December 2007 Volume 3 Issue 3

Trade Science Inc.

Natural Products

An Indian Journal

Full Paper

NPAIJ, 3(3), 2007 [139-143]

Byrsonima crassa triterpenes and antitubercular activity

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Received: 3rd August, 2007 ; Accepted: 8th August, 2007

ABSTRACT

We evaluated the potential antitubercular activity of triterpenes obtained from leaves and barks of *Byrsonima crassa*, a native species of Brazilian central savannah-like belt of vegetation. The structures of compounds were elucidated on the basis of spectroscopic evidence. The antitubercular activity was determined by microplate alamar blue method(MABA). From chloroformic extracts of leaves, by bioassay guided fractionation, we obtained mixtures of known triterpenes: α-amyrin, β-amyrin and their acetates, lupeol, oleanolic acid, ursolic acid and α-amyrinone, which exhibited minimum inhibitory concentration (MIC) ranged of 31.25-312.25μg/mL. β-amyrin and friedelin isolated from chloroformic extract of bark showed MIC of 312,25μg/mL and 125μg/mL respectively. This is the first report of triperpenes present in *B. crassa* and their antimycobacterial activity.

KEYWORDS

Byrsonima crassa;
Antitubercular activity;
Triterpenes;
Oleanolic acid;
Ursolic acid.

INTRODUCTION

Tuberculosis still remains an important public health problem worldwide. According to the World Health Organization there were an estimated 8,8 million new TB cases in 2005[1]. Despite the improvements of the chemotherapeutic, tuberculosis is severely affected by the development of multidrug resistant *M. tuberculosis* strains[2,3,4]. The urgent need for the development of new drugs to reduce the global burden of tuberculosis is very related in the current biomedical literatures[5,6]. Natural products and/or their semi-synthetic derivatives can leads to novel examples of antimycobacterial drugs and may play important roles in the chemotherapy of tuberculosis. A review of plant terpenoids showed moderate to significant biological activity against *M.tuberculosis*[2].

Byrsonima crassa niedenzu(IK) is a member of genus *Byrsonima*(Malpighiaceae) and is a native species from Brazilian Cerrado(savannah-like vegetation). *B.crassa* is popularly known as murici-cascudo or

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murici-vermelho[7]. There are several properties attributed to the bark and leaves of this species. It is used in Brazilian folk medicine for the treatment of diseases related mainly to gastric and peptic ulcer[7]. Previously we have reported the antiulcer activity and the chemical composition of the methanolic and hydromethanolic extracts of *B.crassa*[8,9,10]. Despite the popular use of *B.crassa* as medicinal plant, there are no data based on the antitubercular activity of its leaves and barks extracts, and no relate was observed about the chemical composition of the apolar chloroformic extract from this plant.

The aim of the present study was to identify the apolar compounds present in the leaves and barks of *Byrsonima crassa* and to determine the antitubercular activity of those isolated and or enriched fractions.

EXPERIMENTAL

Plant material

The leaves and barks of *B.crassa* Niedenzu(IK) was collected at Porto National, Tocantins State, Brazil and identified by Dr.Eduardo Ribeiro dos Santos. A voucher specimen(nº 3377) was deposited in the herbarium of the tocantins university.

Extraction and isolation

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

Leaves

The chloroformic extract of leaves(3.34g) was subjected to column chromatography(CC) on silica gel (32.0×3.0cm i.d.) to obtain some fractions according their polarity on *n*-hexane, dichloromethane and finally, methanol. After the solvent evaporation were obtained the hexanic, dichloromethanic and methanolic fractions.

The hexanic fraction(HF, 168mg) was applied to a silica gel column(12cm×2.0cm i.d.) furnished acetates of β-amyrin and α-amyrin.

The dichloromethane fraction(DF)(10.0g) was subjected to column chromatography(silica gel, *n*-hexane -EtOAc and EtOAc, in order of increasing polarity) to obtain 49 fractions. Fractions 1-2(158mg) was submitted to a column chromatography(14.0cm×2.0cm i.d.), which eluted with *n*-hexane–chloroform(75:15) yielded the mixture of α-amyrinone, lupeol and β-amyrin(10.0mg). Fraction 8-10(227mg), which eluted with hexane-chloroform(1:1), on subjecting to column chromatography(6.0cm×2.0cm i.d.) afforded further quantities of a mixture of β-amyrin and α-amyrin (36.0mg).

The methanolic fraction(MF)(4.0g) was separated by CC on silica gel(18cm×3.5cm i.d.) with mixtures of *n*-hexane–EtOAc of increasing polarity. The 56 eluted fractions of 100ml were combined in eight fractions. Fractions 37-56(225mg) were applied to a silica gel column chromatography using as mobile phase n-hexane-EtOAc(70:30) furnished the mixture of ursolic and oleanolic acid(36.0mg) .

Barks

The chloroformic extract of the barks(5.8g) was

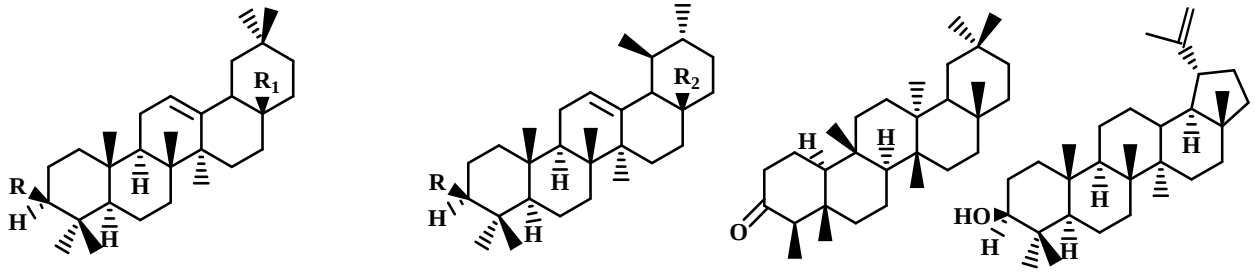

Figure 1: Structure of triterpenes identified in the leaves and bark of *B.crassa*

subjected to CC on silica gel(32cm×5.0cm i.d.) using a gradient of n-hexane–EtOAc, with the 99 eluted fractions(100ml each) being combined to give 21 fractions. Fractions 31-34 yielded the triterpene friedelin as a white crystal(530mg). In the other hand, fractions 39-40 furnished β-amyrin(2.4g) as a pure white crystal.

Structural identification of the compounds

The NMR spectra in $CDCl_3$ were obtained using a Varian INOVA 500 spectrometer, operating at 500 MHz for ^1H and 150MHz for ^{13}C and Chemical shifts were given in δ(ppm) using tetramethylsilane(TMS) as internal standard.

The compounds were identified using NMR experiments according to Olea methodology(1990) and structures were confirmed by comparison against literature spectroscopic data[11].

The spectral data of the ^{13}C NMR of the known triterpenes are given as below:

lupeol: ^{13}C NMR($CDCl_3$, 125MHz): δ 39.0(C-1); 27.4(C-2); 77.4(C-3); 38.5(C-4); 55.3(C-5); 19.6(C-6); 34.2(C-7); 40.0(C-8); 50.0(C-9); 37.1(C-10); 19.7(C-11); 25.9(C-12); 39.0(C-13); 42.3(C-14); 26.9(C-15); 35.4(C-16); 43.0(C-17); 48.3(C-18); 47.6(C-19); 150.9(C-20); 29.7(C-21); 39.7(C-22); 28.1(C-23); 15.5(C-24); 16.7(C-25); 15.5(C-26); 15.2(C-27); 18.0(C-28); 109.3(C-29); 19.7(C-30).

α-amyrin: ^{13}C NMR($CDCl_3$, 125MHz): 38.8(C-1); 27.3(C-2); 79.0(C-3); 38.8(C-4); 55.2(C-5); 18.4(C-6); 32.9(C-7); 40.0(C-8); 47.8(C-9); 37.0(C-10); 23.4(C-11); 124.5(C-12); 139.6(C-13); 42.1(C-14); 28.7(C-15); 26.7(C-16); 33.8(C-17); 59.1(C-18); 46.9(C-19); 31.8(C-20); 34.0(C-21); 37.0(C-22); 28.2(C-23); 22.0(C-24); 15.7(C-25); 16.9(C-26); 26.5(C-27); 28.1(C-28); 33.9(C-29); 24.0(C-30).

β-amyrin: ^{13}C NMR($CDCl_3$, 125MHz): 38.8(C-1); 27.3(C-2); 77.70(C-3); 38.7(C-4); 55.2(C-5); 18.4 (C-6); 32.7(C-7); 39.0(C-8); 47.7(C-9); 37.2(C-10); 23.6(C-11); 121.8(C-12); 145.2(C-13); 41.6(C-14); 26.2(C-15); 26.9(C-16); 32.5(C-17); 47.3(C-18); 46.9(C-19); 31.1(C-20); 34.8(C-21); 37.2(C-22); 28.1(C-23); 15.7(C-24); 15.6(C-25); 16.8(C-26); 25.9(C-27); 28.4(C-28); 33.3(C-29); 23.6(C-30).

α-amyrin acetate: ^{13}C NMR($CDCl_3$, 125MHz): 38.4(C-1); 23.6(C-2); 80.7(C-3); 37.6(C-4); 55.3(C-5); 18.3(C-6); 32.8(C-7); 40.1(C-8); 47.6(C-9); 36.8(C-10); 23.2(C-11); 125.1(C-12); 139.4(C-13); 42.1(C-14); 28.1(C-15); 26.7(C-16); 33.8(C-17); 59.0(C-18); 39.7(C-19); 39.7(C-20); 31.3(C-21); 41.5(C-22); 28.7(C-23);16.8(C-24); 15.7(C-25); 16.8(C-26); 23.2(C-27); 28.1(C-28); 17.5(C-29); 21.4(C-30); 170.5(CH_3COO); 21.3(CH_3COO).

β-amyrin acetate: ^{13}C NMR($CDCl_3$, 125MHz): 38.2 (C-1); 23.6(C-2); 80.7(C-3); 37.6(C-4); 55.3(C-5); 18.3(C-6); 32.6(C-7); 39.7(C-8); 47.5(C-9); 36.8(C-10); 23.4(C-11); 121.5(C-12); 144.9(C-13); 41.7(C-14); 26.9(C-15); 26.2(C-16); 32.5(C-17); 47.2(C-18); 46.8(C-19); 31.1(C-20); 34.8(C-21); 37.1(C-22); 28.3(C-23);16.8(C-24); 15.7(C-25); 16.8(C-26); 26.0(C-27); 27.9(C-28); 33.4(C-29); 23.6(C-30); 170.5(CH_3COO); 21.3(CH_3COO).

Friedelin: ^{13}C NMR($CDCl_3$, 125MHz): 22.4(C-1); 41.6(C-2); 213.1(C-3); 58.4(C-4); 42.1(C-5); 41.3(C-6); 18.1(C-7); 52.9(C-8); 37.5(C-9); 60.0(C-10); 35.7(C-11); 30.3(C-12); 38.3(C-13); 39.8(C-14); 32.3(C-15); 36.1(C-16); 29.9(C-17); 42.6(C-18); 35.1(C-19); 28.0(C-20); 32.9(C-21); 39.4(C-22); 6.9(C-23); 14.8(C-24); 17.5(C-25); 20.1(C-26); 18.4(C-27); 31.5(C-28); 34.9(C-29); 32.0(C-30)

α-amyrinone: ^{13}C NMR($CDCl_3$, 125MHz): 39.3(C-1); 33.9(C-2); 216.5(C-3); 47.0(C-4); 55.1(C-5); 19.3(C-6); 32.5(C-7); 39.9(C-8); 48.6(C-9); 36.8(C-10); 23.2(C-11); 124.0(C-12); 139.4(C-13); 42.1(C-14); 28.1(C-15); 26.7(C-16); 33.8(C-17); 59.0(C-18); 39.7(C-19); 39.7(C-20); 31.3(C-21); 41.5(C-22); 26.6(C-23); 21.1(C-24); 15.3(C-25); 15.3(C-26); 16.6(C-27); 23.2(C-28); 17.3(C-29); 21.4 (C-30)

General experimentation procedures

Analytical and preparative TLC were carried out on Kieselgel 60 precoated Al sheets(0.2 mm, Merck) and column chromatography was performed in silica gel(70-230 mesh, Merck), the spots were visualized by spraying with 10% solution of H_2SO_4, followed by heating at 110 ^{0}C.

Determination of antitubercular activity

Antitubercular activity of extracts and fractions di-

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TABLE 1: MIC determination by MABA of isoniazid, choroformic extracts and fractions of *B.crassa* against *M.tuberculosis.*

Compounds	MIC (μg/mL)
B. crassa(barks) 3340mg	312.25
β-amyrin(2400mg)	312.25
Friedelin(530mg)	ND*
B. crassa(leaves) 5800mg	62.5
mixture of ursolic and oleanolic acid(36mg)	62.5
mixture of lupeol, α-amirenone and β-amyrin (10mg)	312.25
mixture of α-amyrin and β-amyrin(36mg)	31.25
mixture of α-amyrin and β-amyrin acetates (168mg)	31.25
Reference drug	
Isoniazid	0.03

***ND-not done**

luted in DMSO was determined against *Mycobacterium tuberculosis* H37Rv ATCC 27294 using the microplate alamar blue assay(MABA) preconized by Collins & Franzblau[12]. Isoniazid was utilized as reference drug. It was measured the minimal inhibitory concentration(MIC) of these compounds necessary to inhibit 90% of the mycobacterial grow-up using sterile 96-well plates(Falcon 3072; Becton Dickinson, Lincoln Park, NJ). Fluorescense was measured in SPECTRAfluor Plus(Tecan) in botton reading mode with excitation at 530 nm and emission at 590nm. The MIC value of <125μg/mL was defined as active against *M.tuberculosis*[13].

RESULTS AND DISCUSSIONS

Investigation of traditional-used medicinal plants is valuable tools for search potential candidates of chemotherapeutic drugs. Plants extracts are attractive source of new drugs, and the bioassay-guided fractionation facilitate isolation of active principles contained in this crude natural products. In previous study, Cardoso et al verified that methanolic extract had mutagenic activity in *Salmonella typhymurium* TA 98, but no mutagenicity were observed to chloroformic extract[14].

From ECHCl$_3$ extracts(MIC of 312,25μg/mL for barks and 125μg/mL for leaves), we performed phytochemical fractionation and their bioassay. Ten previously known compounds were identified(Figure 1) and the MIC of fractions was demonstrated(TABLE 1). The hexanic fraction(HF) of leaves extracts yielded a mixture of α-amyrin and β-amyrin acetates(MIC of 31.25 μg/mL). The dichloromethane fraction(DF) showed presence of a mixture of lupeol, α-amyrinone and β-amyrin(MIC of 312.5μg/mL) and a mixture of α-amyrin and β-amyrin(MIC of 31.25μg/mL). In the methanolic fraction(MF) it was identified the triterpenes oleanolic and ursolic acids(MIC of 62.5μg/mL). Ninety percent of chloroformic extract from barks was constituted of pure β-amyrin(MIC of 312.25μg/mL) and also yielded 10% of friedelin(MIC of 125μg/mL).

According to Copp, secondary metabolites of terpenoid origin are among the most promising class of natural products with antimycobacterial activity[15].

Akihisa et al, found MIC value higher than 64μg/mL to α-amyrin and to β-amyrin isolated from *Asteraceae* flowers[15]. The MIC value of 31.25μg/mL determined here for mixture of α-amyrin and β-amyrin is better than of each isolated compound, indicating that this better results is probably due to the synergic action of the mixture. *M.tuberculosis* growth inhibition with MIC value of 312.25μg/mL found for pure β-amyrin, reinforces this conclusion. Although the large amount of the triterpene β-amyrin on the barks of *B.crassa* it was not observed a promise antimycobac terial activity.

The mixture of lupeol, α-amyrinone and β-amyrin showed the MIC of 312.25μg/mL. Wachter *et al*, studying the lupeol isolated from *Chuquiraga ulcina* (Argentina) verified MIC value of 64μg/mL[17]. For the mixture of oleanolic and ursolic acids it was demonstrated MIC value of 62.5μg/mL. According to Caldwell *et al*, the oleanolic acid has MIC value of 16 μg/mL[18]. Cantrell et al, presented the MIC value of 50μg/mL for ursolic acid[2]**.** Gu also obtained better MIC value for oleanolic acid(MIC of 28.7μg/mL) than that for ursolic acid(MIC of 41.9μg/ mL)[13]. In this sense each isolated compound shown MIC value better than the mixture.

Our results suggest that the oleanolic acid, lupeol and mixture of α- and β-amyrin could be related with the antitubercular activity obtained at chloroformic leaves extracts of *B.crassa*. The high lipophilicity of terpenes is probably one of the factors that allow their penetration on the mycobacterial cell wall.

ACKNOWLEDGMENTS

We thank the Fundacao de Amparo a Pesquisa do Estado de Sao Paulo(FAPESP) for a grant to Miriam Sannomiya. and for fundings from Biota-Fapesp program.

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