EVALUATION OF ANTI-Mycobacterium tuberculosis ACTIVITY OF Campomanesia adamantium (MYRTACEAE)

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INTRODUCTION

Tuberculosis (TB) has reemerged as one of the leading causes of death in the world, reaching a million deaths annually. The emergence of multidrug-resistant strains of Mycobacterium tuberculosis and co-infections with HIV has aggravated this serious situation and, since resistance decreases the effectiveness of most available antitubercular agents, there is an urgent need to develop new drugs to help reduce the global burden of tuberculosis, as has been well documented.

Natural products and/or their semi-synthetic derivatives can lead to novel antimycobacterial drugs and might have in the future important roles in the chemotherapy of tuberculosis. The first step to obtain these products is its extraction from the plants, following by the achievement of biological tests with the extracts and fractions.

The Campomanesia adamantium (Myrtaceae) species is a small tree with edible fruit, commonly known as guavira or gauabiroba. The fruit is widely used to make liqueurs, juices and sweets. The infusion of the guavira leaves is used in popular medicine as depurative, anti-diarrhoeic, cleanser, anti-rheumatic and to decrease the blood cholesterol.

The phytochemical studies about Campomanesia genus are recent. Chemical investigation of seeds of C. lineatifolia led to the isolation and identification of β-triketone type compounds, named champanones. Studies of the leaves in this Campomanesia species identified quercetin, myricetin and rutin by HPLC.

In this way, the present paper describes the anti-M. tuberculosis activity of extracts, fractions and pure substances from Campomanesia adamantium fruits and the identification and quantification of flavonanes and chalcones in these fractions, as well as their action against M. tuberculosis in vitro.

EXPERIMENTAL

Plant material

The fruits of the C. adamantium were collected in Jardim city, Mato Grosso do Sul State, Brazil (latitude 21° 25’ 02.0” S and longitude 056° 13’ 77.0” W) and identified by M. Sobral of Universidade Federal de Minas Gerais. A voucher specimen (nº 5195 - Jardim) was deposited in the Herbarium Mato Grosso do Sul - HMS, Campo Grande, MS, Brazil.

General procedures

Sephadex LH-20 (Pharmacia, 60 cm x 2.5 cm) was used for GPC. TLC analyses were carried out on silica gel 60G (Merck) (20 cm x 20 cm x 0.2 mm) plates eluted with toluene: ethyl acetate: acetic acid 8:2:0.5 v/v/v. The spots were visualized by spraying with NP/PEG reagent, or 10% H2SO4, followed by heating at 110 °C for 5 min. Column chromatographies were performed on silica gel (Merck, 40-63 μm and 63-200 μm) and Lichroprep RP18 (Merck, 40-63 μm). NMR spectra were recorded on a Bruker DFX 300 spectrometer.

The fractions obtained from the ethyl acetate extract were analyzed by an Analytical HPLC (Varian 210) system, equipped with a diode array detector (DAD). Star WS software was used for chromatograms data treatment. The column was an RP18 (25 cm x 4.6 mm x 5 μm) reversed-phase type, with a small precolumn (2.5 cm x 3 mm) containing the same packing, used to protect the analytical column. Elution was carried out with a gradient solvent program of methanol/water/acetonitrile (40:50:10 in the beginning), taking 40 min to reach 80% methanol, 10% water and 10% acetonitrile, returning after that in 10 min to the initial conditions. The flow rate was 1.0 mL/min and the injected volume of the samples varied from 10 to 20 μL.

Keywords: Campomanesia adamantium; anti-Mycobacterium tuberculosis; flavonoids.
Evaluation of anti-Mycobacterium tuberculosis activity of Campomanesia adamantium

50 μL. All chromatographic analysis was performed at 22 °C.

Spectroscopy-grade acetoniite and methanol were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore). The solvents employed in the other analyses were of analytical-grade.

Extraction and fractionation procedures

Fresh fruits material (852.0 g) was extracted at room temperature sequentially with ethyl acetate and methanol for 48 h with each solvent. Extracts were filtered and concentrated under vacuum, yielding the ethyl acetate (4.5 g) and methanol (11.0 g) extracts. A portion of the ethyl acetate extract (1.2 g) was fractionated by gel permeation column chromatography (GPC) (Sephadex LH-20) using ethyl acetate as solvent at 0.5 mL/min flow rate. Forty-five fractions of 7 mL were collected. The fractions were combined according to their behavior on thin layer chromatography (TLC) and resulted in 9 samples: F1 (48 mg), F2 (45 mg), F3 (97.4 mg), F4 (93 mg), F5 (156 mg), F6 (185.6 mg), F7 (195.0 mg), F8 (292.5 mg) and F9 (78.5 mg). These fractions were submitted to HPLC-DAD-UV analysis and tested against M. tuberculosis.

The compounds employed as standards were isolated from C. adamantium leaves (compounds 1-5) and C. pubescens fruits (compound 6), and used in the form of methanol solutions. The standards used for HPLC analysis were purified until purity suitable for this purpose. The ethanol extract of the leaves from C. adamantium was partitioned with water/hexane (1:1 v/v) and water/ethyl acetate (1:1 v/v), getting the hexane and ethyl acetate phases. The ethyl acetate phase was fractionated by GPC (Sephadex LH-20) by elution with methanol/ethyl acetate isocratic system. The substances 1-5 obtained were further purified by column chromatography on silica gel eluted with toluene/ethyl acetate/ethanol gradient system or on RP 18 column eluted with water/acetonitrile/methanol gradient system. Compound 6 was obtained from the hexanic extract of C. pubescens fruits. This extract was fractionated by column chromatography on silica gel, using hexane/ethyl acetate/ethanol as gradient solvent system. The fraction containing the substance 6 was purified by another column chromatography on silica gel (toluene/ethyl acetate/ethanol gradient system). The structures of the flavonoids 1-6 were unambiguously determined by means of spectroscopic methods (1H, 13C and 2D NMR experiments COSY, HSQC, HMBC and NOESY) and compared to those previously reported3,14. Compounds 5 and 6 were also tested against M. tuberculosis. The 1H and 13C NMR results and peak assignments are given below.

Quantification of flavanones and chalcones

The estimation of flavanones and chalcones content in the samples was performed by external calibration. The standards were dissolved separately in spectroscopy-grade methanol in order to obtain stock solutions, which were diluted into 5 concentrations for each one. Aliquots (50 μL) of all dilutions were analyzed by HPLC-DAD (n=3). For each standard, the mean areas under the chromatogram peaks, for the different concentrations, were plotted versus the concentration values. The equation parameters (slope and interception) of the linear least squares regression of the plots were used to obtain concentration values for the samples. The results are expressed as mg pure substance/g fraction (Table 1).

Stability study

The stability of the working standard solutions was tested at 22 °C (working temperature) and −20 °C (storage temperature). The stability
of relevant components in the samples was evaluated during all the storage steps (i.e. at room temperature and at -20°C). Good stability was defined as less than 2% loss of the initial drug concentration in the stated time.

**Determination of anti-*M. tuberculosis* activity**

The anti-*M. tuberculosis* activity of extracts and fractions was determined using the Microplate Alamar Blue Assay (MABA) as analytical method. Stock solutions of the tested compounds were prepared in dimethyl sulfoxide and diluted in broth medium Middlebrook 7H9 (Difco), supplemented with oleic acid, albumin, dextrose, and catalase (OADC enrichment - BBL/Becton-Dickinson, Sparks, MD, USA), to obtain final drugs concentration ranges of 0.15 to 1600 \( \mu \text{g/mL} \). The isoniazid was solubilized with distilled water according to the manufacturers’ recommendations (Difco laboratories, Detroit, MI, USA) and used as standard drug. Suspensions of *M. tuberculosis* \( \text{H}_3\text{Rv} \text{ ATCC 27294} \) were prepared so that their turbidities matched with standard scale McFarland no. 1. They were further diluted 1:25 in broth medium Middlebrook 7H9 (Difco) supplemented with OADC and added to each well of microtiter plate, together with the essayed compounds. Minimal inhibitory concentration (MIC) of these compounds necessary to inhibit 90% of growth of *M. tuberculosis* \( \text{H}_3\text{Rv} \text{ ATCC 27294} \) were determined in triplicate in sterile 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, NJ), by reading the fluorescence with excitation at 530 nm and emission at 590 nm in a SPECTRAfluor Plus (Tecan) microfluorimeter. For standard test, the MIC value of isoniazid was determined each time. The acceptable MIC of isoniazid ranges from 0.015 to 0.05 \( \mu \text{g/mL} \).

**RESULTS AND DISCUSSION**

The ethyl acetate and methanolic extracts from *C. adamantium* fruits and 9 fractions (F1-F9) of ethyl acetate extract were prepared as described in the Experimental, and analyzed by HPLC (Figure 1). Table 1 shows the identified compounds in the fractions of the ethyl acetate extract of *C. adamantium* fruits.

Identification and quantification of compounds were performed by NMR spectroscopy and HPLC analysis, respectively, resulting in: 7-hydroxy-5-methoxy-6-C-methylflavanone (1), 5,7-dihydroxy-6-C-methylflavanone (2), 5,7-dihydroxy-8-C-methylflavanone (3), 2’,4’-dihydroxy-6’-methoxychalcone (4), 5,7-dihydroxy-6, 8-di-C-methylflavanone (5), 2’,4’-dihydroxy-3’,5’-dimethyl-6’-methoxychalcone (6), with purities of 93 to 97% (Figure 2 and Table 1).

HPLC analysis showed baseline separation for the compounds of interest. The identification of 1-6 was made by comparison with retention times of standards and by adding standards to the samples. A diode array detector was employed in the determination of compounds and in the evaluation of interference in all peaks of the chromatograms. Average standard errors for the peak areas of replicate injections (n=3) were smaller than 2%, showing good repeatability of the calibration curves. The peak areas were determined at different wavelengths, according to the compound: 284 nm for the species 1, 296 nm for 2 and 3, 350 nm for 4, 299 nm for 5, and 340 nm for 6.

The anti-*M. tuberculosis* \( \text{H}_3\text{Rv} \text{ activity} \) was evaluated (Table 1) for extracts, fractions and compounds 5 and 6. Tosun et al. considered inactive the plant extracts that could not prevent growth of *M. tuberculosis* up to concentration of 200 \( \mu \text{g/mL} \), and according to Gu et al. a sample with MIC value \( \leq128 \mu \text{g/mL} \) is defined as active. In this sense, we considered a promising result the MIC of 62.5 \( \mu \text{g/mL} \) found in ethyl acetate crude extract. Within the 9 fractions from ethyl acetate extract, F6 and F7 presented good inhibition activity on the growth of *M. tuberculosis*, with MIC of 39.1 \( \mu \text{g/mL} \) (Table 1).

The fractions F1 to F3, with MICs >250 and equals to 125 and \( \leq25 \) \( \mu \text{g/mL} \), respectively, did not show any of the 1 to 6 quantified compounds. The fraction F4 showed 34.7 mg/g of the compound 6 (2’,4’-dihydroxy-3’,5’-dimethyl-6’-methoxychalcone) and a MIC of 125 \( \mu \text{g/mL} \). The fractions F5, F6 and F7 showed a mixture of 5 and 6 (5,7-dihydroxy-6, 8-di-C-methylflavanone). The MIC of 62.5 \( \mu \text{g/mL} \) was verified for F5 and of 39.1 \( \mu \text{g/mL} \) for F6 and F7. The greater MIC value for F5 may be explained by the presence of compound 5 in lower concentration for F5 than for F6 and F7. The fractions F6 and F7 presented the best results against *M. tuberculosis* \( \text{H}_3\text{Rv} \text{ ATCC 27294} \). Both contain the greatest quantities of compounds 5 and 6, respectively 5,7-dihydroxy-6, 8-di-C-methylflavanone (5) and 2’,4’-dihydroxy-3’,5’-dimethyl-6’-methoxychalcone (6), considered ensemble. The chalcone seems to have the greater activity, because F8, with high content of flavanone and less chalcone, has a MIC greater than F6 and F7.

**Figure 2. Structures of the compounds identified in fractions of ethyl acetate extract from *C. adamantium*. Compounds: 7-hydroxy-5-methoxy-6-C-methylflavanone (1), 5,7-dihydroxy-6-C-methylflavanone (2), 5,7-dihydroxy-8-C-methylflavanone (3), 2’, 4’-dihydroxy-6’-methoxychalcone (4), 5,7-dihydroxy-6, 8-di-C-methylflavanone (5), 2’,4’-dihydroxy-3’,5’-dimethyl-6’-methoxychalcone (6)**

Compounds 5 and 6 were tested separately and exhibited MIC >250 and equal to 62.5 \( \mu \text{g/mL} \), respectively, while their mixtures, in several ratios, showed different MICs according to the ratio. A MIC of 62.5 \( \mu \text{g/mL} \) was verified for the mixture (5+6) in ratio 8:2, a MIC of 15.6 \( \mu \text{g/mL} \) for the ratios 3:7 and 1:1, a value of 31.25 \( \mu \text{g/mL} \) for the ratio 7:3 and the better activity, namely 7.8 \( \mu \text{g/mL} \), for the ratio 3:7. It is clear the occurrence of a synergism between compounds 5 and 6 when mixed, especially in certain ratios.

The F9 showed a MIC of 62.5 \( \mu \text{g/mL} \) and this fraction contains a complex mixture of compounds from 1 to 6, identified as: 7-hydroxy-5-methoxy-6-C-methylflavanone (1), 5,7-dihydroxy-6-C-methylflavanone (2), 5,7-dihydroxy-8-C-methylflavanone (3) and...
Table 1. MIC values and identified compounds of bulk extracts and fractions of C. adamantium fruits. F1 to F9: fractions of ethyl acetate extract

<table>
<thead>
<tr>
<th>Samples</th>
<th>MICs (μg/mL)</th>
<th>Compounds (mg/g of fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol extract</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>ethyl acetate extract</td>
<td>62.5</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>&gt; 250</td>
<td>ND</td>
</tr>
<tr>
<td>F2</td>
<td>125</td>
<td>ND</td>
</tr>
<tr>
<td>F3</td>
<td>125</td>
<td>ND</td>
</tr>
<tr>
<td>F4</td>
<td>125</td>
<td>6 (34.7)</td>
</tr>
<tr>
<td>F5</td>
<td>62.5</td>
<td>5 and 6 (43.7 and 247.3)</td>
</tr>
<tr>
<td>F6</td>
<td>39.1</td>
<td>5 and 6 (123.7 and 330.0)</td>
</tr>
<tr>
<td>F7</td>
<td>39.1</td>
<td>5 and 6 (147.9 and 290.3)</td>
</tr>
<tr>
<td>F8</td>
<td>125</td>
<td>5 and 6 (349.0 and 147.6)</td>
</tr>
<tr>
<td>F9</td>
<td>62.5</td>
<td>1, 2, 3, 4 (53.7, 175.0, 60.4 and 12.3)</td>
</tr>
</tbody>
</table>

ND= Not detected. Compounds: 7-hydroxy-5-methoxy-6-C-methylflavanone (1), 5,7-dihydroxy-6-C-methylflavanone (2), 5,7-dihydroxy-8-C-methylflavanone (3), 2', 4'-dihydroxy-6'-methoxychalcone (4), 5,7-dihydroxy-6, 8-di-C-methylflavanone (5), 2', 4'-dihydroxy-3', 5'-dimethyl-6'-methoxychalcone (6).

2', 4'-dihydroxy-6'-methoxychalcone (4).

In respect of the identified compounds, chalcones have been reported to possess many therapeutic properties, including anti-inflammatory, antimicrobial, antifungal, antioxidant, cytotoxic, anti-tumor and anticancer activities. M. tuberculosis, M. avium and M. bovis are strongly inhibited by licochalcone obtained from Glycyrrhiza inflata (Fabaceae), with MICs in the range of 5-20 μg/mL. A number of Glycyrrhiza species are used as throat demulcents.

The literature reports that 2', 4'-dihydroxy-3', 5'-dimethyl-6'-methoxychalcone, isolated from Dalea versicolor, exhibit, alone and in synergy with known antibiotics (berberine, erythromycin and tetracycline), activity towards the human pathogen Staphylococcus aureus and the opportunistic Bacillus cereus.

Salem and Werbovetz described leishmanicidal and trypanocidal activities of this same chalcone isolated from Psorothamnus polydenius. Lin et al. synthesized and evaluated a series of flavonols, flavonones and chalcones for inhibitory activity against M. tuberculosis H₃₇Rv. The authors also relate that the presence of a halogen substituent on ring A of 2'-hydroxychalcone led to an increase in anti-tubercular activity. Compounds with a halogen at the 3-position demonstrate stronger anti-M. tuberculosis activity than those with a halogen substituent at the 2- or 4-position. Chalcones with a 2'-hydroxyl group in ring B and a 3-chloro- or 3-iodo-group in ring A have the highest activity, inhibiting 90 to 92% growth of M. tuberculosis H₃₇Rv at a drug concentration of 12.5 mg/mL. The activity of 2'-hydroxychalcone (61% inhibition) was enhanced by adding a chlorine atom (89%) or a methoxy group (78%) to 4'-position of ring B. Introduction of an additional substituent, such as methoxy, amino, bromo or carboxyl groups, on ring B led to a dramatic decrease or a complete loss of activity.

According Cantrell et al., compounds that exhibit a MIC of 64 μg/mL or lower are considered promising. In this sense, MIC of 39.1 μg/mL for F6 and F7 and 62.5 μg/mL for F5 and F9 are very interesting, because these samples are enriched fractions, not isolated compounds. On the other hand, the mixture of pure compounds 5 and 6 in a ratio of 2:8 showed a very promising antitubercular activity with MIC of 7.8 μg/mL.

These MIC values are very larger than the MIC of 0.03 μg/mL of the reference drug Isoniazid, but are comparable or better than the MIC of pyrazinamide (20-100 μg/mL), another first-line anti-tuberculosis drug.

In conclusion, the bioassay-directed fractionation of the ethyl acetate extract from C. adamantium fruits yielded fractions which promising anti-TB activity. The compounds identified within these fractions, namely, 7-hydroxy-5-methoxy-6-C-methylflavanone (1), 5,7-dihydroxy-6-C-methylflavanone (2), 5,7-dihydroxy-8-C-methylflavanone (3), 2', 4'-dihydroxy-6'-methoxychalcone (4), 5,7-dihydroxy-6, 8-di-C-methylflavanone (5), 2', 4'-dihydroxy-3', 5'-dimethyl-6'-methoxychalcone (6), probably contribute for this activity against M. tuberculosis. The synergism between the compounds 5 and 6 depends on their concentration ratio.

ACKNOWLEDGEMENTS

We thank to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for grants to F. R. Pavan (process n°06/50680 and 2008/10390-2) and for funding from the Biota-Fapesp Program (process n°02/0550-6). We thank to CNPq for grants to R. G. Coelho. We thank to FUNDECT (Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul) for financial support to C. A. L. Cardoso and R. G. Coelho. We also thank M. Sobral for the identification of C. adamantium.

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