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Antimycobacterial activity of Brazilian Amazon plants extracts

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Abstract

Tuberculosis (TB) is one infectious disease responsible for more than 2 million of deaths worldwide. The increase of TB cases resistant to drugs normally used in treatment has reinforced the necessity of development of new antimicrobials, which should be active to resistant strains and latent bacilli, further it should reduce the treatment duration. Thus fifty-six plants extracts obtained from Brazilian Amazon forest were tested in three strains of *Mycobacterium tuberculosis*, being one pan-susceptible strain ($H_{37}R_v$), one isoniazid resistant and one rifampicin resistant. Twenty-nine plants extracts were active against pan-susceptible strain, twenty-four against isoniazid resistant and thirteen against rifampicin resistant. These results indicate the potential of the Brazilian Amazon plants products as source of new antimicrobials.

Keywords: *Mycobacterium tuberculosis*; microbial resistance; plant extract.

Introduction

One third of the worldwide population is infected with the *Mycobacterium tuberculosis*, etiological agent of tuberculosis (TB)[1].In 2010, there were about 8.8 million incident cases of TB, around 128 cases/100,000 habitants, including 1.1 million deaths in HIV-negatives and 350 thousand in HIV-positives[1]. In Brazil, were reported 81.570 new TB cases in 2011[2]. These data corroborates with the affirmative that TB is a serious problem of public health[1].

In Brazil, the treatment regimen has duration of six months and consists in a drug combination including isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and pyrazinamide (PZA) for two months and INH and RMP, for more four months[2]. However, the lengthy six month therapy makes patient compliance difficult, and noncompliance is frequently associated with drug-resistant, multidrug-resistant (MDR, resistant at least to INH and RMP) and extensively drug-resistant (XDR, a MDR resistant also to a fluoroquinolone and an injectable drug, as amykacin, kanamycin or capreomycin) strains[1].This picture added to the fact that no new specific antimicrobial for TB were introduced in the last 40 years, reinforce the necessity to develop new drugs that must be more efficient against resistant strains, reduce the treatment duration and act against latent bacilli[3].

Most of XIX century pharmacopoeia were constituted of animal, mineral and specially plant products, responsible for the majority of drugs which were just a little bit different from those used in popular medicine and were qualitatively the more important part of therapeutical researches in that period[4].In the beginning of last century, the study of active substances presents in plants boosted a scientific and technological revolution.

The development of drugs derived from natural products became possible with the advancement of technologies associated with combinatorial chemistry, biotechnology and genomics knowledge[5].Even with the development in the areas of organic synthesis, industrial microbiology and molecular biology, many drugs continued to be obtained from natural sources, maybe because the difficulty of obtain molecules with same stereochemistry or the economic viability of producing them synthetically[6,4].

A large number of plant species are founded in South America equatorial areas.[7] In Brazil is the largest plant genetic diversity of the world, once in the Amazon, can be founded 17% of Brazilian diversity[8]. Data of 2002 indicate that plants medicinal therapy moves a market around 500 million U.S. dollars only in Brazil[9] and the demand for new drugs derived from plant genetic resources arouses great interest[10].

Given the need for news drugs that act more effectively to control TB and chemical diversity of plant origin products, this study evaluated the antimycobacterial activity of various plants extracts belonging to Brazilian Amazon flora.

Material and Methods

Plant species collected

The plants were collected near the Manaus city- Brazil and were chosen to be of plant families for which there are reports in the literature to produce substances with biological activities (Table 1).

Table 1: Plant species collected

Family	Plant Specie	Collect site	Date
Fabaceae	*Campsiandracomosa*	Lago do Catalão, AM	02/2005
	*Campsiandra*sp	Lago do Catalão, AM	02/2005
		Volta Grande do Xingu	02/2007
	Degueliaduckeana	Praia Dourada, AM	09/2005
Moraceae	*Ficus*sp	Araguanã, MA	01/2007
Olacaceae	*Minquartiaguianensis*	ReservaDucke, AM	04/2005
Rubiaceae	*Duroiamacrophylla*	PresidenteFigueiredo, AM	05/2005
	Duroiasaccifera	ReservaDucke, AM	10/2005
	Ferdinandusa hirsute	ReservaDucke, AM	10/2005
	Ferdinadusarudgeoides	ReservaDucke, AM	10/2005
	*Ferdinadusa*sp	ReservaDucke, AM	10/2005
	Palicoureacorymbifera	ReservaDucke, AM	10/2005
	Palicoureaguianensis	ReservaDucke, AM	10/2005
Rutaceae	*Zanthoxilum*sp	Lago do Catalão, AM	02/2005
Salicaceae	*Salix martiana*	Lago do Catalão, AM	07/2004
Verbenaceae	*Lippiamicrophylla*	Boa Vista, RR	09/2005
	Stachytarphetacayennensis	Volta Grande do Xingu	02/2007
	Vitexcymosa	Lago do Catalão, AM	07/2004
Zingiberaceae	*Zingiberzerumbet*	Manaus, AM	not determined

Preparations of extracts

Each plant fraction was separated, powder and extracted, first with dichloromethane, using ultrasound for 20 minutes. The material was filtered and re-extracted with dichloromethane to exhaustive extraction. After this extraction, the plant material was dried at room temperature and then extracted with methanol, under the same conditions by exhaustive extraction. Finally, the plant material was extracted also with distilled water until exhaustive extraction. The dichloromethane and methanol extracts were concentrated on rota-evaporator and the aqueous in lyophilizer, totaling fifty-six extracts.

The extracts were prepared by the Laboratory Bioprospecting in the National Research Institute of Amazonian Research (INPA). The extracts were stored at 4 ^{0}C, solubilized in dimethylsulfoxide 99.5% at 10 mg/mL in the day of assay.

Preparation of inoculums

The activity of the extracts was tested against three strains of *M. tuberculosis*: a pan-susceptible H37Rv (ATCC 27294), a mono resistant INH (ATCC 35822) with a *kat*GS315T (AGC-ACC) mutation and other mono resistant RIF (ATCC 35338), with a *rpo*B H526Y (CAC-TAC) mutation. These strains were grown in Ogawa-Kudoh for approximately 14 days at a temperature of 37 C. A suspension of each bacterial strain was prepared in a sterile tube containing glass beads and homogenized with distilled water for adjusting equal to the McFarland standard 1. Then, the bacterial suspension was added to Middlebrook 7H9 broth in the proportion of 1:20[11].

Initial screening of extracts

The antimycobacterial activity of extracts was determined preliminarily by the Resazurinmicrotiter assay method (REMA), in a fixed concentration of 200 µg/mL, for the three strains of *M. tuberculosis* cited previously. To performed the screening were used a 96-well microplate, in each well was added 75 µL of Middlebrook 7H9 broth supplemented with OADC (oleic acid, albumin, dextrose, catalase), 75 µL of extract at 200µg/mL and 75µL of inoculum. The plates were incubated for seven days at 37 C and then 30 µL of resazurin were added to each well. The plates were reincubated at 37 C for two days and after this period the plates were readed[12]. For all tests were used antimicrobial, strains and medium controls.

Determination of Minimum Inhibitory Concentration

The extracts that showed less or equal activity to 200 µg/mL in the screening had minimal inhibitory concentration (MIC) determined also by the REMA method[12] as cited previously at a 1:2 dilution from the concentration of 200 µg/ml to 6.25µg/mL.

Results and Discussion

Among 56 extracts tested, 29 were active against the *M. tuberculosis* $H_{37}R_v$ strain with MIC between 50 and 200µg/mL. Against INHr strain, 24 extracts were active, with MIC between 12.5 and 200µg/mL, and 13 extract were active against RMPr strain with MIC between 25 and 200 µg/mL (Table 2).

Table 2: MIC of extracts actives against the strains of *M. tuberculosis* pan-susceptible, INHr and RMPr.

	Number of extracts with MIC of (µg/mL)					
	12,5	25	50	100	200	>200*
Pan-susceptible			3	7	19	27
INHr	2	1	5	8	8	32
RMPr		2	3	6	2	43

*extract considered inactive

The extracts of *Duroiamacrophylla, Ferdinandusarudgeoides, Ferdinadunsa* sp and *Palicoureaguianensis*(Rubiaceae) were active against *M. tuberculosis* RMPr strain, with MIC between 25 and 50µg/mL. The activity of these extracts may be associated with the possible presence of flavonoids, since this substance is present in these species, which is commonly found in Rubiaceae family species, whose antimicrobial activity against *Escherichia coli* has been demonstrated previously[13].

The extracts of Ficussp (Moraceae), Campsiandracomosa, Campsiandrasp (Fabaceae), Duroiamacrophylla, Duroiasaccifera, Ferdinandusahirsuta, Ferdinandusarudgeoides, Palicoureacorymbifera, Palicoureaguianensis (Rubiaceae) showed activity against *M. tuberculosis* INHr strain, with MIC between 50 and 200µg/mL.

Table 3: MIC of all plants extracts tested against the strain *M.tuberculosis* pan-susceptible, INHr and RMPr

Family	Specie	Part of plant	Solvent	Pan-susceptible [µg/mL]	INHr [µg/mL]	RMPr [µg/mL]
Fabaceae						
	Campsiandracomosa	Seed	H_2O	>200	>200	>200
		Bark	H_2O	>200	>200	>200
		Leaves	H_2O	>200	>200	>200
		Branch	H_2O	>200	>200	>200
		Bark	DCM	>200	>200	>200
		Leaves	DCM	>200	>200	>200
		Branch	DCM	>200	>200	>200
		Seed	DCM	>200	>200	>200
		Bark	MeOH	200	200	>200
		Leaves	MeOH	>200	>200	>200
		Seed	MeOH	>200	>200	>200
	Campsiandra sp	Branch	DCM	200	100	>200
		Branch	MeOH	200	100	>200
	Degueliaduckeana	Leaves	H_2O	>200	>200	>200
		Roots	H_2O	>200	>200	>200
		Leaves	DCM	200	>200	>200
		Branch	DCM	100	>200	>200
		Leaves	MeOH	>200	>200	>200
		Branch	MeOH	>200	>200	>200
Moraceae						
	Ficus sp	Bark	H_2O	200	>200	>200
		Leaves	H_2O	>200	>200	>200
		Branch	DCM	50	50	100
		Bark	MeOH	50	>200	>200
		Leaves	MeOH	>200	>200	>200
		Branch	MeOH	100	>200	>200
Olacaceae						
	Minquartiaguianensis	Leaves	DCM	200	>200	>200
		Branch	MeOH	200	>200	>200
Rubiaceae						
	Duroiamacrophylla	Leaves	H_2O	200	50	50
		Branch	DCM	200	100	>200
		Leaves	MeOH	100	100	>200

	Duroiasaccifera	Leaves	DCM	200	200	100
		Leaves	MeOH	>200	>200	>200
		Branch	DCM	200	100	>200
	Ferdinandusahirsuta	Leaves	DCM	100	50	>200
	Ferdinandusarudgeoides	Leaves	DCM	200	200	50
		Leaves	MeOH	>200	200	100
	Ferdinandusa sp	Leaves	DCM	200	200	25
		Leaves	DCM	100	100	100
		Branch	DCM	>200	200	100
		Leaves	MeOH	>200	>200	>200
	Palicoureacorymbifera	Leaves	DCM	200	100	100
	Palicoureaguianensis	Leaves	H_2O	>200	>200	>200
		Leaves	DCM	>200	>200	50
		Branch	DCM	200	200	>200
Rutaceae						
	Zanthoxilum sp	Bark	DCM	200	25	25
		Bark	MeOH	100	100	200
Salicaceae						
	Salix martiana	Branch	DCM	50	50	200
			MeOH	>200	>200	>200
Verbenaceae						
	Lippiamicrophylla	Leaves	DCM	200	200	>200
	Vitexcymosa	Flower	DCM	100	>200	>200
	Stachytarphetacayennensis	Leaves	H_2O	>200	>200	>200
		Roots	H_2O	>200	>200	>200
Zingiberaceae						
	Zingiberzerumbet	Roots	Hex	>200	12,5	>200
		Roots	H_2O	200	50	>200
		Leaves	DCM	200	12,5	>200
		Leaves	MeOH	>200	>200	>200

H_2O= wather ; DCM= dichloromethane; Hex= hexane; MeOH= methanol

Species of the families (*Piptadeniagonoacanth*(Fabaceae), *Brosimumguanense,* *Ficusgomelleria*(Moraceae), *Guettardavirburnoides*(Rubiaceae) were previously described with antimycobacterial activity with similar MIC found in this study[14].Some species of Fabaceae family have, among others compounds, flavonoids that can be responsible for the activity observed[15]. Others studies also analyzed the antimicrobial action of these family against *Cladosporiumsphaerospermum, Staphylococcus aureus, Bacillus cereus* and *Pseudomonas aeruginosa*[16].

With MIC between 25 and 200 µg/mL, the extracts of *Zanthoxilum*sp(Rutaceae) also were active against RMPr strain and may have the antimicrobial activity justified due to the presence of alkaloids and flavonoids[17]. These substance have already had their bioactivity previously proved as trypanocidal[18,19]. Others species of Rutaceae, also showed antimicrobial activity, that confirm the tendency of this family to synthesize secondary metabolites that are biologically active[20].

The extracts of *Zingiberzerumbet*(Zingiberaceae) were active against *M. tuberculosis* $H_{37}R_v$ and INHr strains with MIC of 200 and 12.5 µg/mL, respectively (Table 3). This family havecurcuminoids such as curcumina, which is commonly found in species of Zingiberaceae and can be responsible for the antimicrobial activity of these extracts. The curcumina is responsible for several pharmacological activities, including anti-inflammatory, antibacterial, antioxidant and nematicidal[21].

The Verbenaceae family, represented in this study by Vitexcymosa and Lippiamicrophylla showed activity against M. tuberculosis$H_{37}R_v$ and INHr strains, with MIC of 100 and 200g/mL. The extract activity of Verbenaceae family, against M. tuberculosis$H_{37}R_v$ strain has been observed in other studies based on etnopharmacology, which authors suggested that substances such as phytosterols may be involved in the antimycobacterial activity of extracts of the genus Vitex[22].Likewise, extracts of the genus Lippia, also showed antimicrobial activity against Staphylococcus aureus, Candida albicans and M. smegmatis[23].

The extracts of Minquartiaguianensis that were active only against M.tuberculosis$H_{37}R_v$ strain, with MIC of 200 µg/mL, ethnopharmacological are used in order to treat infections caused by intestinal parasites[24]. From this same specie has been isolated the minquartinoic acid, which was active against leishmaniasis and malaria[25], as well as triterpenoids that are known for their inflammatory[26] and probably antibacterial activity[27,28].

The extracts of *Salix martiana* were active against three *M. tuberculosis* strains with MIC of 50 and 200µg/mL. Recent studies isolated from *Salix martiana* and others species of this genus, substance such as salicin, which has antioxidant properties[29]and has an inhibitory action of tumor cells, since this substance induces apoptosis these cells[30]. From one specie of the genus Salix, was also isolated acetylsalicylic acid, which after some modifications gave origin to para-aminosalicylic acid, an agent with antibacterial activity used in combination with other drugs for the treatment of TB[31,2].

Extracts from Rutaceae and Rubiaceae families, were more active against strains of *M. tuberculosis* INHr and RMPr than against the $H_{37}R_v$ strain (Table 3). However, few extracts were active against the *M. tuberculosis* RMPr strain (Table 2). Mutations in H526Y of the *rpoB* gene from *M. tuberculosis* are among the most frequent in relation to high-level resistance to rifampicin. Strains with mutations in this codon are resistant not only to rifampicin, as well as their analogues[32,33].

The bioactivity of the extracts studied instigates research model for plant products as precursors of new drugs. Added to this the fact, Brazil is rich in plant biodiversity, which offers a wide range of products of economic importance, especially the herbal origin of plant genetic resources. However, the extracts may have active substance that are antagonized or potentiated in presence of other ones[34]. Thus, isolating the active substance, or a fraction of the crude extract could be obtained an higher activity of a given extract.

Further studies of these extracts, in order to identify the active substance and the mechanism of action are needed to confirm the potential of these plants as antimicrobial therapy candidates. Moreover, the molecular diversity of plants that confer a variety of structures with biological potential is an advantage of the natural products when compared to synthetic, as this generally complicates the process of synthesis.

Conclusions

Among fifty-six extracts tested against all strains of *M. tuberculosis*, twenty-nine were active against the H37Rv strain, twenty-four against the INHr strain and thirteen against RMPr strain. Products of plant origin are an important source of bioactive substances and Brazil has a rich plant biodiversity, it reinforces the need for studies to identify the potential of these products of plant origin, as well as the active ingredient responsible for its activities.

Authors' contributions

All authorsconceived of the study, participated in its design, read and approved the final manuscript. The Laboratório de Bioprospecção e Biotecnologia, InstitutoNacional de Pesquisas da Amazônia, carried out the plants collection and extraction. The Laboratório de Micobactérias, Faculdade de Medicina, Universidade Federal do Rio Grande contributed with the biological assays.

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