TRITERPENES AND ANTITUBERCULAR ACTIVITY OF *Byrsonima crassa*

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We evaluated the potential antitubercular activity of triterpenes obtained from leaves and bark of *Byrsonima crassa*. From chloroform extracts of the leaves, by bioassay-guided fractionation, we obtained mixtures of known triterpenes: α-amyрин, β-amyрин and their acetates, lupenol, oleanonic acid, ursolic acid and α-amyrinone. Tested against *Mycobacterium tuberculosis*, the triterpenes exhibited minimum inhibitory concentrations (MICs) of 31.25 - 312.25 µg/mL, β-amyrin and friedelin, isolated from the chloroform extract of bark, showed MICs of 312.25 and 125 µg/mL respectively. This is the first report of the identification and determination of the activity of *B. crassa* triterpenes against *M. tuberculosis*.

Keywords: *Byrsonima crassa*; antitubercular activity; triterpenes.

INTRODUCTION

Tuberculosis (TB) remains an important public health problem worldwide, causing the deaths of about 1.6 million people each year. According to the World Health Organization, it is estimated that 8.8 million new TB cases occurred in 2007.1 Despite the improvements in chemotherapy, TB control is severely affected by the development of multidrug resistant *M. tuberculosis* strains.2,3 The urgent need to create or find new drugs to reduce the global burden of tuberculosis is much discussed in the current biomedical literature.5,6 Natural products and/or their semi-synthetic derivatives can lead to compounds and may play important roles in the treatment of TB.7 A review of plant terpenoids showed moderate to significant biological activity against *M. tuberculosis*.2

*Byrsonima crassa* Niedenzu (IK) (Malpighiaceae) is a native specie of the Brazilian Cerrado (savannah-like vegetation). *B. crassa* is popularly known in Brazil as “murici-cascudo” or “murici-vermelho”.8 Several medicinal properties are attributed to the bark and leaves of this species. It is used in Brazilian folk medicine for the treatment of diseases related mainly to peptic ulcer.9 In earlier papers we have reported the antitulcer activity and the chemical composition of the methanol and hydromethanol extracts of *B. crassa*.9,10 Despite its popular use as a medicinal plant, no data have been published on the antitubercular activity of leaf and bark extracts of this species, and we have found no report on the chemical composition of the nonpolar chloroform extract of this plant.

The aim of the present study was to identify the nonpolar com-

pounds present in the leaves and bark of *Byrsonima crassa* and to determine the antitubercular activity of their enriched fractions and isolated compounds.

EXPERIMENTAL

General experimentation procedures

Analytical and preparative thin layer chromatography (TLC) were carried out on Kieselgel 60 precoated Al sheets (0.2 mm, Merck) and column chromatography was performed on silica gel (70–230 mesh, Merck). The TLC spots were visualized by spraying with 10% H2SO4 in water, followed by heating at 110 °C.

The NMR spectra in CDCl3 were acquired with a Varian INOVA 500 spectrometer, operating at 500 MHz for 1H and 150 MHz for 13C. Chemical shifts were given in δ (ppm), using tetramethylsilane (TMS) as internal standard.

The compounds were identified by NMR as described by Olea and Roque11 and structures were confirmed by comparison against literature spectroscopic data13-16 (Figure 1).

Plant material

Leaves and bark of *B. crassa* Niedenzu (IK) were collected at Porto National, Tocantins State, Brazil (10° 42’ S, 48° 24’ W), and identified by Dr. E. R. dos Santos. A voucher specimen (nº 3377) was deposited at the herbarium of the Universidade de Tocantins.

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Extraction and isolation

The ground materials (1.8 kg bark; 2.0 kg leaves) were subjected to exhaustive extraction with chloroform at room temperature for 48 h. Solvent was evaporated at 60 °C under reduced pressure, affording the crude leaf extract (53.8 g) and the crude bark extract (14.1 g).

Leaves

The chloroform leaf extract (33.4 g) was subjected to column chromatography (CC) on silica gel (32.0 x 3.0 cm i.d.) to obtain fractions of rising polarity by elution with n-hexane, dichloromethane, and methanol, in this order. Evaporation of the solvents resulted in fractions of increasing polarity. 56 fractions were collected, of which 37-56 yielded a mixture of ursolic and oleanolic acid (MIC 31.25 µg/mL). The dichloromethane fraction (DF) gave a mixture of lupeol, α-amyrinone and β-amyrin (MIC 312.5 µg/mL). The methanol fraction (MF) yielded the triterpenes oleanolic and ursolic acids (MIC 31.25 µg/mL). The dichloromethane fraction (DF) gave a mixture of lupeol, α-amyrinone and β-amyrin (MIC 312.5 µg/mL) and a mixture of α-amyrin and β-amyrin (MIC 31.25 µg/mL). The methanol fraction (MF) yielded the triterpenes oleanolic and ursolic acids (MIC of 62.5 µg/mL). In the 5.8 g of chloroform bark extract, 2.4 g of pure β-amyrin (MIC 312.25 µg/mL) and 530 mg of friedelin (MIC 125 µg/mL) were found.

Bark

The chloroform extract of the bark (5.8 g) was subjected to CC on silica gel (32 x 5.0 cm i.d.) using a gradient of n-hexane–EtOAc of increasing polarity. The 99 eluted fractions (100 mL each) were combined to give 21 fractions. Fractions 31-34 yielded the triterpene friedelin as a white crystal (530 mg), while fractions 39-40 furnished β-amyrin (2.4 g) as a pure white crystal.

Determination of antitubercular activity

Antitubercular activity of extracts and fractions diluted in DMSO was determined against Mycobacterium tuberculosis H₃Rv ATCC 27294, using the microplate Alamar Blue assay (MABA), as recommended and validated by Collins and Franzblau. The minimal inhibitory concentration (MIC) of the compound was defined as the concentration necessary to inhibit 90% of the mycobacterial growth in sterile 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ). Fluorescence was measured in a Spectrafluor Plus microfluorimeter (Tecan) in bottom reading mode, with excitation at 530 nm and emission at 590 nm. A sample with a MIC value <125 µg/mL was defined as active against M. tuberculosis. As a standard control, the MIC value of isoniazid was determined on each microplate. The acceptable MIC of isoniazid ranged from 0.015 to 0.05 µg/mL.

RESULTS AND DISCUSSION

Investigation of traditionaly used medicinal plants is an efficient way of searching for new candidate chemotherapeutic drugs. Plant extracts are an attractive source of new drugs, and bioassay-guided fractionation facilitates the isolation of active principles contained in crude natural products.

In a previous study of B. crassa, Cardoso et al. showed that its methanol extract was mutagenic to Salmonella typhimurium TA 98, but the chloroform extract was devoid of this activity.

The B. crassa chloroform extracts (MICs of 312.25 µg/mL for bark and 125 µg/mL for leaves) were fractionated resulting in the identification of nine previously known compounds (Figure 1), whose MICs were measured (Table 1). The hexane fraction (HF) of the leaf extract yielded a mixture of α-amyrin and β-amyrin acetates (MIC 31.25 µg/mL). The dichloromethane fraction (DF) gave a mixture of lupeol, α-amyrinone and β-amyrin (MIC 312.5 µg/mL) and a mixture of α-amyrin and β-amyrin (MIC 31.25 µg/mL). The methanol fraction (MF) yielded the triterpenes oleanolic and ursolic acids (MIC of 62.5 µg/mL). In the 5.8 g of chloroform bark extract, 2.4 g of pure β-amyrin (MIC 312.25 µg/mL) and 530 mg of friedelin (MIC 125 µg/mL) were found.

Table 1. MIC values of isoniazid, chloroform extracts and fractions of B. crassa against M. tuberculosis, determined by MABA

<table>
<thead>
<tr>
<th>Samples</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bark chloroform extract</td>
<td>312.25</td>
</tr>
<tr>
<td>β-amyrin</td>
<td>312.25</td>
</tr>
<tr>
<td>friedelin</td>
<td>ND*</td>
</tr>
<tr>
<td>leave chloroform extract</td>
<td>62.5</td>
</tr>
<tr>
<td>mixture of ursolic and oleic acid</td>
<td>62.5</td>
</tr>
<tr>
<td>mixture of lupeol, α-amyrinone and β-amyrin</td>
<td>312.25</td>
</tr>
<tr>
<td>mixture of α-amyrin and β-amyrin</td>
<td>31.25</td>
</tr>
<tr>
<td>mixture of α-amyrin and β-amyrin acetates</td>
<td>31.25</td>
</tr>
<tr>
<td>Reference drug</td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*ND – not done

According to Copp, secondary metabolites of terpenoid origin are among the most promising classes of natural products with antimycobacterial activity.

Akihisa et al. found a MIC against M. tuberculosis higher than 64 µg/mL for α-amyrin and for β-amyrin isolated from Asteraceae flowers. The MIC value of 31.25 µg/mL determined here for the mixture of α-amyrin and β-amyrin is better than the MIC of either

Figure 1

R1 = CH, R2 = CH3, R3 = H : β-amyrin
R1 = OAc, R2 = CH3, R3 = H : β-amyrin acetate
R1 = OH, R2 = H, R3 = CH3 : α-amyrinone
R1 = OAc, R2 = H, R3 = CH3 : oleanolic acid
R1 = OH, R2 = H, R3 = CH3 :ursolic acid
R1 = CH, R2 = CH3, R3 = O-amyrene

R2 = H, R3 = CH3 : α-amyrene
compound in isolation, probably indicating their synergist action in the mixture. The MIC of 312.25 µg/mL for pure β-amyrin, found in the current work, reinforces this conclusion. This high MIC value disqualifies this compound, present in large amounts in the bark of *B. crassa*, as a tuberculostatic drug. However, considering the great intensification of its tuberculostatic effect when mixed with its α isomer, it should be possible to increase its activity by appropriate structural alterations.\(^7\)

The mixture of lupeol, α-amyrinone and β-amyrin had the MIC of 312.25 µg/mL. Wachter et al.,\(^2\) studying the lupeol isolated from *Chuaquiraga ulicina* (Argentina), found a MIC of 64 µg/mL. The MIC of the mixture of oleanolic and ursolic acids in the present work was of 62.5 µg/mL. According to Caldwell et al.,\(^2\) oleanolic acid has a MIC of 16 µg/mL. Cantrell *et al.*\(^2\) obtained a MIC of 50 µg/mL for ursolic acid. Gu *et al.*\(^18\) also obtained a better MIC value for oleanolic acid (MIC of 28.7 µg/mL) than for ursolic acid (MIC of 41.9 µg/mL).

In these last examples, each isolated compound exhibited a better MIC than the mixture.

According to Cantrell *et al.*\(^2\), isolated compounds that exhibit a MIC of 64 µg/mL or lower are considered promising. For crude extracts, the MIC should be equal to or lower than 125 µg/mL.\(^16\) Thus, the values of 62.5 µg/mL for the mixture of oleanolic and ursolic acids and of 31.25 µg/mL for the mixture of α-amyrin and β-amyrin and its acetates, obtained here, are as good as a promising isolated compound. Although the MIC values obtained here are larger than that of isoniazid (0.03 µg/mL), these inhibitory concentrations are comparable to the MIC of pyrazinamide (another first-line antituberculosis drug), 20-100 µg/mL.\(^24\)

Concluding, our results suggest that oleanolic acid, lupeol and the mixture of α- and β-amyrin are involved in the antitubercular activity of the chloroform extract of *B. crassa* leaves. The high lipophilicity of terpenes is probably the main factor that allows their penetration through the mycobacterial cell wall.

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