has worsened in the last years because of the increase in tuberculosis cases caused by resistant \textit{M. tuberculosis} strains (Okunade and Elvin-Lewis, 2004). Therefore, the search for new drugs from natural origin is urgent and extremely important in developing countries like Brazil, which are the richest countries in terms of biodiversity.

The results obtained in this study suggest a potential application of (-)-cubebin derivatives in the treatment of \textit{Mycobacterium} species. Further investigations should be conducted in order to explore this application.

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