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ORIGINAL ARTICLE

Hyptis brevipes AND *Paullinia pinnata* EXTRACTS AND THEIR FRACTIONS PRESENTING ACTIVITY AGAINST *Mycobacterium abscessus* SUBSP. *massiliense*

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ABSTRACT

Medicinal plants are of great interest for the discovery of new biomolecules with diversified effects. Over the last decade different outbreaks caused by *Mycobacterium abscessus* subsp. *massiliense* have been reported, evidencing it as an important emerging pathogen in underdeveloped countries. This study investigated the antimycobacterial activity of six Brazilian medicinal plant extracts and their fractions. *Hyptis brevipes, Tocoyena formosa, Randia armata, Paullinia pinnata, Lafoensia pacari* and *Anadenanthera colubrina* were evaluated against *M. a. massiliense*. Total extracts from the medicinal plants *H. brevipes, T. formosa, P. pinnata* and *L. pacari* presented a minimal bactericidal concentration of 1 mg/mL. After fractioning, the ethanolic fractions from *H. brevipes* and *P. pinnata* presented bactericidal activity, and the ethyl acetate fraction from *H. brevipes* and *T. formosa* presented antimycobacterial action. The best bactericidal function of all plant fractions was the ethanolic, which contained rutin and rosmarinic acid that were shown to have microbicidal activity.

KEY WORDS: *Mycobacterium abscessus*; medicinal plants; Brazilian medicinal plants; HPLC and biodiversity.

INTRODUCTION

Medicinal plants and herbs have been used for several centuries to treat and reduce symptoms of various diseases. Therapies based on these plants have motivated growing interest regarding the discovery of new natural biomolecules with broad biological effects, such as anti-inflammatory, antitumor, antimicrobial and disinfectant action (Alves et al., 2013).

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According to the World Health Organization (WHO), in developing countries an estimate of about 65-80% of the population relies on phytotherapy as the primary source of treatment (Mazzari & Prieto, 2014). In Brazil, between 2013 and 2015, there was an increase in the use of medicinal plants mainly due to their low cost (Brasil, 2016).

The practice of traditional medicine and use of natural products to control infections caused by mycobacteria is little known (Dutra et al., 2016). Although approximately 180 plant species are used to reduce the symptoms of tuberculosis in South Africa (McGaw et al., 2008), to our knowledge, only one study evaluated plant extracts for bactericidal function against *Mycobacterium abscessus* subsp. *massiliense* (Pereira et al., 2018).

M. a. massiliense has been increasingly reported as the cause of soft tissue infection outbreaks primarily after surgery or accidental trauma and can occur in both immunocompetent and immunocompromised patients (de Carvalho et al., 2018). The *M. a. massiliense* strains isolated in the municipalities of Belém do Pará, Goiânia, Rio de Janeiro and Vitoria were shown to be a single genetic clone disseminated in Brazil (Cardoso et al., 2008; Viana-Niero et al., 2008; Cardoso et al., 2011).

Medicinal plants of the genus *Hyptis* are also commonly used in alternative medical practices (Dutra et al., 2016). Plants of this genus present compounds such as diterpenes, flavonoids, and lignans (Deng et al., 2009; Xu et al., 2013). There are reports regarding the use of *H. brevipes* species for the treatment of asthma, malaria, conservation of cereals and as mosquito repellent (Bhuiyan et al., 2010). The essential oil from some species of *Hyptis* also has strong antimicrobial activity with considerable antioxidant power (Bhuiyan et al., 2010).

The genus *Paullinia* encompasses some plant species of considerable commercial importance, among them *P. cupana* (guaraná) used in food and beverages. Another species, *P. pinnata L.*, is used in the manufacture of a phytotherapic medication for the treatment of human malaria and erectile dysfunction (Lunga et al., 2014). Studies revealed that glycosidic compounds present in the stem of plants of this genus are also capable of acting as antimicrobials against enteric bacteria (Lunga et al., 2015).

With a view to increasing the number of alternatives for the treatment of infections, the purpose of this study was to investigate the antimycobacterial activity of the vegetal extracts and their fractions from six Brazilian medicinal plants against *M. a. massiliense.* These compounds are known as antibactericidal, but to date, have not been tested against rapidly growing mycobacteria.

MATERIAL AND METHODS

Study area

All plant materials were collected from March to July 2012 in the Pantanal, municipality of Corumbá, State of Mato Grosso do Sul, Brazil. Pantanal is a wetland presenting a seasonal climate with a rainy period from October to April, and a pronounced dry season from May to September. During the rainy season, many regions of the Pantanal are flooded and some plant species remain underwater (Junk et al., 2006; Santos et al., 2014).

Different parts of the plants were collected (SISBIO license number 33165 and SisGen license number A15EB96), such as the bark and aerial parts including leaves, flowers and fruit when available. For this study *Hyptis brevipes, Tocoyena Formosa, Randia armata, Paullinia pinnata, Lafoensia pacari* and *Anadenanthera colubrina* were used.

All plants were identified by Prof. Dr. Geraldo Alvez Damasceno Junior, and vouchers were deposited in the herbarium CGMS of UFMS, Campo Grande, Brazil. Other characteristics of the plant materials are described in Table 1.

Table 1. Information about plants collected in Pantanal, Corumbá, Mato Grosso do Sul, Brazil.

Species	Family	Location Coordinates	Part of plant analyzed	ID Voucher
Hyptis brevipes Poit	Lamiaceae	19°34'8"S; 57°1'10"O	Aerial part	CGMS 41030
Tocoyena formosa (Cham. & Schltdl.) K.Schum	Rubiaceae	19°29'2"S; 57°2'16"O	Leaves	CGMS 41026
Randia armata (Sw.) DC	Rubiaceae	19°29'2"S; 57°2'16"O	Aerial parts including flowers	CGMS 41020
Paullinia pinnata L.	Sapindaceae	19°36'30"S; 57°2'8"O	Aerial parts/ Leaves	CGMS 41013
Lafoensia pacari A.St.-Hil	Lythraceae	23°12'25"S; 55°18'41"O	Bark	CGMS 41002
Anadenanthera colubrina (Vell.) Brenan	Fabaceae	19°40'13"S; 57°0'20"O	Bark	CGMS 38928

Plant extraction

Plant powders of all species analyzed in this study were extracted in an accelerated solvent extractor-ASE™ 150-DIONEX™ and each plant part was initially fractioned into two extracts: non-polar and polar. The non-polar fractions were extracted with hexane and acetone (8:2) but were not included in this study due to low solubility. The polar fractions, which were utilized in microbiological tests, were extracted using a mixture of ethanol and water (7:3) at 125°C, with a static extraction time of 5 minutes and 80% rinsing across 2 cycles under a nitrogen atmosphere followed by concentration and lyophilization. When needed, the extracts were solubilized in 3% DMSO at a concentration of 1 mg/mL, and used in antimicrobial assays, after performing a serial dilution of two factors in a 96 well plate.

The polar extracts that presented potential activity against *M. abscessus* subsp. *massiliense* were fractioned again using accelerated solvent extractor ASE-150 DIONEX™. About 1 g of extract was fractioned using silica gel 70-230 mesh Macherey-Nagel (39 g) extracted with hexane (EtOH-Frhex), Chloroform (EtOH-FrCHCl$_3$), ethyl acetate (EtOH-FrAcEt) or ethanol (EtOH-FrEtOH). The parameters applied in ASE 150 fractioning were temperature of 100°C, static extraction cycle of 1 minute, washing with 150% of the volume, and one purging cycle for 100 seconds. All fractions were dried and used in microbiological tests starting at 1mg/mL concentration.

Microorganism

The strain used in the prospection of plant extracts and fractions was *M. a. massiliense* GO06, cryopreserved in the Laboratory of Molecular Bacteriology of the Institute of Tropical Pathology and Public Health of the Federal University of Goiás. This isolate was randomly chosen among isolates collected from patients who had infections caused by contaminated instruments following invasive procedures (Cardoso et al., 2008). This isolate was stored in a freezer at –80 °C.

Antimycobacterial assay of extracts and fractions

The *M. a. massiliense* culture was reactivated in 5 mL of Muller Hinton (MH) broth for approximately three days at 37°C. It was then adjusted to an optical density of 1.0 at 590 nm, corresponding to a concentration of approximately 3 x 10^8 CFU/mL. The culture was diluted so that 1.5 x 10^3 CFU/mL was added to each well of a microplate containing 200 µL of the serially diluted plant extracts or their fractions. As mycobacterial growth control, the strain was incubated with the extracts and fractions diluent,

and as inhibition control, mycobacteria were incubated with serial dilutions of clarithromycin (8 - 0.06 μg/mL). The 96-well plates were incubated at 37°C at 120 rpm for 3 days. Resazurin suspension (0.01% w/v) was added followed by a further incubation. The optical densities of each assay were determined at OD 595 nm. As growth control, a bacteria culture without any additive was used and this culture was utilized as a 100% (0% inhibition) growth. The percentage of growth inhibition induced by each treatment was determined in relation to the growth control culture. For determination of CFU counts after the incubation period, a 1:4 dilution was performed, plated on MH agar, and incubated for 5 days at 37°C, to determine the number of CFU. Minimum bactericidal concentration (MBC) was determined to be the lowest concentration capable of inhibiting mycobacterial growth by 99.9%. As a control, clarithromycin at a concentration of 8 - 0.06 μg/mL was used.

Identification and characterization of the phenolic components of fractions by HPLC-DAD-MC

Fractions underwent liquid chromatography evaluation and the concentrations of the main constituent were determined according to the Carvalho et al. (2018) methodology. The fractions were analyzed by high-performance liquid chromatography coupled to a diode array detector (DAD) and an ESI-qTOF microTOF-Q III mass spectrometer. Analyses were performed using a Kinetex column (C-18 - 2.6μ, 150 x 2.2 mm, Phenomenex) protected by a pre-column with the same material. The mobile phase was solvent A: ultrapure water and solvent B: solvent B, both with 0.1% formic acid (v/v); with the gradient starting at 3% B (0–2 min); 3-25% B (2-25min); 25-80% B (25-40 min), followed by 8 minutes to wash and recondition the column. Flow rate: 0.3 mL/min; column oven: 50 °C and injection volume: 1 μL. The UV analyses were performed at the 240-800 nm range and mass spectrometer in negative and positive mode (m/z 120–1200). To identify the compounds' accurate mass spectrometry, fragmentation pathway, UV data, and literature information (Carvalho et al., 2018) were used.

Statistical Analysis

The data were tabulated using Excel software and the average and standard deviation values were calculated. The CFU values of the growth control were compared with the CFU values of each treatment concentration with extracts and fractions.

RESULTS

Table 1 presents relevant information on the aspects of plant collection used in the experiments. *H. brevipes* (HB), *T. formosa* (TF), *R. armata* (RA) and *P. pinnata* (PP) were collected in the same geographical region, while *L. pacari* (LP) and *A. colubrina* (AC) were collected in another setting close to the former.

The plants were collected and processed to produce a crude extract, which was incubated with *M. a. massiliense*; Table 2 shows the antimicrobial activity of the crude extracts. In the tested concentration of 1 mg/mL, *H. brevipes, T. formosa* and *P. pinnata* extracts presented 100% inhibition of bacterial growth. These are the results for the minimal bactericidal concentration (MBC) as all results from subsequent serial dilutions were ineffective in killing 100% of *M. a. massilense* (data not shown).

H. brevipes, P. pinnata and *T. formosa* extracts were fractioned and evaluated against *M. a. massiliense.* The *H. brevipes* fractions presented 100% inhibitory action on the Hx and EtOH parts and to a lesser extent on EtAC. The *Tocoyena formosa* tests with the fractions showed a 50% inhibition for EtOH and EtAc, while Hx presented no action. Finally, in the fractions of the *Paullinia pinnata* plant, there was no action on the EtOH, the EtAC or the Hx. The ethanolic fractions of *H. brevipes* and *P. pinnata* evidenced better activity against *M. a. massiliense* than the *T. formosa* ethanolic fraction (Figure).

Table 2. Antimicrobial activity of crude extracts of Pantanal plants[a]

	HB[b]	TF	RA	PP	LP	AC
M. a. massiliense[c]	+ + +	+ + +	+ +	+ + +	+ + +	+ +

[a] Extracts antimycobacterial activities were evaluated by the resarzurin method.
[b]HB, *Hyptis brevipes*; TF, *Tocoyena formosa*; RA, *Randia armata*; PP, *Paullinia pinnata*; LF, *Lafoensia pacari* and AC, *Anadenanthera colubrina*.
[c] + Considered when equal to or greater than 25% inhibition; + +, Equal to or greater than 50% and + + +, greater than 75% inhibition.

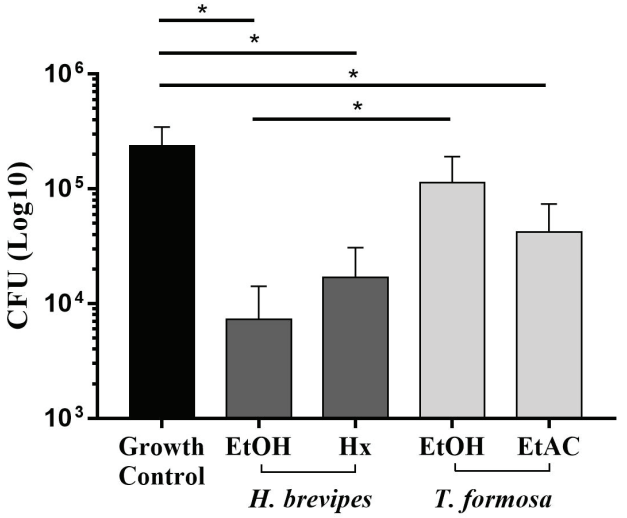

Figure. Antimycobacterial activity of *H. brevipes* and *T. formosa* fractions. The ethanolic (EtOH) and hexane (Hx) fractions (1 mg/mL) were evaluated against *M. a. massiliense*. CFU counts from each assay were plotted. As control, bacteria were incubated with 1µg/mL of clarithromycin that eliminated all bacteria. Bacteria grown without any antimicrobial agent is shown (Growth Control). CFU count significantly different from the growth control group ($p < 0.05$) is shown with an asterisk. This results is one of three independent experiments.

The ethanol fractions that presented the best mycobactericidal activity were characterized by HPLC for phytochemical profile identification. In the *H. brevipes* ethanol fraction, 14 different components were observed, which were classified into 3 families of compounds, namely, sugar, phenol and flavonoid; the major components identified were rutin and rosmarinic acid (Table 3). In the *P. pinnata* ethanol fraction, 17 flavonoid compounds were identified including quinic acid and rutin (Table 4). The analysis of the *T. formosa* ethanolic fraction showed 8 triterpenoid saponin molecules and a Kaempherol-di-deoxy-hexoside-hexoside. The *T. formosa* ethanolic fraction proved different from the other two plant fractions evaluated (data not shown).

Table 3. Compound identified in ethanolic fractions from *H. brevipes*

Compounds	RT (min)	UV (nm)	$[M-H]^-$ m/z	Molecular formula $[M-H]^-$ m/z	$[M-H]^-$ MS/MS (m/z)	Class
Dihexosyl	1.3	-	341.1042	$C_{12}H_{21}O_{11}$	-	Sugar
Salvianic acid A	3.8	280	197.0447	$C_9H_9O_5$	179 ($C_9H_7O_4$)	
Caffeic acid	10.2	294/322	179.0345	$C_9H_7O_4$	-	Phenol
Hidroxyjasmonic acid glucoside	13.3	-	387.1661	$C_{18}H_{27}O_9$	207 ($C_{12}H_{15}O_3$); 163 ($C_{11}H_{15}O$)	
Hidroxyjasmonic acid methyl ester glucoside	17.5	-	401.1818	$C_{19}H_{29}O_9$	195 ($C_{12}H_{19}O_2$); 177 ($C_{12}H_{17}O$)	
Rutin	17.9	255/351	609.1448	$C_{27}H_{29}O_{16}$	300 ($C_{15}H_8O_7$)	Flavonoid
Isoquercitrin	18.3	266/352	463.0877	$C_{21}H_{19}O_{12}$	300 ($C_{15}H_8O_7$)	Flavonoid
Kaempferol-O-rutinoside	19.6	265/345	593.1512	$C_{27}H_{29}O_{15}$	285 ($C_{15}H_9O_6$)	Flavonoid
Rosmarinic acid	20.4	285/327	359.0766	$C_{18}H_{15}O_8$	197 ($C_9H_9O_5$); 179 ($C_9H_7O_4$); 161 ($C_9H_5O_3$)	
Flavonol-trihidroxy-dimethoxy	24.0	278/342	345.0629	$C_{17}H_{13}O_8$	315 ($C_{15}H_7O_8$); 287 ($C_{14}H_7O_7$)	Flavonoid
Brevipolide C derivative	24.6	298/327	401.1224	$C_{21}H_{21}O_8$	161 ($C_9H_5O_3$)	
Brevipolide derivative	27.3	298/324	403.1396	$C_{22}H_{24}O_8$	161 ($C_9H_5O_3$)	
Flavonol-trihidroxy-trimethoxy	28.8	278/347	359.0763	$C_{18}H_{15}O_8$	329 ($C_{16}H_9O_8$)	Flavonoid

[a] RT: retention time

Table 4. Compounds identified in ethanolic fractions from *P. pinnata.*

Compounds	RT (min)	UV (nm)	$[M-H]^-$ m/z	Molecular formula $[M-H]^-$ m/z	$[M-H]^-$ MS/MS (m/z)	Class
Quinic acid	1.2	-	191.0555	$C_7H_{11}O_6$	-	
Unknown	2.6	-	265.093	$C_{10}H_{17}O_8$	-	-
p-Coumaroylglucose	10.2	224/281	325.0915	$C_{15}H_{17}O_8$	163 ($C_9H_7O_3$)	
Unknown	11.2	-	451.2174	$C_{20}H_{35}O_{11}$	-	
Unknown	11.3	-	451.2174	$C_{20}H_{35}O_{11}$	-	
Unknown	11.4	-	363.0722	$C_{17}H_{15}O_9$	-	
Hidroxyjasmonic acid glucoside	13.3	-	387.1661	$C_{18}H_{27}O_9$	207 ($C_{12}H_{15}O_3$); 163 ($C_{11}H_{15}O$)	
Kaempferol tetraglucoside	16.1	265/347	901.2449	$C_{39}H_{49}O_{24}$	575 ($C_{27}H_{27}O_{14}$); 285($C_{15}H_9O_6$)	Flavonoid
Quercetin triglucoside	16.7	255/355	755.2055	$C_{33}H_{39}O_{20}$	300 ($C_{15}H_8O_7$)	Flavonoid
Quercetin-O-rutinoside	17.3	265/351	609.1439	$C_{27}H_{29}O_{16}$	300 ($C_{15}H_8O_7$)	Flavonoid
Rutin	17.9	255/351	609.1448	$C_{27}H_{29}O_{16}$	300 ($C_{15}H_8O_7$)	Flavonoid
Kaempferol di-deoxy-hexoside-hexoside	17.9	264/347	739.2084	$C_{33}H_{39}O_{19}$	284 ($C_{15}H_8O_6$)	Flavonoid
Kaempferol pentosil rutinoside	18.6	265/350	725.1915	$C_{32}H_{37}O_{19}$	284 ($C_{15}H_8O_6$)	Flavonoid
Kaempferol rutinoside	18.7	266/349	593.1488	$C_{27}H_{29}O_{15}$	284 ($C_{15}H_8O_6$)	Flavonoid
Kaempferol rutinoside	19.8	266/347	593.1495	$C_{27}H_{29}O_{15}$	285 ($C_{15}H_9O_6$)	Flavonoid
O-methylquercetin-O-rutinoside	20.4	265/353	623.1599	$C_{28}H_{31}O_{16}$	315 ($C_{16}H_{11}O_7$)	Flavonoid
Unknown	20.5	-	405.1187	$C_{20}H_{21}O_9$	-	

[a] RT: retention time

DISCUSSION

This study was the first to evaluate the activity of different plant species collected from the biodiversity region of Mato Grosso Pantanal against *M. a. massiliense.* Extracts from the medicinal plants *H. brevipes, T. formosa, P. pinnata* and *L. pacari* investigated in this study presented therapeutic potential against fast growing pathogenic mycobacteria as they presented an MBC of 1 mg/mL. When the fractioned compounds of the *Hyptis brevipes* and *Paullinia pinnata* crude extracts were evaluated, the best bactericidal activity was detected in the EtOH fraction.

The site of the collection as well as the part of the plant used for the extracts is important to determine the molecular profile of the extract. The climate, temperature and humidity modify phytochemical profiles (Pilatti et al., 2018), therefore the plant parts were collected at the end of the rainy season, where green plants produce more secondary metabolites presenting a broad spectrum of activity in the extracts (Huang et al., 2017).

H. brevipes plant extract inhibited mycobacteria in 1 mg/mL. Other studies with *H. brevipes* extract (Xu et al., 2013) and its essential oils (Asekun et al., 1999) evidenced that at a 100 mg/mL concentration, a strong antibacterial activity against *Staphylococcus aureus* and a low activity against *Escherichia coli* were present. These results might indicate that the extracts of *H. brevipes* act against gram-positive bacteria. The results in this study show that *H. brevipes* extracts presented increased antimycobactericidal activity compared to *S. aureus* and *E. coli*.

P. pinnata crude extract from a plant collected in the Republic of Cameroon showed MICs ranging from 781 to 390 μg/mL against *E. coli* and *S. aureus*, respectively (Lunga et al., 2015). The high *P. pinnata* MBC found for *M. a. massiliense* (1 mg/mL) may be due to the mycobacteria cell walls presenting high mycolic acid contents possibly interfering with the penetration of the extract into the cytoplasm and or reaching its target (Jankute et al., 2017).

H. brevipes, P. pinnata and *T. formosa* fractions were evaluated against *M. a. massiliense*. The *H. brevipes* and *P. pinnata* ethanolic fractions presented better activity against *M. a. massiliense* than the *T. formosa* ethanolic fraction. The literature shows that the ethanolic fractions of different plant extracts have high antimicrobial activity. Usually, these fractions contain tannin, which presents significant bactericidal properties (Carvalho et al., 2018). They also contain flavonoids, glycosides, phytosterols, saponins, steroids, triterpenoids, known for their antimicrobial effect (Cushnie & Lamb, 2005; Sati et al., 2019). The combination of these molecules contained within the ethanolic fraction should cause the inhibition of *M. a. massiliense* growth. However, the *T. formosa* ethanolic fraction did not inhibit as efficiently as the *H. brevipes* and *P. pinnata* fractions. These two fractions probably contain specific compounds which present enhanced mycobactericidal activities.

14 different components were observed in the *H. brevipes* ethanolic fraction, which were classified into 3 families of compounds: sugar, phenol and flavonoid, the major components identified were rutin and rosmarinic acid. In the *P. pinnata* ethanol fraction 17 flavonoid compounds were identified, among them, quinic acid and rutin.

Rutin was the most common compound found in the *H. brevipes* and *P. pinnata* ethanolic fractions, in accordance with the literature and known to have antifungal and antibacterial activity, mainly against *Escherichia coli* and *Bacilli* sp (Pereira et al., 2007; Maddox et al., 2010; Cetin-Karaca et al., 2011; Santos et al., 2016; Strada et al., 2017). Arima et al. (2002) combined rutin with other plant compounds such as flavonoids and showed that the antimicrobial activity of rutin depends on its association with other plant components (Arima et al., 2002). Rutin was previously shown to have antimycobacterial activity against *M. smegmatis* and *M. fortuitum* (Lechner et al., 2008), presenting a MIC of 128 and 256 µg/mL, respectively.

This study evaluated the antimycobacterial activity of different plant species collected from the biodiversity region of Mato Grosso Pantanal against *M. a. massiliense*. Extracts from the medicinal plants *H. brevipes, T. formosa, P. pinnata* and *L. pacari* investigated in this study presented therapeutic potential against rapidly growing pathogenic mycobacteria as they presented an MBC of 1 mg/mL. When the fractioned compounds of the crude extracts of *H. brevipes* and *P. pinnata* were evaluated, the best bactericidal activity was detected in the EtOH fraction, which contains mostly rutin, rosmarinic acid and quinic acid.

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