

## 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:  $\alpha$ -amyrin,  $\beta$ -amyrin and their acetates, lupeol, oleanolic acid, ursolic acid and  $\alpha$ -amyrinone. Tested against *Mycobacterium tuberculosis*, the triterpenes exhibited minimum inhibitory concentrations (MICs) of 31.25 - 312.25  $\mu$ g/mL.  $\beta$ -amyrin and friedelin, isolated from the chloroform extract of bark, showed MICs of 312.25 and 125  $\mu$ g/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.<sup>1</sup> Despite the improvements in chemotherapy, TB control is severely affected by the development of multidrug resistant *M. tuberculosis* strains.<sup>2-4</sup> The urgent need to create or find new drugs to reduce the global burden of tuberculosis is much discussed in the current biomedical literature.<sup>5,6</sup> Natural products and/or their semi-synthetic derivatives can lead to compounds and may play important roles in the treatment of TB.<sup>7</sup> A review of plant terpenoids showed moderate to significant biological activity against *M. tuberculosis*.<sup>2</sup>

*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”.<sup>8</sup> 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.<sup>8</sup> In earlier papers we have reported the antiulcer activity and the chemical composition of the methanol and hydromethanol extracts of *B. crassa*.<sup>9-11</sup> 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% H<sub>2</sub>SO<sub>4</sub> in water, followed by heating at 110 °C.

The NMR spectra in CDCl<sub>3</sub> were acquired with a Varian INOVA 500 spectrometer, operating at 500 MHz for <sup>1</sup>H and 150 MHz for <sup>13</sup>C. Chemical shifts were given in  $\delta$  (ppm), using tetramethylsilane (TMS) as internal standard.

The compounds were identified by NMR as described by Olea and Roque<sup>12</sup> and structures were confirmed by comparison against literature spectroscopic data<sup>13-16</sup> (Figure 1).

### Plant material

Leaves and bark of *B. crassa* Niedenzu (IK) were collected at Porto Nacional, Tocantins State, Brazil (10° 42' S, 48° 24' W), and identified by Dr. E. R. dos Santos. A voucher specimen (n° 3377) was deposited at the herbarium of the Universidade de Tocantins.

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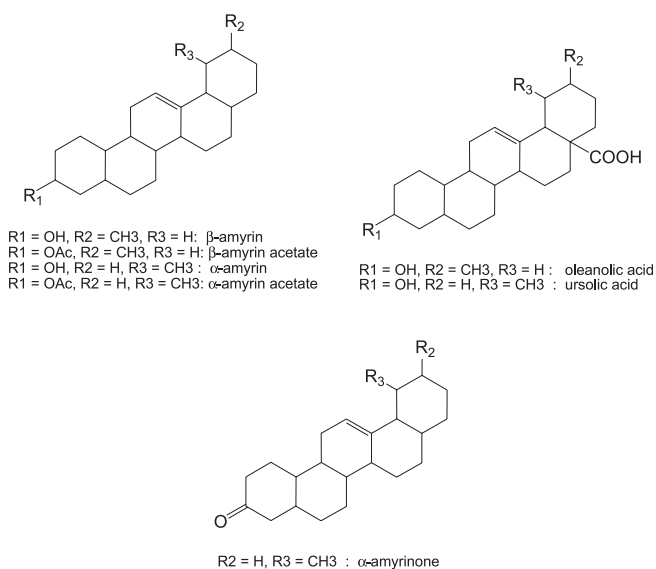


Figure 1

### 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 hexane (168 mg), dichloromethane (10.0 g), and methanol (4.0 g) dry fractions. The hexane fraction (HF, 168 mg), applied to a silica gel column (12 x 2.0 cm i.d.) and eluted with *n*-hexane, furnished the acetates of  $\beta$ -amyrin and  $\alpha$ -amyrin (108 mg). The dichloromethane fraction (DF, 10.0 g), also subjected to CC (silica gel, *n*-hexane–EtOAc mixtures, in order of increasing polarity), afforded 49 fractions. Fractions 1-2 (158 mg) were loaded on a new silica gel column (14.0 x 2.0 cm i.d.) and eluted with *n*-hexane–chloroform (75:15), yielding a mixture of  $\alpha$ -amyrinone, lupeol and  $\beta$ -amyrin (10.0 mg). Fractions 8-10 (227 mg) were also rechromatographed on a silica gel column (6.0 x 2.0 cm i.d.), yielding a mixture of  $\beta$ -amyrin and  $\alpha$ -amyrin (36.0 mg).

The methanol fraction (MF, 4.0 g) was fractionated by CC on silica gel (18 x 3.5 cm i.d.), eluted with mixtures of *n*-hexane and EtOAc of increasing polarity. 56 fractions were collected, of which 37-56 were combined (225 mg), applied to a silica gel CC and eluted with *n*-hexane–EtOAc (70:30). This last yielded a mixture of ursolic and oleanolic acids (36.0 mg).

### 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 rising 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  $\beta$ -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* H<sub>37</sub>Rv ATCC 27294, using the microplate Alamar Blue assay (MABA), as recommended and validated by Collins and Franzblau.<sup>17</sup> 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  $\mu$ g/mL was defined as active against *M. tuberculosis*.<sup>18</sup> 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  $\mu$ g/mL.<sup>17</sup>

### RESULTS AND DISCUSSION

Investigation of traditionally 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.*<sup>19</sup> 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  $\mu$ g/mL for bark and 125  $\mu$ g/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  $\alpha$ -amyrin and  $\beta$ -amyrin acetates (MIC 31.25  $\mu$ g/mL). The dichloromethane fraction (DF) gave a mixture of lupeol,  $\alpha$ -amyrinone and  $\beta$ -amyrin (MIC 312.5  $\mu$ g/mL) and a mixture of  $\alpha$ -amyrin and  $\beta$ -amyrin (MIC 31.25  $\mu$ g/mL). The methanol fraction (MF) yielded the triterpenes oleanolic and ursolic acids (MIC of 62.5  $\mu$ g/mL). In the 5.8 g of chloroform bark extract, 2.4 g of pure  $\beta$ -amyrin (MIC 312.25  $\mu$ g/mL) and 530 mg of friedelin (MIC 125  $\mu$ g/mL) were found.

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

Samples	MIC ( $\mu$ g/mL)
bark chloroform extract	312.25
$\beta$ -amyrin	312.25
friedelin	ND*
leave chloroform extract	62.5
mixture of ursolic and oleanolic acid	62.5
mixture of lupeol, $\alpha$ -amyrinone and $\beta$ -amyrin	312.25
mixture of $\alpha$ -amyrin and $\beta$ -amyrin	31.25
mixture of $\alpha$ -amyrin and $\beta$ -amyrin acetates	31.25
Reference drug	
Isoniazid	0.03

\*ND – not done

According to Copp,<sup>20</sup> secondary metabolites of terpenoid origin are among the most promising classes of natural products with antimycobacterial activity.

Akihisa *et al.*<sup>21</sup> found a MIC against *M. tuberculosis* higher than 64  $\mu$ g/mL for  $\alpha$ -amyrin and for  $\beta$ -amyrin isolated from *Asteraceae* flowers. The MIC value of 31.25  $\mu$ g/mL determined here for the mixture of  $\alpha$ -amyrin and  $\beta$ -amyrin is better than the MIC of either

compound in isolation, probably indicating their synergist action in the mixture. The MIC of 312.25 µg/mL for pure β-amyryn, 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.<sup>7</sup>

The mixture of lupeol, α-amyrynone and β-amyryn had the MIC of 312.25 µg/mL. Wachter *et al.*,<sup>22</sup> studying the lupeol isolated from *Chiquiraga ulcina* (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.*,<sup>23</sup> oleanolic acid has a MIC of 16 µg/mL. Cantrell *et al.*<sup>2</sup> obtained a MIC of 50 µg/mL for ursolic acid. Gu *et al.*<sup>18</sup> 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.*,<sup>2</sup> 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.<sup>18</sup> 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-amyryn and β-amyryn 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 antitubercular drug), 20-100 µg/mL.<sup>24</sup>

Concluding, our results suggest that oleanolic acid, lupeol and the mixture of α- and β-amyryn 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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