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Investigation of the antimycobacterial activity of 36 plant extracts from the brazilian Atlantic Forest

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Thirty-six plant extracts from the brazilian Atlantic Forest were tested for their antimycobacterial activity against Mycobacterium tuberculosis *$H_{37}Rv$ and* M. kansasii*, using the method REMA in seriate concentrations of 100 to 0.20 µg/mL. Among the thirty six extracts tested, five were active against* M. tuberculosis*, and three of these extracts also showed activity against* M. kansasii*. Cytotoxicity test with VERO cells was performed with the five extracts active against* M. tuberculosis*. Only the extract of* Peschiera affinis *was identified as non-toxic in the concentration of 100µg/mL.*

Uniterms
- *Mycobacterium tuberculosis*
- *Mycobacterium kansasii*
- Plant extracts/ antimycobacterial activity
- Antimycobacterials
- Cytotoxicity

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INTRODUCTION

Tuberculosis (TB) is considered one of the main causes of death worldwide. There were nine million new TB cases and approximately two million TB deaths in 2004 (WHO, 2006). The available treatment requires a long lasting (at least six months) multi-drug scheme, which causes difficulties to the patient's adhesion. Furthermore the available drugs do not assure the sterilization of the lesion, being possible that, in spite of the clinical cure the bacillus stays in latent state inside the macrophages. Associated to these factors is the increase of the number of cases of TB with resistant and multi-drug resistant (MDR) strains and the insufficient therapeutic arsenal available for the treatment, which stimulates the development of new anti-TB drugs (Silva *et al.,* 2006).

In addition to that, some species of non-tuberculous mycobacteria (NTM) have increased their clinical importance in the last years. *M. kansasii* causes both pulmonary and extra-pulmonary diseases and ranked second behind *Mycobacterium avium* complex as the most common MNT infections (Graybill, Bocanegra, 2001; Telles *et al.,* 2005). The current treatment of pulmonary diseases caused by *M. kansasii* in non-HIV-infected patients includes isoniazid, rifampicin and ethambutol. Nevertheless, alternative drugs have been proposed for patients infected with *M. kansasii* resistant to rifampicin and AIDS patients treated with HIV protease inhibitors, since rifampicin accelerates the hepatic metabolism of these drugs, rendering them potentially ineffective (Guna *et al.,* 2005).

Since ancient times, natural products notably those from plant origin, have consistently been an important source of therapeutic agents. Currently, about 25-30% of all drugs available as therapeutics are derived from natural products (plants, microbes and animals). In spite of that, in the last decades, due mainly to the advance of combinatorial chemistry, research into natural products in

the pharmaceutical industry has experienced a slow decline (Calixto, 2005; Newman *et al.,* 2003). The search for new pharmacologically active agents obtained by screening natural sources such as microbial and plant extracts has led to the discovery of many clinical useful drugs that play a major role in the treatment of human diseases (Shu, 1998).

Several compounds with antimycobacterial activity have been found among plants, fungi and marine organisms (Lewis, 1999; Pauli *et al.,* 2005). Over 350 natural products, mainly plant species, which have been used in traditional medicine, have been assessed for their antimycobacterial activities (Newton *et al.,* 2000). A number have been shown to demonstrate significant *in vitro* antimycobacterial activities and active plant-derived compounds belonging to various chemical classes have been isolated. These findings have therefore stimulated further search towards the isolation of new antimycobacterial agents from natural products (Newton *et al.,* 2002). This products form one avenue in the search for new antitubercular agents, with many groups undertaking screening of natural product extracts as the preliminary step to finding new lead compounds, as for combinations of cost, health and throughput issues, there are a wide range of mycobacterial test organisms and biological assay methodologies utilized in current day antimycobacterial drug discovery (Copp, 2003).

Likewise, different kinds of studies on the mechanisms of action, interactions with antibiotics or other medicinal plants or compounds, and the pharmacokinetic profile of the extracts should be given high priority (Ríos, Recio, 2005).

Natural products chemistry is a research area with unlimited potential, important to countries rich in plant species and which are abundant in native vegetation like Brazil. This country is one of the two richest countries on earth in terms of biodiversity and has two of the 25 world's hotspots threatened of disappear (Mittermeier *et al.,* 1998). The Atlantic Forest region ranks among the top five of these hotspots and only 7.5% of the original extent of the Atlantic Forest remain intact. Given the fast devastation of the environment the screening of plant secondary metabolites for the search of biological activities becomes an urgent task (Calixto, 2005).

In this study we evaluated the antimycobacterial activity of 36 plant extracts belonging to 19 different botanical families (Anacardiaceae, Apocynaceae, Bignoniaceae, Bombacaceae, Caesalpiniaceae, Euphorbiaceae, Fabaceae, Flacourtiaceae, Lecythidaceae, Mimosaceae, Meliaceae, Moraceae, Myristicaceae, Nyctaginaceae, Rubiaceae, Rutaceae, Sapotaceae, Siparunaceae, Tiliaceae) selected from the Atlantic Forest.

MATERIALS AND METHODS

Plant material

Plants were collected from two Atlantic Forest fragments (Bela Fama Forest - Santana do Deserto city, MG and Boa Vista Forest - Levy Gasparian city, RJ), Brazil. Taxonomic identifications were done by Sebastião J. da Silva Neto from Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, Brazil. Voucher specimens are deposited at the Herbarium of the Federal University of Rio de Janeiro.

Preparation of extracts

The air-dried and powdered leaves (20 g of each) were exhaustively extracted with ethanol 96° GL. The obtained extracts were filtered and evaporated under reduced pressure on a rotary evaporator.

Isolates and strain preparation

The antimicrobial activity of the extracts was evaluated against *M. tuberculosis* H_{37}Rv (ATCC 27294) and *M. kansasii* (ATCC 12478) maintained on Ogawa medium for about 14 days. The bacterial suspensions were prepared in sterile water containing beads of glass of 3 mm. The suspension was homogenized by vortex agitation and the turbidity was adjusted in agreement with tube one of the scale of McFarland (3,2 x 10^6 cfu/mL). The inoculum was prepared diluting the bacterial suspension in the proportion of 1:25 in medium 7H9 broth (4.7 g of Middlebrook 7H9 broth base [Difco - Becton Diskinson], 2 mL of glycerol [Vetec ltda.] in 900 ml water) enriched with 10% oleic acid, albumin, dextrose and catalase (OADC -BBL) (Franzblau *et al.,* 1998).

Evaluation of the antimycobacterial activity of the extracts

The method used for the determination of the antimycobacterial activity was the REMA (Palomino *et al.,* 2002). The screening assay was, in brief, accomplished in microplates (96 wells) using the resazurin as indicator of cellular viability. Seventy-five microlitres of medium 7H9 enriched with 10% OADC was used in each well, after was added 75 µL of the extracts were weighted dissolved in DMSO at a concentration of 100 µg/mL and 75 µL of the *Mycobacterium* inoculum. The Minimal Inhibitory Concentration (MIC) was determined for extract serial dilutions (starting for 100 µg/mL) using 100 µL of medium

7H9, 100 µL of extract and 100 µL of inoculum. The MIC was did just for those extracts presenting antimycobacterial activity at 100 µg/mL in the screening assay. We establish MIC 100 µg/mL as active because the MIC of a crude natural extract may or may not be a reliable indicator of the chances for success in isolating a potent antimycobacterial agent from that extract. The possibility exists that (i) an extract with a relatively low MIC (high activity) may contain large quantities of only very few moderately active major constituents, while (ii) moderately active crude materials could lead to minor compounds with high activity (Pauli *et al.,* 2005).

Cytotoxicity assay

The citototoxicity of the extracts with antimycobacterial activity were determined with VERO cells (Case *et al.,* 2006). Concisely, the VERO cells were cultivated at 37 ºC in a humid atmosphere with 5% CO_2 (Sánchez *et al.,* 2002) in modified DMEM medium (10,4 g DMEM, 900 ml water MilliQ, 2,0g $NaHCO_3$ - Sigma D5671) complemented with 10% Bovine Fetal Serum (FCS), in order to obtain about 1 to $2x10^5$ cell/mL (Sivropoulou *et al.,* 1997). The extracts were serial diluted (1:2) from an initial concentration of 100µg/ml to a final concentration of 3µg/mL. The number of cells was determined in a Neubauer's chamber, while the percentage of viable cells, was measured with trypan blue. Statistical analysis to determine LD_{50} was accomplished through the PROBIT method.

RESULTS AND DISCUSSION

Thirty six plant extracts from the Brazilian Atlantic Forest (*Anadenanthera colubrina, Aparisthmium cordatum, Apuleia leiocarpa, Astronium fraxinifolium, Bathysa australis, Bombacopsis stenopetala, Brosimum guianense, Cariniana estrellensis, Carpotroche brasiliensis, Casearia sylvestris, Cedrela fissilis, Croton floribundus, Dalbergia nigra, Ficus gomelleria, Guapira opposita, Guettarda virburnoides, Luehea grandiflora, Mabea fistulifera, Malouetia arborea, Melanoxylon brauna, Pera heteranthera, Pera leandri, Peschiera affinis, Piptadenia gonoacantha, Plathymenia foliolosa, Pouteria filipes, Psychotria vellosiana, Senifeldera multiflora, Simira glaziovii, Simira sampaioana, Siparuna guianensis, Siparuna reginae, Sorocea bonplandii, Sparattosperma leucanthum, Zanthoxylum rhoifolium, Virola oleifera)* were tested for their antimycobacterial activity at the concentration of 100 µg/mL in order to select the most active ones (Table I). Five extracts: *Psychotria vellosiana; Pouteria filipes, Cedrela fissilis, Plathymenia foliolosa* and *Peschiera affinis* were active against *M. tuberculosis* with MIC between 0.2 and 3.12 µg/mL (Table II). Concerning *M. kansasii,* three of these extracts (*Cedrela fissilis, Plathymenia foliolosa* and *Peschiera affinis*) were also active, however with MIC 100 µg/mL (Table II). Other ten extracts were active only against *M. kansasii*: with MIC of the 100 µg/mL (*Siparuna arianeae* (Siparunaceae); *Guettarda virburnoides* (Rubiaceae)*; Cariniana estrellensis* (Lecythydaceae); *Sparattosperma leucanthum* (Bignoniaceae); *Brosimum guanense* (Moraceae)), 50 µg/mL (*Siparuna reginae* (Siparunaceae); *Piptadenia gonoacantha* (Fabaceae); *Ficus gomelleria* (Moraceae)); 25 µg/mL (*Aparisthmium cordatium* (Euphorbiaceae)) and 1,56 µg/mL (*Senifildera multiflora* (*Euphorbiaceae*)). The five extracts active against *M. tuberculosis* belong to plants used in Brazilian Folk Medicine (1, 3, 10, 12). Even though a larger number of extracts showed activity against *M. kansasii*, in general, their MIC were higher than those for *M. tuberculosis* (Table II). In fact, environment microorganisms show a higher level of natural resistance than classical pathogens.

The cytotoxicy of the five extracts active against *M. tuberculosis* was evaluated showing that one (*Peschiera affinis*) was not toxic to VERO cells in the concentration of 458 µg/mL (Figure 1). The extract of *Peschiera affinis*, besides not being toxic for the VERO cells in a higher concentration, also presented a low MIC against *M. tuberculosis.*

The presence of indol alkaloids, commonly found in plants of the families Rubiaceae and Apocynaceae (Schripsema *et al.,* 2003) could explain the activity of the extracts of *Psychotria vellosiana* and *Peschiera affinis*

TABLE I - MIC of active extracts of the Atlantic forest against to *M. tuberculosis* and *M. kansasii*

	Nº of the extracts with MIC of the (µg/mL)							
	0.20	0.78	1.56	3.12	25	50	100	>100*
M. tuberculosis	2	2		1				31
M. kansasii			1		1	3	8	23

* No active extract.

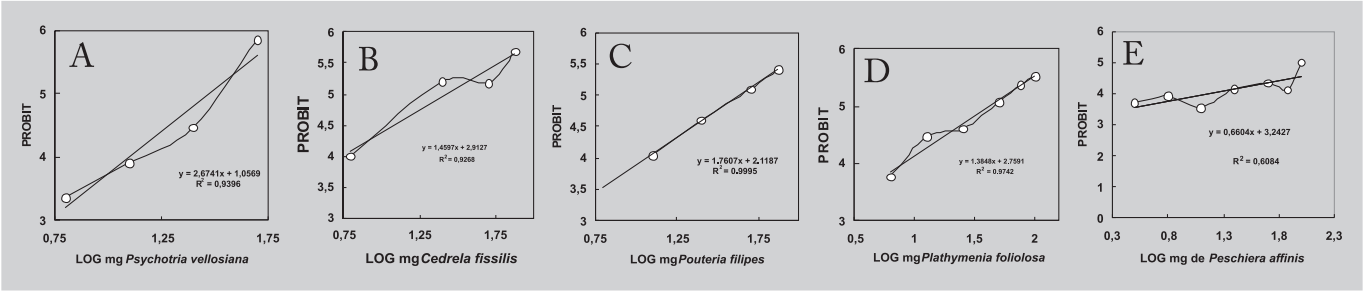

FIGURE 1 - Cytotoxicity graphs, (A) Cytotoxicity test of *Psychotria vellosiana,* (B) Cytotoxicity test of *Cedrela fissilis*, (C) Cytotoxicity test of *Pouteria filipes*, (D) Cytotoxity test of *Plathymenia foliolosa*, (E) Cytotoxicity test of *Peschiera affinis*.

TABLE II - Results of MIC of five vegetable extracts of the Atlantic forest against to *M. tuberculosis* and *M. kansasii*

	M. tuberculosis	*M. kansasii*
Psychotria vellosiana (Rubiaceae)	≤ 0.20 µg/mL	> 100 µg/mL
Cedrela fissilis (Meliaceae)	3.12 µg/mL	100 µg/mL
Pouteria filipes (Sapotaceae)	0.78 µg/mL	> 100 µg/mL
Plathymenia foliolosa (Fabaceae)	0.78 µg/mL	100 µg/mL
Peschiera affinis (Apocynaceae)	≤ 0.20 µg/mL	100 µg/mL

against mycobacteria. Phytochemical analyses of plants of the genus *Psychotria* demonstrated the presence of psychotridines, which have been associated with dose-dependent analgesic effects (Amador *et al.,* 2001). *Peschiera affinis* as well as other Apocynaceae plants are used in northeast Brazil as antitumoral and spasmolytic drugs (Miranda *et al.,* 2003).

The family Maliaceae, represented in this study by *Cedrela fissilis* presents modified triterpenes (limonoids) and inhibitory activity has been demonstrated in the enzyme Leishmania APRT (Adenine Phosphoribosyl Transferase), present also in *M. tuberculosis* genome (Ambrozin *et al.,* 2005). This enzyme has an important function in the pathway of purine salvation, which turns this enzyme an interesting target to antimicrobial agents (Zottis *et al.,* 2006).

Recently, an extract of *Plathymenia foliolosa* showed antimicrobial activity against *E. coli* (Alves, 2000), allowing to infer that this plant could be active against other microorganisms.

These results suggest that *Peschiera affinis* has active compounds against *M. tuberculosis* and *M. kansasii* which are not toxic to human cells. Further purification studies on the extract of this plant are being carried out in order to identify the active compounds.

RESUMO

Análise da atividade antimicobacteriana de 36 extratos vegetais da Mata Atlântica brasileira

Trinta e seis extratos vegetais originários da Mata Atlântica foram testados quanto à sua atividade antimicobacteriana frente ao M. tuberculosis $H_{37}Rv$ *e* M. kansasii*, utilizando o método REMA em concentrações seriadas de 100 a 0,20 µg/mL. Dentre os trinta e seis extratos testados, cinco mostraram atividade frente ao* M. tuberculosis*, e destes apenas, três mostraram atividade ao* M. kansasii*, que apresentou susceptibilidade a outros dez. O teste de citotoxicidade com células VERO foi realizado com os cinco extratos ativos frente ao* M. tuberculosis *em que identificou-se a não toxicidade em apenas um extrato (*Peschiera affinis*) na concentração de 100 µg/mL.*

UNITERMOS: Mycobacterium tuberculosis. Mycobacterium kansasii. *Extratos vegetais/atividade antimicobacteriana. Antimicobacterianos. Citotoxicidade.*

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