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ORIGINAL ARTICLE

Metabolites from endophytic *Aspergillus fumigatus* and their *in vitro* effect against the causal agent of tuberculosis

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ABSTRACT

Tuberculosis (TB) remains one of the most deadly communicable infectious diseases, causing 1.4 million deaths in 2015 worldwide due to many conditions, including the inadequate treatment and the emergence of multidrug-resistant strains of the causal agent, *Mycobacterium tuberculosis*. Therefore, drugs developed from natural sources, as microorganisms and plant extracts, are a frequent target for the research and discovery of antimicrobial compounds. The current study started the characterization of compounds produced by an *Aspergillus fumigatus* isolated from copaíba (*Copaifera multijuga*) that efficiently inhibits *M. tuberculosis* by releasing the compounds into the fermentation broth under specific culture conditions. A preliminary assay was carried out with a correlate species, *M. smegmatis*, aiming to detect an antimicrobial effect related to *A. fumigatus* fermentation broth. The direct use of this substrate in antibiosis assays against*M. tuberculosis* H_{37}Rv strain (ATCC 27294) allowed the detection of antimicrobial activity with a minimal inhibitory concentration of 256 μg mL^{-1}, demonstrating that purification processes developed by the Biotage Flash Chromatography System are robust and reliable techniques for purification of compounds from natural sources. Also, this chromatographic system can be used in combination with specific biochemical tests, improving the search for reliable results. We conclude that this fraction can express a broad action range, inhibiting both *Mycobacterium* species used as target organisms.

KEYWORDS: *Mycobacterium* spp., antimicrobial activity, copaíba, chromatography

Metabolitos de *Aspergillus fumigatus* endofítico e seu efeito *in vitro* contra o agente causal da tuberculose

RESUMO

A tuberculose continua a ser uma das doenças infecciosas transmissíveis mais mortais, causando 1,4 milhão de mortes em 2015 em todo o mundo devido a vários fatores, incluindo o tratamento inadequado e o surgimento de cepas multirresistentes do agente causal, *Mycobacterium tuberculosis*. Portanto, as drogas desenvolvidas a partir de fontes naturais, como micro-organismos e extratos de plantas, são um alvo freqüente para a pesquisa e descoberta de compostos antimicrobianos. O presente estudo foi um ponto de partida para caracterizar compostos produzidos por um *Aspergillus fumigatus* isolado de copaíba (*Copaifera multijuga*) que inibe eficientemente *M. tuberculosis*, liberando os compostos no caldo de fermentação em condições de cultura específicas. Realizou-se um ensaio preliminar com uma espécie correlata, *M. smegmatis*, com o objetivo de detectar um efeito antimicrobiano relacionado ao caldo de fermentação de *A. fumigatus*. O uso direto deste substrato em ensaios de antibiose contra a estirpe H37Rv de *M. tuberculosis* (ATCC 27294) permitiu a detecção de atividade antimicrobiana com uma concentração inibitória mínima de 256 μg mL^{-1}, demonstrando que os processos de purificação desenvolvidos pelo *Biotage Flash Chromatography System* são técnicas robustas e confiáveis para purificar compostos de fontes naturais. Além disso, este sistema cromatográfico pode ser usado em combinação com testes bioquímicos específicos, melhorando a busca de resultados confiáveis. Concluímos que esta fração pode expressar uma ampla gama de ação, inibindo ambas as espécies de *Mycobacterium* utilizadas como organismos-alvo.

PALAVRAS-CHAVE: *Mycobacterium* spp., atividade antimicrobiana, copaíba, cromatografia

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INTRODUCTION

Mycobacterium tuberculosis is the microorganism that causes tuberculosis (TB), one of the infectious diseases with the highest levels of morbidity and lethality in the world, with 1.4 million TB deaths in 2015 (WHO, 2016), especially in developing countries (Grutzmacher *et al*. 2012), where the health care is precarious to most of the population. The high level of mortality is mainly due to the emergence of multidrug-resistant strains, coupled with inadequate use of antibiotics, such as inappropriate choices, inadequate dosing, poor adherence to treatment guidelines and self-medication, which makes this disease a major concern of the World Health Organization (WHO)(Petrini and Hoffner 1999; Prestinaci *et al*. 2015). With the emergence of drug-resistant strains of *M. tuberculosis*, and the necessarylong-term treatment that is detrimental to a patient's health due to the high levels of drug toxicity and various adverse effects, it has become of critical importance to search for new effective anti-*M. tuberculosis* agents (Gemechu *et al*. 2013). Antimicrobial resistance (AMR) is a major public health problem that undermines the effective prevention and treatment of an ever-increasing range of infections caused by pathogens, such as bacteria, which are no longer susceptible to common antibiotics (Prestinaci *et al*. 2015).

There is a great interest in the search for new drugs, strengthened by the hope of selecting the most effective for treating TB. In fact, many medications available and currently used in clinical practice are derived from secondary metabolites produced by microorganisms or by fermentation processes associated with them (Ferrara 2006).It is estimated that approximately 25% of known biologically active products obtained from natural sources were obtained from fungi (Kongsaeree *et al.* 2003). In addition to their high metabolic rate, fungi have advantages over other sources, since they consist on a renewable and easy to maintain resource. Also, the technology for the production and purification of bioactive metabolites is already stablished(Cafêu 2007).

Aspergillus is a well-known fungi genus, with potential forthe synthesis of biologically active compounds, such as brefeldin A (from*A. clavatus*), with antitumor activity (Wang *et al.* 2002), aspernigrin A (from*A. awamori*) and naphthoquinoneimine(from *A. niger*),with antifungal potential (Zhang *et al.* 2007), asporyzin C (from *A. oryzae*), which inhibits the growth of *Escherichia coli* (Qiao *et al.* 2010)) and antioxidants from*Aspergillus* spp. (Li *et al.* 2004). *Aspergillus* fungi are associated with plants as endophytes, especially intropical regions(Isaka *et al.* 2001; Oliveira 2008; Prince 2008). Endophytic *Aspergillus* areconsidered promising for the production of novel antimicrobial compounds, since they can secrete these moleculeswhen cultured in laboratory-controlled conditions. Carvalho (2005, 2010) isolated 45 endophytic fungi associated with *Copaifera multijuga* Hayne (Fabaceae), a tree species typical of the Amazon region, and nine of those were identified and showedvariable levels of antimicrobial activity against *M. tuberculosis*.

Biologically active substances of natural origin may have a broad and effective spectrum, but their purification process is one of the most difficult steps, justifying the search for more robust purification systems aimingat the obtention of compounds for biotechnological interest in a less delayed way.

In the current study, thefractions from *A. fumigatus*strain CBA2743fermentation broth wereobtained by chromatographic techniques and tested against *M. smegmatis* and *M. tuberculosis* to prove the efficacy of these protocols as first steps for screening of compounds for biotechnological purposes.

MATERIAL AND METHODS

Strains

Aspergillus fumigatus strain CBA 2743 is an endophytic fungus isolated from *Copaifera multijuga* Hayne by Carvalho (2005) and already known for its ability to inhibit *Mycobacterium tuberculosis* (Carvalho 2010). The stock culture was maintained and reactivated in PDA medium (Himedia) at 28°C for 5 days. *Mycobacterium smegmatis* was kindly provided by PhD Maria Francisca Simas Teixeira of the Universidade Federal do Amazonas. The bacterial strainwas maintained on TSA (Difco™) and reactivated in TSB (Difco™) at 35°C for 48 hours. *Mycobacteriumtuberculosis*H37Rv (ATCC 27294) was stored at the Mycobacteria Collection (Mycobacteriology Laboratory, Instituto Nacional de Pesquisas da Amazônia) and was reactivated in Lowenstein-Jensen Broth (Difco™) at 35°C for 21 days.

Fractionation of *A. fumigatus* fermentation broth

Glycerol medium proved to be the best substrate for the production of metabolites by *A. fumigatus* (unpublished data). A mycelium disc (10 mm diameter) of *A. fumigatus* CBA 2743grown on Potato Dextrose Agar – PDA (Himedia), was inoculated into aflask containing glycerol broth (2g KNO_3,0.3g casein; 2g NaCl, 2g K_2HPO_4, 1g KH_2PO_4, 0.1g $MgSO_4$, 0.1g $CaCO_3$, 0.01g $FeSO_4$, 0.01g $ZnSO_4$, 10 mLglycerol, to a total volume of 1000 mL), the pH of the medium was adjusted to 5.0 before sterilization. The culture was incubated at 28°C for 28 days, the fungus was grown without agitation and then the fermentation broth was recovered by vacuum pump filtration. Fermentation brothfiltrated was used for antibiosis agar diffusion method(Silva *et al.*2012)against *M. smegmatis*and for partitioning with n-butanol (1:1). Afterwards, the sample was concentrated on a rotary-evaporator.

Chromatographic analysis

The concentratedsample was solubilized in 5 mL methanol, resulting in an alcoholic solution of 14 mg mL^{-1}, which was subjected to High Performance Thin Layer Chromatography (HPTLC) to determine the elution system through the selectivity test in a normal phase (Alugram Xtra SIL G

/ UV254) using methanol, ethyl acetate, acetonitrile, dichloromethane, hexane, n-butanol, toluene, ammonium hydroxide, acetic acid and ethanol. Combinations of eluents were also used: dichloromethane:ethanol (8:2); hexane:ethyl acetate (8:2);toluene:ethanol (8:2); toluene:ethanol (8:2) + 100µL of acetic acid; hexane:ethyl acetate:methanol (8:1:1).

Also, the concentrate sample of *A. fumigatus* was subjected to Medium Pressure Liquid Chromatography (MPLC)in a preparative scaleusing the Isolera One System (Biotage). Extract was evaluated using two wavelengths (200 and 366 nm), adjustable flow rates of 20 mL min^{-1} and two solvent combinations (hexane:ethyl acetate and ethyl acetate:methanol) on an exploratory gradient to obtain fractions with highest purity and yield.For sample purification in solid phase extraction (SPE), we used a normal phase silica cartridge(SNAP 25g).

Additionally, the concentrated samples were subjected to High-Performance Liquid Chromatography (HPLC) on analytical scale connected to a diode array detector (SPD). The samples were solubilized in acetonitrile (Sigma®, HPLC grade), then filtered through a Millipore membrane (0.22 µm), and diluted in a mixture of acetonitrile:water (1:1). This sample was analyzed using the following settings: Shim-pack CLC-ODS column and pre-column (internal diameter and length - 15 cm x 4.6 mm; particle diameter - 5 µm, pore diameter - 100 Å) maintained at 40 °C; acetonitrile and water gradient starting at 5% and ending at 100% in an hour. The monitoring wavelength was between 190-400 nm.

Antibiosis bioassays against *M. smegmatis*

Sterile paper discs (6mm diameter) were embedded with 10µL of the fractionresuspended in ethanol and dried in a biological safety cabinet for 15 minutes. *Mycobacterium smegmatis* suspension, standardized toMcFarland turbidity scale nº. 1, corresponding to approximately 3 x 10^8 $CFUmL^{-1}$, was spread on Petri dishes containing TSA medium (Difco™) with a sterile swab, the 30 fractions obtained from the MPLC (Fig. 2) were tested. Then, paper discs were carefully placed onto the surface of the culture medium. Petri dishes were maintained at 4°C for 4 hours, and then incubated at 35°C for 48 hours for further observations and measurement of the inhibition halos (Silva *et al.* 2012). A disc embedded in ethanol was used as negative control.

Antibiosis bioassays against *M. tuberculosis*

Approximately 5 mg of *Mycobacterium tuberculosis*cells were transferred into a tube containing glass beads andstirred vigorously. After stirring, 1.5 mL solution of 0.04% Tween/0.2% albumin (v/v) was added and the tube wasstirred again. Then, 100 µLof this solution was transferred to a new tube containing 5 mL of 0.04% tween solution/0.2% albumin (v/v) and then stirred. The turbidity of the bacterial suspension was adjusted to match the McFarland scalenº 1.

A 25X diluted mycobacterial suspension in Middlebrook 7H9 broth (BD Difco™) was prepared. To check for extracellular mycobacterial antagonism activity, Alamar Blue solution was used as a redox developer, according to the technique described by Franzblau *et al.* (1998). Minimum Inhibitory Concentration (MIC) was establishedin triplicates in a decreasing range of 512 to 4 μgmL^{-1},these values being a concentration range of most antibiotics. Two successful fractions against *M. smegmatis* (04 and 13) were evaluated on the microplates incubated at 37°C for 5 days. After this period, 50 µL of Alamar Blue:Tween 80 (1:1) were added to the wells and the plates were incubated for 24 hours. The blue color on the wells shows the inhibitory activity.

RESULTS

In a first screening, the use of different chromatographic systems showed better results with dichloromethane, ethanol, ethyl acetate and n-butanol. Then, these solvents were used together in different combinations and concentrations to achieve more satisfactory separation of compounds. More reliable combinations for separation of the substances were obtained with toluene-ethanol (8:2).Both combinations allowed the separation of *A. fumigatus* CBA 2743 n-butanol fraction,in a larger number of chromatographic bands, reflecting on a better separation of the substances in two wavelengths (254 and 366nm).

By using solid phase extraction (SPE) with a normal phase cartridge, four soluble fractions in hexane, ethyl acetate, methanol and water:methanol (9:1) were obtained and two of these showed the antibiosis activity (hexane and ethyl acetate fractions). At a normal phase chromatography, these fractions were better separated using toluene:ethanol 8:2 + 100 µL of acetic acid (Figure 1).

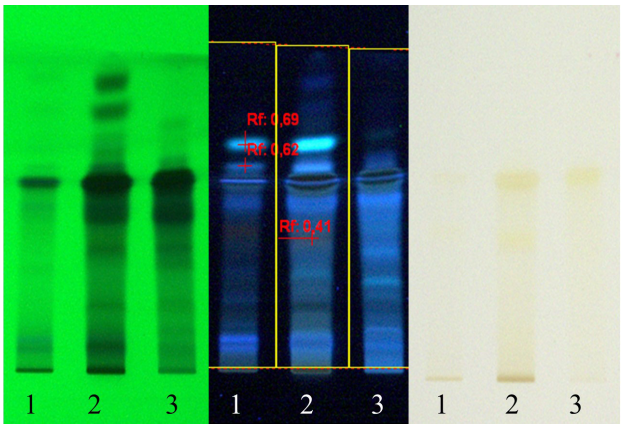

Figure 1. Profile of the fractionsof *A. fumigatus* obtained in SPE(1 : n-butanol; 2 : hexane; 3 :ethyl acetate) with mobile phase of toluene:ethanol 8:2 + 100 µL acetic acid, solubilized in methanol. From left to right: Green UV light 366nn, UV light 254nn and white light. This figure is in color in the electronic version.

The best result for FLASH chromatographyinMPLC was obtained with thegradient of ethyl acetate:methanol, resulting in 30 fractions (F1 to F30) (Figure 2), which were tested for biological activity against *M. smegmatis*. Two fractions (F4 and F13) were active in the antibiosis bioassays (Figure 3) and were used for the oxidation reaction of Alamar Blue assay against *M. tuberculosis*, by which mycobacterial development is detected by the pink color and absence of bacterial growth is detected by the bluish color. Positive antimycobacterial activity was detected in blue wells of F4 fraction with 256 μg mL^{-1} MIC.

The F4 fraction submitted to an analytical HPLC indicated two defined peaks, possibly indicating the presence of two different substances (Figure 4). Several wavelengths were tested to achieve the best one at 254 nm.

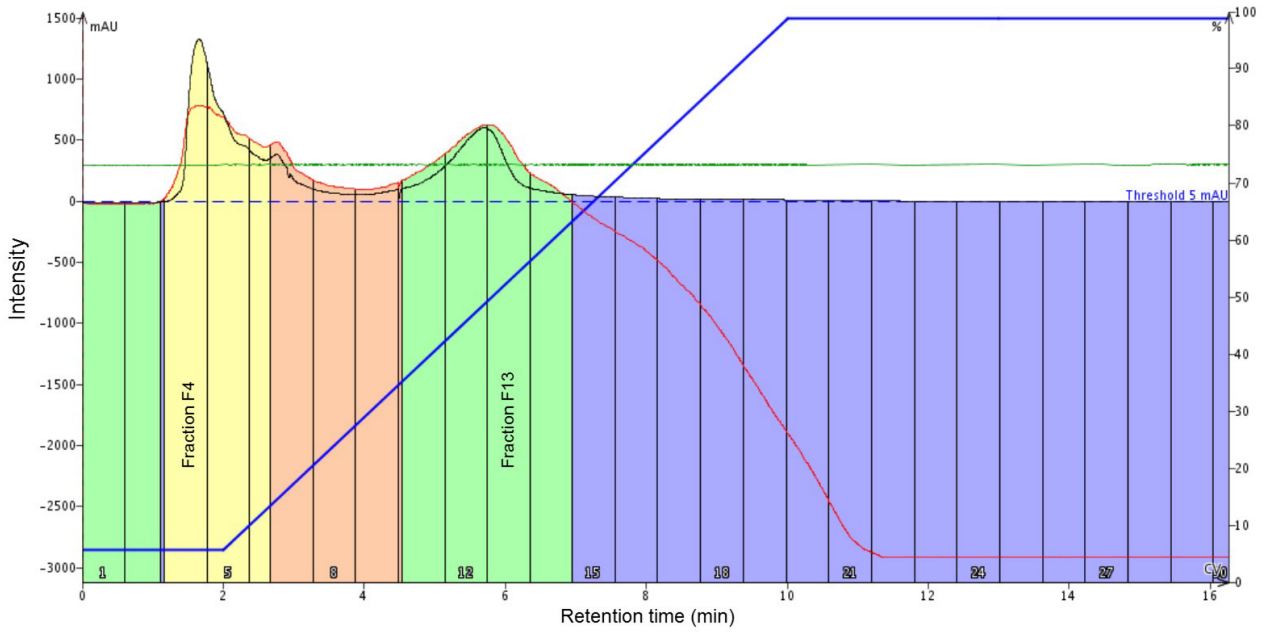

Figure 2. FLASH chromatogram in MPLC of the fraction of *A. fumigatus* obtained from liquid-liquid partition in n-butanol. Thegradient was performed with ethyl acetate: methanol, resulting in 30 fractions (F1 to F30). This figure is in color in the electronic version.

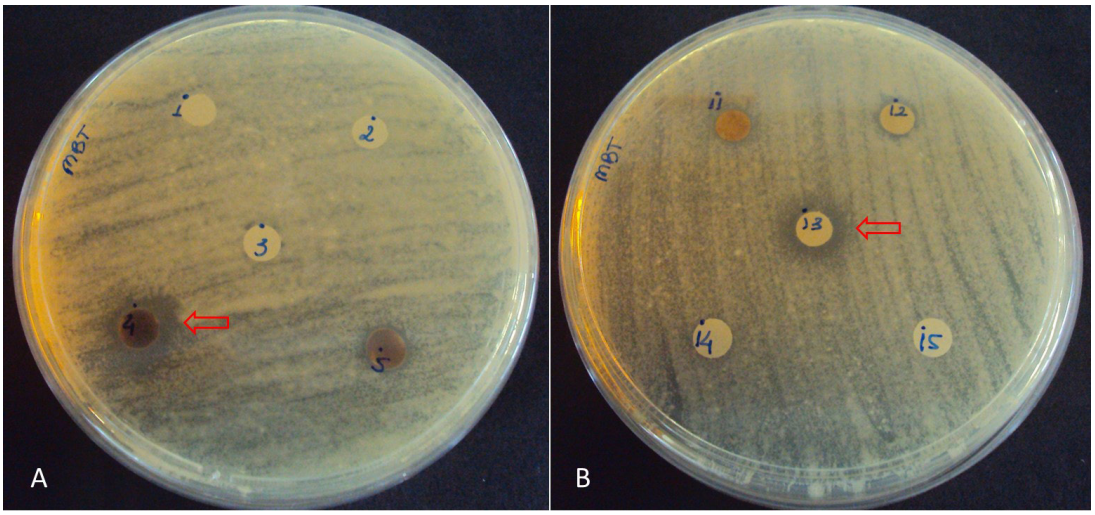

Figure 3. Antibiosis test of the fractions of *A. fumigatus* in n-butanol against *M. smegmatis*. Arrows indicate fractions F4 (plate A) and F13 (plate B), with positive inhibitory results on *M. smegmatis*. This figure is in color in the electronic version.

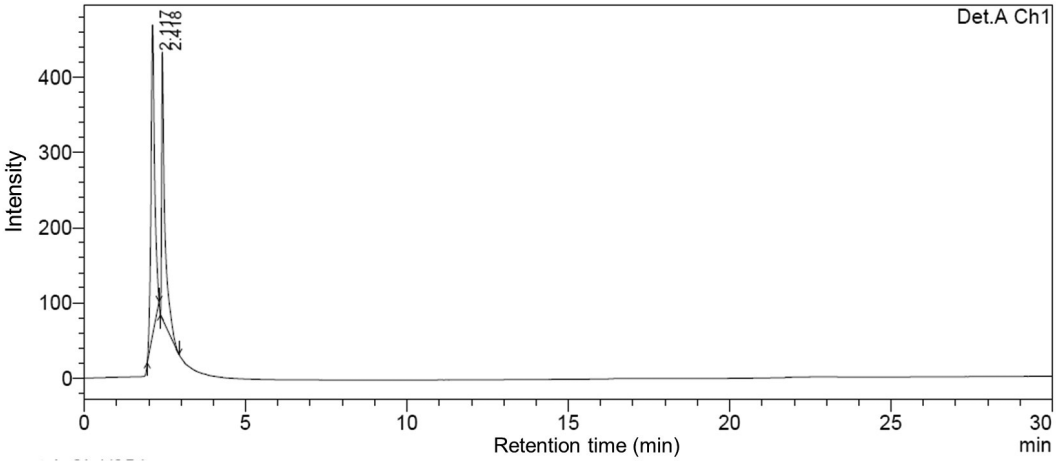

Figure 4. Chromatogram in analytical HPLC of the F4 fraction of *A. fumigatus* selected from bio-guided monitoring against *M. smegmatis*. The profile was obtained by wavelength of A_{254nm}.

DISCUSSION

The FLASH purification system using an exploratory gradient proved to be efficient in separating substances by combining satisfactory results with the possibility to obtain a higher amount of the sample (20mg). This is important because the obtention ofa suitable sample amount for fractioning samples by chromatographic techniques is laborious and time consuming.

Thus, MPLC proved to be a suitable system for assembling chemical compound libraries for the search for new drugs, as observed by other authors (Edwards and Hunter 2003; Pottorf and Player 2004; Liu *et al.* 2012). In the current study, monitoring the fractioning techniques by antibiosis bioassays allowed the selection and purification of target compounds in gradually increasing degrees. Although there was detection of antimycobacterial activity with the Alamar Blue test, a 256 µg mL^{-1} MIC is still high when compared with the current standards for TB therapy. Gu *et al.* (2004) consider as promising results those with MIC ≤ 125 µg mL^{-1}.

Sandoval-Montemayor *et al.* (2012) evaluated 19 constituents of *Citrus aurantifolia* and 10 were active at concentrations below 200 µgmL^{-1}. Palmitic acid, a saturated fatty acid, exhibited higher activity against multidrug-resistant *M. tuberculosis* strains (MIC = 50 µg mL^{-1}) than oleic acid and linoleic acid, unsaturated fatty acids, which demonstrated less activity (MICs = 100 µg mL^{-1}). From 28 extracts of the leaves of *Annona sylvatica*, the methanol extract demonstrated antimycobacterial activity with a MIC = 184.33 µgmL^{-1}, and the ethyl acetate fraction, resulting from the liquid-liquid partitioning of the *A. sylvatica* extract, showed a MIC of 115.2 µgmL^{-1} (Araujo *et al.*2014).

Despite these significant pharmacological activities, this native medicinal plant has not yet been explored for the production of bioactives from its ownendophytes. An example of such bioactives can be found in the endophytic fungus *Phomopsis stipata*, isolated from *Styrax camporum* Pohl (Styracaceae), in whichits secondary metabolites showed some promising results, with significant *in vitro* antimycobacterial activity of 31.25 µg mL^{-1} (Prince *et al.* 2012).In our continuous screening for biologically active secondary metabolites from plant endophytes, we investigated the ones produced by *A. fumigatus*isolated from *Copaifera multijuga*, an important Amazonian medicinal plant. Although*A. fumigatus*is an etiologic agent, responsible for fungal infections in immunosuppressed patients (Latagé, 1999), it is also known to produce a vast plethora of bioactives (Magotra *et al.* 2017), somewith significant antimicrobial activity in initial bioactivity screens. Fractions from the extract were able to inhibit the growth of *Staphylococcus aureus*, including the methicillin-resistant strain, and *Mycobacterium tuberculosis* H37Ra (Flewelling *et al.* 2015).The potential of *A. fumigatus* as a producer of secondary metabolites has already been shown in other studies, where it produced antibiotics (Waksman and Geiger 1944; Furtado *et al.* 2005), antifungals (Mukhopadhyay *et al.* 1987; Schulz *et al.* 2002; Liu *et al.* 2004), and substances with anti-acetyl-CoA activity (Tomoda *et al.* 1994), antitumor activity (Cui *et al.* 1995) and anti-trypanosomal activity (Watts *et al.* 2010).

As they areubiquitous fungi, it is interesting to isolate and purify *Aspergillus* spp. from different sources in order to obtain promising isolates with significant antimicrobial activity, not only for *M. tuberculosis*, but for other pathogens as well. In this study, this activity was observed in an *A. fumigatus* endophyte isolated from *Copaifera multijuga* (copaíba), which showed inhibitory activity against *M. smegmatis* and *M. tuberculosis*. Thus, this genus of plant could become an important source of new antimicrobial compounds, including for other target

pathogens, and may contribute to the supply of new active substances. Screenings such as those carried out in the present studyareessential to start the search and purify new compounds of biotechnological interest.

To establish a successful fermentation process it is necessary to make the environmental and nutritional conditions favorable for over-production of the desired metabolite by the microorganism (Ismaie, 2017). Therefore more tests should be done in cultivation, including selection of the cultivation medium, agitation rate, fermentation time, incubation temperature, pH value, inoculum nature, and medium volume, in order to increase the production of secondary metabolites.

CONCLUSIONS

The endophytic *Aspergillus fumigatus* CBA 2743 isolated from copaíba, *Copaifera multijuga* showed considerable antimicrobial activity, since it demonstrated an inhibitory activity against both *Mycobacterium smegmatis* and *M. tuberculosis*. Its secondary metabolites should be better investigated for applied purposes, since they requirea potential cytotoxic effect assessment. Chromatographic methods allied to antibiosis tests, although laborious, were an excellent complement to the study of new antimicrobial compounds. This bio-guided system wasthe most suited for this type of prospection, since each step of purification producedmore fractions and the FLASH chromatography system wasthe choice for purification of large amounts of substances.

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