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Artigo Original

Plants from the Brazilian Cerrado with antimycobacterial effect

Plantas do Cerrado com efeito antimicobacteriano

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Abstract

Tuberculosis constitutes a serious public health problem because it has multi-resistant forms that require treatment that is both difficult and extensive. There is a need to develop new antimycobacterial compounds, and plants represent a source of therapeutic resources. This study analyzed antimycobacterial action in eight extracts from plants found in the Brazilian Cerrado. The results showed significant inhibitory concentrations in relation to *Mycobacterium tuberculosis*, especially from the extracts of *Apuleia molaris* and Ouratea spectabilis, both of which presented reduced cytotoxic effects. Fractionation revealed a hexane fraction of *A. molaris* with significant and promising activity regarding future in vivo assays.

Keywords: Cerrado; Public health; Tuberculosis

Resumo

A tuberculose representa um sério problema de saúde pública diante as formas multirresistentes, tratamentos extensivos e dificuldades à adesão. Há necessidade em desenvolver novos compostos antimicobacterianos, sendo as plantas uma fonte de recursos terapêuticos. Este estudo analisou ação antimicobacteriana em oito extratos de plantas presentes no Cerrado. Os resultados demonstraram expressivas concentrações inibitórias para *Mycobacterium tuberculosis* com destaque para *Apuleia molaris* e Ouratea spectabilis, ambos apresentaram reduzidos efeitos citotóxicos, cujo fracionamento relevou uma fração hexânica de *A. molaris* com atividade significativa e promissora para futuros ensaios in vivo.

Palavras-chave: Cerrado; Saúde Pública; Tuberculose

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1 Introduction

Tuberculosis (TB) is mainly caused by the *Mycobacterium tuberculosis* bacillus (PANDIT et al., 2015) and it represents a serious public health problem worldwide (BOTTGER, 2011). There were 10.4 million new ¬¬cases and 1.4 million deaths due to TB reported in 2015, which reflects an infectious disease with high mortality rates (WHO, 2016).

The high rates of incidence and prevalence of TB in developing countries have an impact on morbidity and mortality, particularly because the incidence of the human immunodeficiency virus (HIV) is also high (ALFFENAAR et al., 2017).

Increased numbers of cases of MDR-TB (multidrug-resistant tuberculosis) (PANDIT et al., 2015) and XDR-TB (extensively drug-resistant tuberculosis) (PARIDA et al., 2015) have made it difficult to control this disease because the latter do not adequately correspond to treatment (SINGH et al., 2015), as well as presenting higher toxicity and treatment costs (TIBERI et al., 2017b).

There has been scant development of new drugs to treat TB. This represents a challenge and new research is required in relation to factors such as new mechanisms of action (SINGH and MIZRAHI, 2017), reduced treatment period (SANDGREN et al., 2009), increased effectiveness regarding resistant strains (TIBERI et al., 2017a), and reduced adverse effects of treatment (KUETE, 2010).

Natural products, especially plants, are capable of providing innovative structures for the formulation of new drugs (SANTHOSH and SURIYANARAYANAN, 2013). They are important sources of bioprospecting for antimycobacterial compounds (PAULI et al., 2005), and trials involving plant extracts are increasingly more frequent (FIDELIS et al., 2014; MOLINA-SALINAS et al., 2010).

The Brazilian Cerrado is a promising biome because it contains specimens with pharmacological potential (NOVAES et al., 2013), expressive phytophysiological heterogeneity, and floristic richness (SANO et al., 2010). The Cerrado contains more than 12,000 plant species, approximately 30% of which are endemic to the region (FORZZA et al., 2010).

The Cerrado is considered to be the planet's richest tropical savannah in terms of biodiversity (SANO et al., 2010); it occupies an area of approximately 1,783 million square kilometers, which represents 22% of the Brazilian territory (JEPSON, 2005). However, increasing levels of agribusiness have had negative impacts on this biodiversity and resulted in changes in the water cycle (SPERA et al., 2016).

The Cerrado is considered to be a hotspot in terms of biodiversity. It is a region with an exceptional concentration of endemic species which results from a great loss of natural areas (MITTERMEIER et al., 2004, MYERS et al., 2000). Its preservation is a priority, although in Brazil only 3% of this biome is legally protected by preservation areas (FRANÇOSO et al., 2015).

Due to this urgent scenario, and the estimated pharmacological potential within the Cerrado, this study analyzed specimens found in the Cerrado. There are currently few scientific studies regarding the antimycobacterial activity of such specimens.

2 Materials and Methods

2.1 Botanical Material

All the botanical material was collected from the Cerrado biome in the Brazilian state of Tocantins. The specimens were identified in the herbarium of the Federal University of Tocantins (UFT), Porto Nacional campus.

2.2 Preparation of extracts

The samples were dried at room temperature, ground in a knife mill, and packed in amber flasks to percolate in 70% (v/v) ethanol for 72 hours at 25 °C, protected from light.

The solutions were filtered and their solvents were removed using a rotary evaporator to concentrate the crude extracts. The extracts were lyophilized and stored at -20°C.

2.3 Phytochemical screening

The processes of phytochemical identification, fractionation and the production of the extracts were conducted at the UFT Phytochemical Laboratory. For the phytochemical analysis, a qualitative, preliminary, prospection methodology was followed, based on the detection of some important constituents of the secondary metabolite groups (SIMÕES et al., 2007).

2.4 Fractionation

The extracts with potential activity against *Mycobacterium tuberculosis* were fractionated. An aliquot of 5.0 g of each extract was solubilized in 100 mL of methanolic solution (MetOH:H_2O) in a 4:1 ratio and then partitioned into a separatory funnel using solvent of increasing polarity: n-hexane (5 x 30 mL) and dichloromethane (5 x 30 mL). The solvents were removed using a rotary evaporator and the extracts were lyophilized and packaged at -20°C.

2.5 Microbiological assays

The analysis of antimycobacterial activity was performed at the Central Laboratory of Public Health of Tocantins (LACEN-TO) using the MABA (microplate alamar blue assay) method described by Franzblau et al. (1998). The assays used the *Mycobacterium tuberculosis*, H_{37}Rv standard strain, ATCC (American Type Culture Collection) 27294, which was provided by the Professor Hélio Fraga Reference Center/Fiocruz, Rio de Janeiro, Brazil.

The mycobacterial suspension (1:25), in 7H9 medium (4.7g of Middlebrook 7H9 broth base - Difco) with 10% ADC (albumin, dextrose and catalase), was prepared from the inoculum (H37Rv) on the No. 1 McFarland standard scale (3.2 x 10^6 CFU/mL). Rifampicin -RIF- (Sigma-Aldrich)

was chosen as the standard antibiotic.

The dried extracts were dissolved in Type I water containing 1% DMSO (dimethylsulfoxide) and subjected to sterile filtration (0.22 µm). These were adjusted to an initial concentration of 250 µg/mL, followed by two-fold, serial dilutions to a final concentration of 0.49 µg/mL. For the RIF, an initial concentration of 32 µg/mL and final concentration of 0.0625 µg/mL were used.

The microbiological assays were run in triplicate using sterile, 96-well microplates, which were sealed and incubated for seven days at 37 °C. After this period, 30 µL of 0.01% (w/v) resazurin was added to all the wells and re-incubated for 24 hours before the readings were taken.

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of extract that prevented a change of color to pink. Extracts with MIC <100 µg/mL were considered to be promising regarding antimycobacterial activity according to the classification criteria adopted by Kuete (2010).

2.6 Cytotoxicity Study

The extracts that were identified as having significant antimycobacterial activity (MIC <100 µg/mL) were evaluated for cytotoxicity. For these assays, the following two cell lines were used: LLC-MK_2 (ATTC CCL-7) and Vero (ATCC CCL-81). Mosmann's methodology (MOSMANN, 1983) was followed, using MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide). The experiments were performed at the Laboratory of Parasitology of the Department of Clinical, Toxicological and Bromatological Analysis at the University of São Paulo (USP) in the city of Ribeirão Preto.

The cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 µg/mL), penicillin (100 IU/mL) and amphotericin B (25 µg/mL). The cells were then incubated in a 5%, CO_2 oven, under a humid atmosphere of 37 °C until 80% confluence; they were subsequently diluted in phosphate-buffered saline (PBS) for 1 x 10^6 cells/mL. Cell viability was assessed using the Trypan blue exclusion method.

In sterile, 96-well microplates, 200 µL of cell suspension (106 cells/mL) was pipetted into all orifices; after 48 hours incubation half of this volume was discarded. The extracts then underwent serial, two-fold dilutions (500-3.9 µg/mL): 100 µL of each concentration was pipetted into the wells using cellular carpet and then re-incubated for 24 hours. Subsequently, 50 µL of MTT (2.5 mg/mL) was added to all the wells, followed by a wait of three hours. Then, 50 µL of DMSO was added to dissolve the formazan blue crystals the absorbance was read at 570 nm using a Stat Fax 2100 microplate spectrophotometer (Awareness Technology, Palm City, FL, USA). In the case of the untreated cells, the extracts were replaced with water and represented 100% viability, while Triton X-100 solution was used as a positive control.

2.7 Statistical Analysis

The experiments were conducted in triplicate on alternate days. The IC_{50} (inhibitory concentration) value corresponds to 50% inhibition of cell growth, which was determined by a dose-response curve prepared using GraphPad Prism, version 5.0, Windows software. The analysis between the different groups was performed using the Student's t-test method and the differences were considered significant when $p \leq 0.05$.

3 Results

Eight hydroalcoholic extracts of plants from the Cerrado biome in Tocantins, Brazil were selected and evaluated for antimycobacterial activity, as shown in Table 1.

The extracts of *Apuleia molaris* and *Ouratea spectabilis*

Table 1 – Relationship of Cerrado plants used in the microbiological assays against M. tuberculosis H_{37}Rv (ATCC 27294) and their respective inhibitory concentrations.

Botanical species	Vernacular	Herbal registration[a]	Part extracted[b]	MIC[c] (µg/mL)
Plathymenia reticulata Benth	Vinhático	10.072	Bark	(–)[d]
Ouratea spectabilis (Mart. Ex Engl.)	Cabeça de negro	9.547	Bark	62.5
Galactia glauscescens Kunth	Três folhas	10.070	Branches and leaves	(–)[d]
Apuleia molaris Spruce ex Benth	Grapia	9.614	Branches and leaves	62.5
Dipteryx alata Vogel	Baru	9.687	Bark	125.0
Brosimum gaudichaudii Trécul.	Inharé	10.068	Bark	125.0
Tabebuia caraíba (Mart.) Bureau	Paratudo	9.680	Bark	125.0
Terminalia fagifolia Mart.	Camaçari	10.842	Bark	(–)[d]

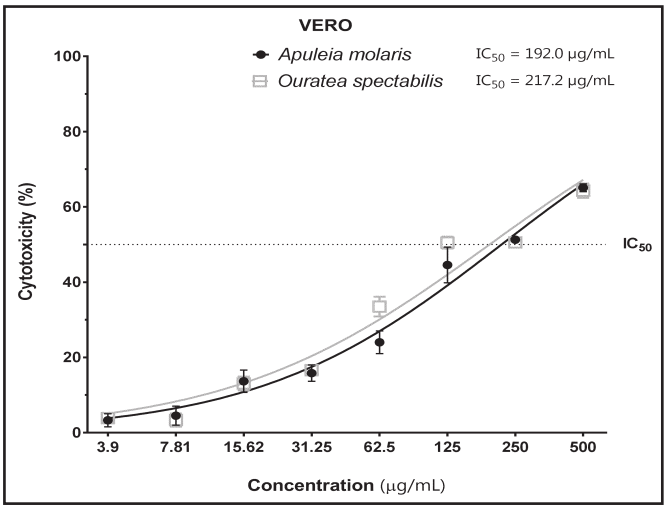
a - specimens were identified in the herbarium of the UFT; b – 70% alcoholic extract (v/v); c – minimum inhibitory concentration; d - absence of antimycobacterial effect at a concentration of 250 µg/mL. Note: RIF presented MIC = 0.5 µg/mL.

showed the most significant inhibitory concentrations (MIC <100 µg/mL), with promising antimycobacterial potential according to the classification criteria adopted by Kuete (2010). The other extracts produced moderate activity (100 <MIC ≤ 625 µg/mL) against the M. tuberculosis strain. Only the extracts with significant MICs were selected for the processes of phytochemical identification, cytotoxicity and partitioning, i.e. those lower than 100 µg/mL.

The phytochemical assays for *A. molaris* and *O. spectabilis* showed positive indications in both extracts of the qualitative presence of saponins, tannins, phenols, flavonoids, steroids and triterpenoids.

In relation to the cytotoxicity experiments involving Vero cells, the graph (FIGURE 1) of the log scale showed a growth curve of cell death as the concentration of the extracts increased.

Figure 1 – Cytotoxicity (IC_{50}) for the crude extracts of *O. spectabilis* and *A. molaris* in relation to Vero cells (1.0 x 10^6 cells/mL).



The IC_{50} values were 192.0 µg/mL for *A. molaris* and 217.2 µg/mL for *O. spectabilis* (FIGURE 1). There was no statistical difference between the paired data (t = 1.23, p = 0.26) and their curves showed a similar performance; thus, both extracts demonstrated mild levels of cytotoxicity in relation to Vero cells.

The *A. molaris* extract presented a mild cytotoxic effect (IC_{50} = 95.2 µg/mL) and *O. spectabilis* presented moderate cytotoxicity (IC_{50} = 49.8 µg/mL) in relation to the LLC-MK_2 cell line (FIGURE 2). Although there were distances between some points of the curve, in the case of the statistical analysis of the t-student test there was no significant difference between the assays (t = 2.23; p = 0.06).

In the partitioning step, three different fractions were obtained for each sample, which were evaluated again for their antimycobacterial effect (TABLE 2). The fractions (HEX, DCM, MetOH) of *O. spectabilis* did not provide as good results as the crude extract, demonstrating a possible synergistic effect between the substances present in its composition.

The hexanic fraction of A. moralis showed the best results regarding the ability to inhibit the H_{37}Rv strain. It is likely that this fraction isolated apolar compounds of the steroid and terpenoid type, demonstrating efficacy against M. tuberculosis.

Figure 2 – Cytotoxicity (IC_{50}) for the crude extracts of *O. spectabilis* and *A. molaris* in relation to LLC-MK_2 cells (1.0 x 10^6 cel/mL).

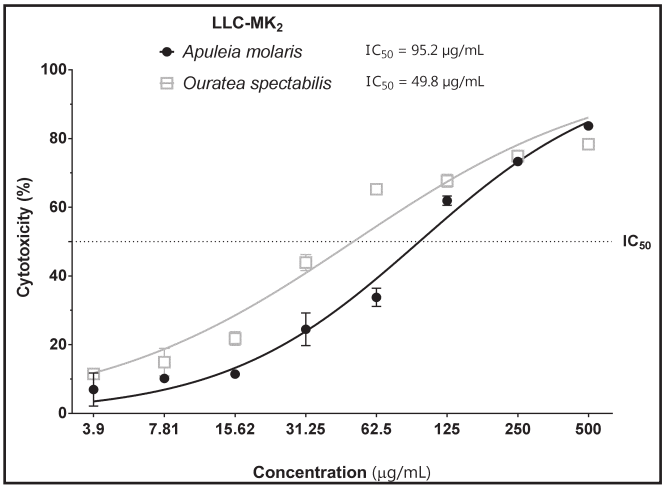


Table 2 – Minimal inhibitory concentrations (MIC) of different fractions obtained from the crude extracts of *Ouratea spectabilis* and *Apuleia molaris* against the *M. tuberculosis strain.*

Extract	Fraction*	Yield (%)	MIC (µg/mL)
O. spectabilis	HEX	10.3	250.0
	DCM	14.5	250.0
	MetOH	22.7	125.0
A. molaris	HEX	16.2	31.25
	DCM	22.8	250.0
	MetOH	30.4	250.0

4 Discussion

The Fabaceae family is widely distributed in the Cerrado biome (NOVAES et al., 2013) and its representativity was also reflected in the percentage of species collected for this study; 50% of the samples belong to this family.

The significant antimycobacterial performance of the crude extracts of *A. molaris* and *O. spectabilis* are considered to be relevant (MIC ≤ 64 µg/mL) for pharmacology (CANTRELL et al., 2001). This activity may be due to the presence of tannins, triterpenoids (NAYYAR and JAIN, 2005), flavonoids (MOLINA-SALINAS et al., 2010) and saponins (WINK, 2008), as these are structural classes of metabolites known for their broad spectrum of antimicrobial activity (KUETE, 2010; SANTHOSH and SURIYANARAYANAN, 2013).

The presence of different functional groups in plant extracts allows a variety of mechanisms of action to occur, which reduces bacterial resistance (SINGH et al., 2015).

The cell membrane is the main target and the majority of mutations are associated with its permeability (PARIDA et al., 2015).

The methanolic fraction of *O. spectabilis* did not out-perform the results of its crude extract; however, it was the fraction with the best performance. This sample had high polarity and it is very likely to have contained isolated compounds of the flavonoid type. Extracts may contain substances that facilitate the adsorption of polar compounds (WINK, 2008), which would explain the performance of this fraction.

The genus Ouratea, which is present in the Ochnacea family, is characterized as a source of flavonoids and biflavonoids (D'ARC FELICIO et al., 2001). Extracts of the Ouratea species contain biflavonoids that perform important biological activities, with antitumoral (FIDELIS et al., 2014), antiviral (BRANDÃO et al., 2011) and antimicrobial (GANGOUÉ-PIÉBOJI et al., 2006) effects. Extracts of the Ouratea species have demonstrated moderate effects regarding the inhibition of different gram positive, cocci species, especially for Staphylococcus aureus, as well as Candida albicans yeasts.

Previous studies (MECINA et al., 2014; SIMONI et al., 2002; VALADARES et al., 2003) have identified the presence of several types of flavonoids in relation to O. spectabilis, particularly biflavonoids (6,6´´-bigenkwanin; 7-7´´-dimetoxi-agasthisflavona), which reflects a probable association between the antimycobacterial effect for this metabolic group in the methanolic fraction in the present study.

Regarding the evaluation of cytotoxicity, Simoni et al. (2002) and Brandrão et al. (2011) reported that extracts from *O. spectabilis* leaves had low cytotoxicity. This corroborates with the results of the present study regarding the alcoholic extract of the bark, which was found to have a mild cytotoxic effect.

The hexane, apolar fraction of *A. molaris* showed a more significant effect in the present study: it was able to penetrate the mycobacterial cell wall, inhibit its growth and cause cell death. Mycobacteria have a hydrophobic cell wall that is rich in lipids (mycolic acids), which are generally susceptible to apolar compounds (NAYYAR and JAIN, 2005; PAULI et al., 2005), and this might explain the good performance of this fraction.

There are few studies regarding the *A. molaris* species (synonym: A. leiocarpa), most of which are associated with morphological characterization (SOUZA REIS et al., 2016). Some of the existing studies have described antimalarial (MUÑOZ et al., 2000), antibiofilm (SILVA et al., 2015), anti-inflammatory and analgesic (RUPPELT et al., 1991) biological effects, whose properties are correlated with the presence of tannins, flavonoids (WOLLENWEBER and H. DIETZ, 1981), triterpenes and β -amyrin (MUÑOZ et al., 2000).

Experiments conducted by Silva et al. (2015) in relation to A. leiocarpa fruit (synonym: *A. molaris*) demonstrated antibiofilm potential against Staphylococcus epidermidis, as well as a low toxicity in relation to Vero cells. This reinforces the promising character of this specimen and highlights the need for further studies to provide better chemical and biological characterization.

Conclusion

The extracts of *Ouratea spectabilis* and *Apuleia molaris* showed chemical variation of secondary metabolites, which changed from polar (flavonoids) to apolar (steroids and triterpenes), suggesting different physiological actions in the face of mycobacterial attack.

The results of this study were expressive, especially when confirming that *Apuleia molaris* has potential antimycobacterial action and possesses low cytotoxic effect. Such characteristics suggest that it may be used as a future alternative in the treatment of tuberculosis.

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